THIRD EDITION

Clinical Microbiology Procedures Handbook

VOLUME 1

THIRD EDITION

Clinical Microbiology Procedures Handbook

EDITOR IN CHIEF, Third Edition and 2007 Update

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Henry D. Isenberg

(Deceased)

ASM PRESS

Washington, DC

VOLUME 1

Address editorial correspondence to ASM Press, 1752 N St., N.W., Washington, DC 20036-2904, USA

Send orders to ASM Press, P.O. Box 605, Herndon, VA 20172, USA
Phone: 800-546-2416; 703-661-1593
Fax: 703-661-1501
E-mail: books@asmusa.org
Online: http://estore.asm.org

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American Society for Microbiology
1752 N St., N.W.
Washington, DC 20036-2904

Library of Congress Cataloging-in-Publication Data

Clinical microbiology procedures handbook.—3rd ed. / editor in chief, third edition and 2007 update, Lynne S. Garcia.
p. ; cm.
"Editor in chief, original and second editions Henry D. Isenberg."
Includes bibliographical references and index.
ISBN-13: 978-1-55581-527-1
ISBN-10: 1-55581-527-8
1. Diagnostic microbiology—Laboratory manuals. I. Garcia, Lynne Shore. II. Isenberg, Henry D.
[DNLM: 1. Microbiological Techniques—methods—Laboratory Manuals. WQ 25 C6415 2010]
QR67.C555 2010
616.9′041—dc22

2010014355

10 9 8 7 6 5 4 3 2 1

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Printed in the United States of America

Dedication

We dedicate the third edition of the *Clinical Microbiology Procedures Handbook* to Henry D. Isenberg. Henry was a pioneer in clinical microbiology who spearheaded the field of microbial diagnosis for more than a half century. He was a gifted mentor, scholar, and scientist who inspired several generations of clinical microbiologists. We are very fortunate to have worked with such an outstanding microbiologist and colleague.

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How To Use This Handbook

General Format

The third edition of this handbook has been divided into three volumes containing the front matter, 16 sections (composed of "procedures"), and an index. Volume 1 contains the front matter of the handbook plus sections 1 through 4. Volume 2 contains sections 5 through 9. Volume 3 contains sections 9 through 16 and the index.

Included at the front of each volume is a short table of contents listing the items contained in the front and back matter and the 16 sections of the handbook. In addition to the table of contents for the entire handbook, each section is immediately preceded by a detailed table of contents for that section, giving the section editors' names, the procedure titles included in that section, and the authors' names for each procedure.

Sections

The content of the handbook has been organized into 16 sections as follows:

Section 1: Procedure Coding, Reimbursement, and Billing Compliance
Section 2: Specimen Collection, Transport, and Acceptability
Section 3: Aerobic Bacteriology
Section 4: Anaerobic Bacteriology
Section 5: Antimicrobial Susceptibility Testing
Section 6: Aerobic Actinomycetes
Section 7: Mycobacteriology and Antimycobacterial Susceptibility Testing
Section 8: Mycology and Antifungal Susceptibility Testing
Section 9: Parasitology
Section 10: Viruses and Chlamydiae
Section 11: Immunology
Section 12: Molecular Diagnostics
Section 13: Epidemiologic and Infection Control Microbiology
Section 14: Quality Assurance, Quality Control, Laboratory Records, and Water Quality
Section 15: Biohazards and Safety
Section 16: Bioterrorism

Procedures

Each section listed above consists of procedures. The procedures have been numbered and are referred to by number in cross-references in the text. The procedure number consists of the section number plus the number of the procedure (plus the number of a subprocedure if applicable). For example, "procedure 5.6" is the sixth procedure in section 5; "procedure 7.4.2" is the second subprocedure of the fourth procedure in section 7.

Page Numbers

The page number within a procedure is the procedure number followed by the number of the page within the procedure. Thus, from the examples given above, “page 5.6.10” is the 10th page within procedure 5.6, and “page 7.4.2.3” is the 3rd page within procedure 7.4.2. In all cases, the last number is the page number within a procedure.

The index is numbered beginning with an “I” followed by the number of the page within the index. For example, “page I.3” is the third page in the index.

Abbreviations

Abbreviations

In this handbook, most abbreviations have been introduced in parentheses after the terms they abbreviate on their first occurrence, e.g., "a central nervous system (CNS) specimen." Some exceptions to this rule are explained below and given in Tables 1 to 4.

Because of their frequent use in this handbook and/or their familiarity to readers, the terms listed in Table 1 have been abbreviated in the procedures; i.e., they have not been spelled out or introduced. Based on the editorial style for books and journals published by the American Society for Microbiology (ASM), the abbreviations listed in Table 2 have also been used without introduction in this handbook. Table 3 lists abbreviations that have been used without introduction in the bodies of tables. Abbreviations for commonly accepted units of measurement have been used without definition if they appeared with numerical values. Table 4 lists some common units of measurement appearing in this handbook. These last two items are also based on ASM style.

As readers use the various procedures in this handbook and see unfamiliar abbreviations that are not defined in the procedures themselves, they should refer to these tables for definitions.

Table 1 Common abbreviations used without introduction in this handbook

Abbreviation	Definition
ATCC	American Type Culture Collection
BAP (*not* SBA)	5% Sheep blood agar plate
BHI	Brain heart infusion
BSL	Biosafety level
CAP	College of American Pathologists
CDC	Centers for Disease Control and Prevention
CHOC	Chocolate agar
CLSI	Clinical and Laboratory Standards Institute (formerly NCCLS)
CMPH	*Clinical Microbiology Procedures Handbook* (first edition)
CSF	Cerebrospinal fluid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EMB	Eosin-methylene blue
EPCM	*Essential Procedures for Clinical Microbiology*
GLC	Gas-liquid chromatography
JCAHO	Joint Commission on Accreditation of Healthcare Organizations
MAC	MacConkey agar
MSDS	Material safety data sheet

(continued)

Table 1 Common abbreviations used without introduction in this handbook *(continued)*

Abbreviation	Definition
N.A.	Numerical aperture
NBS	National Bureau of Standards (pertaining to a special calibrated thermometer)
NCCLS	National Committee for Clinical Laboratory Standards
NIH	National Institutes of Health
OSHA	Occupational Safety and Health Administration
PMNs	Polymorphonuclear leukocytes
PPE	Personal protective equipment
QA	Quality assurance
QC	Quality control
RBCs	Red blood cells *or* erythrocytes
TCBS	Thiosulfate citrate bile salt sucrose agar
THIO	Thioglycolate broth
TSA	Trypticase soy agar *or* tryptic soy agar
TSB	Trypticase soy broth *or* tryptic soy broth
WBCs	White blood cells *or* leukocytes

Table 2 Additional abbreviations used without introduction (according to ASM style)

Abbreviation	Definition
AIDS	Acquired immunodeficiency syndrome
AMP, ADP, ATP, GTP, dCMP, ddGTP, etc.	Adenosine 5′-monophosphate, adenosine 5′-diphosphate, adenosine 5′-triphosphate, guanosine 5′-triphosphate, deoxycytidine 5′-monophosphate, dideoxyguanosine triphosphate, etc.
ATPase, dGTPase, etc.	Adenosine triphosphatase, deoxyguanosine triphosphatase, etc.
cDNA	Complementary deoxyribonucleic acid
CFU	Colony-forming unit(s)
cRNA	Complementary ribonucleic acid
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetate, ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ethyl)-*N,N,N′,N′*-tetraacetic acid
HEPES	*N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid
MIC	Minimal inhibitory concentration
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NAD^+	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
oligo(dT), etc.	Oligodeoxythymidylic acid, etc.
PCR	Polymerase chain reaction
PFU	Plaque-forming unit(s)
poly(A), poly(dT), etc.	Polyadenylic acid, polydeoxythymidylic acid, etc.
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
Tris	Tris(hydroxymethyl)aminomethane
tRNA	Transfer ribonucleic acid
UV	Ultraviolet

Table 3 Abbreviations used without introduction in the bodies of tables

Abbreviation	Definition	Abbreviation	Definition
amt	Amount	SD	Standard deviation
approx	Approximately	SE	Standard error
avg	Average	SEM	Standard error of the mean
concn	Concentration	sp act	Specific activity
diam	Diameter	sp gr	Specific gravity
expt	Experiment	temp	Temperature
exptl	Experimental	tr	Trace
ht	Height	vol	Volume
mo	Month	vs	Versus
mol wt	Molecular weight	wk	Week
no.	Number(s)	wt	Weight
prepn	Preparation	yr	Year

Table 4 Some common units of measurement used in this handbook

Abbreviation	Definition
°C	Degree Celsius
h	Hour
µg	Microgram
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
s	Second

Icons

The three icons listed below are used throughout this handbook. The icons direct the readers to follow important directions as they carry out the procedures. As a reminder, an explanation of the icon appears next to it at each appearance in the text.

It is imperative that these cultures be handled in a biological safety cabinet.

Include QC information on reagent container and in QC records.

Observe standard precautions.

Preface

The third edition of the *Clinical Microbiology Procedures Handbook* (CMPH) is based on the value and user requirements following the first and second editions of CMPH, the companion volume *Essential Procedures for Clinical Microbiology*, and the 2007 update of the second edition.

Almost all of the sixteen sections of the second edition of CMPH have been revised and updated; sections that did not require extensive revision will be updated during the next cycle of changes.

The purpose of the third edition of CMPH remains constant. That is to provide everyone engaged in the microbiological analysis of clinical specimens with procedures that will enable them to correctly perform the appropriate tasks. CMPH remains a cookbook that provides step-by-step descriptions of the numerous procedures used by workers at the bench.

As with the second edition of CMPH and the 2007 update of the second edition, there is increased emphasis on molecular approaches, bioterrorism, infection control in medical facilities, and the host's immunological responses to microbial challenges. Also, continued emphasis is placed on the need to respond to governmental regulations and fiscal constraints. Highly experienced workers with many years of bench experience have written these procedures, and the format adheres to CLSI document GP02-5A (5th ed., 2006).

All procedures have been reviewed extensively by section editors, the editor in chief, and the ASM Press editors. We continue to encourage the users of these documents to bring new methods of universal relevance to our attention so they can be incorporated into the next update and shared with the clinical microbiology community.

Readers are reminded that naming any specific product in CMPH is not intended as an endorsement of that specific product or a suggestion to exclude other equally acceptable products. CMPH is for laboratory use only by qualified, experienced individuals or by personnel under the direct supervision of qualified, experienced individuals. Every effort has been made to ensure that the contents of this update are comprehensive, accurate, reliable, and reproducible.

Not all existing microbiological protocols are included; however, the editors and authors are familiar with all commonly used protocols. As newer protocols become more widely accepted and used, they will be incorporated into future CMPH revisions.

The third edition of CMPH is available in print, on CD, and as a downloadable PDF.

Lynne S. Garcia

Acknowledgments

I thank each of the section editors and authors for their tremendous efforts in planning and completing the third edition of CMPH. Special thanks go to everyone who participated in the original version, the second edition, and the 2007 update of the second edition for their comprehensive contributions in developing and updating these diagnostic procedures for all microbiologists. Each new edition and update builds on the expertise of the previous editors and authors, all of whom provided outstanding contributions to CMPH. We continue to acknowledge and thank the late Dr. Henry D. Isenberg for his outstanding guidance and leadership during the development and updating of CMPH. All microbiologists owe him a tremendous debt of gratitude; he will always be known as the "father of CMPH" and its greatest supporter.

Our editors and authors join me in thanking the officers of ASM, the Publications Board, and, especially ASM Press. As editor in chief, I particularly want to acknowledge John Bell, Cathy Balogh, Susan Birch, and Jeff Holtmeier of ASM Press for their efforts in supporting this and former editions of CMPH.

It has been a great privilege to work with this current group of editors and authors on the third edition, and we all continue to support CMPH in memory of Dr. Isenberg.

Lynne S. Garcia

Reader Response Form

Dear Reader:
We solicit your help in improving the *Clinical Microbiology Procedures Handbook* (CMPH). Updates will be published periodically to keep CMPH current, accurate, and reliable. Your guidance will play an important role in achieving our objective of making CMPH the most useful laboratory procedures guide available. *Please copy this page for your continued use.*

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2. Please list procedures that you deem to be outdated, confusing, or inadequately presented. List the section, procedure, and page number; explain.

3. Indicate the topics that you would like to see added. Please list your reasons for the selection.

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Disclaimer

Microbiological analysis of clinical specimens is a constantly changing discipline; new methods and technologies emerge. The contributors to the third edition of CMPH believe that the procedures and guidelines suggested here are from reliable sources and in line with the practices accepted at the time of publishing. Readers are reminded that the naming of any specific product is not intended as an endorsement of that specific product by ASM Press or any other agency, nor is it a suggestion to exclude other equally acceptable products. CMPH is for laboratory use only by qualified, experienced individuals or by personnel under the direct supervision of qualified, experienced individuals. Every effort has been made to ensure that the contents of this update are comprehensive, accurate, reliable, and reproducible.

SECTION 1

Procedure Coding, Reimbursement, and Billing Compliance

SECTION EDITOR: *Alice S. Weissfeld*
ASSOCIATE SECTION EDITOR: *Vickie S. Baselski*

1.1. Introduction
Alice S. Weissfeld and Vickie S. Baselski 1.1.1

1.2. Procedure Coding, Reimbursement, and Billing Compliance
Alice S. Weissfeld and Vickie S. Baselski 1.2.1

1.1 Introduction

When the first edition of this handbook was published, regulatory billing compliance for laboratory tests was not a major laboratory issue. Most hospital laboratories generally performed services for their own inpatients (and, occasionally, affiliated outpatients), and tests were billed according to a formula established by the business office. In the setting of prospective payment (e.g., diagnosis-related groups), payment credit was allocated based on a different formula. Today, however, microbiologists must be aware not only of the scientific basis of infectious-disease diagnostics but also of the costing, coding, billing, and reimbursement for individual tests for patients seen in a broad spectrum of health care settings with coverage by an enormous number of health care plans. Jargon previously unknown in the clinical laboratory, such as reflex testing, upcoding, downcoding, local coverage decision (LCD), and national coverage decision (NCD), is now so extensive that a glossary of common terminology is included in this section (Appendix 1.1–1). The goal is to be reimbursed adequately for all appropriate work performed in a manner that is in compliance with all regulations.

The issues discussed in this section are complex. In keeping with the mission of this handbook, a model compliance procedure has been written. As with any procedure, we expect some customization to occur. The model procedure deals primarily with some of the more important regulatory principles. It should *not,* in any situation, take the place of guidance established by your own compliance committee or of legal advice from your own institution's legal counsel. It simply represents a starting point for review of salient issues.

A brief historical overview of the economic challenges faced by clinical microbiology laboratories is provided for background. This may be reviewed in detail in the Institute of Medicine's report *Medicare Laboratory Payment Policy, Now and in the Future* (2). The history of reimbursement and compliance begins with Title XVIII, commonly known as the Social Security Act. This act outlines the principles of the Medicare program, specifying broad benefit categories, including physician and hospital services. In accordance with section 1862 (a)(1)(A), the Medicare program provides payment *only* for diagnostic laboratory tests "that are reasonable and necessary for the diagnosis or treatment of illness or injury." It does *not,* however, authorize payment for screening diagnostic services. Over the ensuing 35 years since Title XVIII became law, the interpretive determination of whether a test meets the criteria of being reasonable and necessary to justify payment by Medicare has become known as "medical necessity." Most other third-party payers have established similar payment guidelines with which the laboratory must be familiar. It is notable that both Medicare and other payers may make specific exceptions to allow payment for screening services (e.g., coverage of Pap smears or prostate-specific antigen testing). In the event that a coverage policy disallows payment for a service on the basis of medical necessity, it is possible to transfer financial liability to the patient providing an advanced beneficiary notice (ABN) has been properly executed which informs the patient of their financial responsibility. In 2009, a laboratory-specific ABN, CMS-R-131-L, was discontinued and replaced with a generic ABN, CMS-R-131. Information may be accessed at http://www.cms.hhs.gov/BNI/02_ABN.asp.

The Social Security Act is known as a statute, i.e., a piece of legislation voted into law by Congress and signed by the President. Statutes form the basis for subsequent regulations that are rules established by a federal agency in response to its interpretation of a statute that it is its duty to enforce. A number of federal agencies are directly or indirectly involved with laboratory reimbursement and compliance. The CDC is responsible for the scientific and quality aspects of laboratory testing under the Clinical Laboratory Improvement Act (1967) and Clinical Laboratory Improvement Amendment (CLIA) (1988). The CDC is advised in this process by the Clinical Laboratory Improvement Advisory Committee. The Food and Drug Administration (FDA) is responsible for new product clearance and, since 2003, for test complexity categorization under CLIA. Medicare regulators have historically interpreted tests that are subject to FDA approval or clearance but that have not obtained, such as "not reasonable or necessary" for payment purposes. The FDA is assisted by its Microbiology Medical Device Advisory Committee. The Centers for Medicare & Medicaid Services (CMS), previously known as the Health Care Financing Administration (HCFA), administers the Medicare Program. CMS interprets statutes and regulations and issues these interpretations through a variety of official documents (e.g., *The Medicare Program Integrity Manual* [1], program memoranda and transmittals, and change requests) to define coverage criteria, establish national limitation amounts, and establish national coverage decisions (NCDs). CMS also contracts with independent insurers known generically as contractors and specifically as carriers (for part B outpatient services), as fiscal intermediaries (FIs; for part A inpatient services or part B services through part A providers), and, more recently, as Medicare administrative contractors (MACs; for part A and part B ser-

vices) to administer claims to reimburse physicians or laboratories. CMS is advised in developing coverage policies by the Medicare Carrier Advisory Committee (MCAC) and other contractor committees such as technology assessment groups. Individual carriers, Fls, and MACs may also set payment policies, known as LCDs, for their own geographic regions. Examples of LCDs in infectious-disease diagnostics include syphilis testing, blood cultures, antimicrobial susceptibility testing, TORCH (*Toxoplasma*, rubella, cytomegalovirus, and herpes simplex virus) testing, and human papillomavirus testing. Any information on institutional billing and reimbursement should include a search for LCDs specific to infectious-disease diagnostic testing. For this, a specific website exists at http://www.cms.hhs.gov/mcd/overview.asp (Appendix 1.1–2). Medicare generally requires that laboratories submit billings electronically, but it may occasionally accept handwritten bills on HCFA form 1500 for part B and HCFA for UB92 for part A, both universal insurance billing forms. Payment denials may be appealed through a formal process and ultimately referred to an administrative law judge. As a result of deliberations by a Negotiated Rulemaking Committee on clinical laboratory diagnostic tests mandated by the Balanced Budget Act of 1997, 23 NCDs were developed which take precedence over any existing LCDs for the same tests. Implementation for these NCDs occurred in 2002, a process for review and update was implemented in 2003, and under the Medicare Improvements for Patients and Providers Act of 2008, CMS can now propose new NCDs for preventive (screening) services. Three NCDs were developed in infectious-disease diagnostics by a committee cochaired by the American Society for Microbiology: urine culture, human immunodeficiency virus (HIV) diagnosis, and HIV monitoring and prognosis. The Negotiated Rulemaking Committee also developed a number of administrative policies which became effective in 2003. A database index for current NCDs may be accessed at http://www.cms.hhs.gov/transmittals/downloads/r17ncd.pdf. Again, billings to other payers may require submission of other specific forms, but coverage criteria are often modeled after Medicare billing guidelines.

Coding is the process of assigning a procedure code such as a Current Procedural Terminology (CPT), or a diagnosis or condition, code, usually the International Classification of Diseases, Clinical Modification (ICD-9-CM), code, for the purpose of submitting a claim for reimbursement. CPT codes are property of the American Medical Association but have been named as the official CMS HCFA Common Procedure Coding System (HCPCS). CPT codes are updated annually by a lengthy, systematic process. Most codes are Category I and represent generally accepted procedures, but Category III codes for emerging technologies were introduced in 2002. Category III codes are generally not reimbursable. In some cases, newer emerging technologies may also be assigned an interim HCPCS code. The ICD-9-CM system is a public domain consensus document developed by the World Health Organization. It includes specific diagnoses as well as signs and symptoms or medical conditions that represent the physician's reason for ordering a test. It also has a series of preventive-medicine screening codes (V codes) that in general do not support medical necessity. It is of note that a major update, ICD-10, should be implemented by October 2013. Correct coding is the cornerstone of reimbursement and compliance.

Finally, in 1997, in response to a major antifraud and abuse campaign in the Medicare program (Operation Restore Trust), the Office of the Inspector General published a series of compliance documents, including one for clinical laboratories. One such document, the Office of Inspector General's Compliance Program Guidance for Clinical Laboratories, published in August 1998, contains an outline for the design of individual institutional compliance plans to ensure correct coding, appropriate billing, and honest and ethical laboratory business practice (3). This document, although more than a decade old, still forms the basis for laboratory billing compliance and for the model compliance plan presented in this section.

REFERENCES

1. **Centers for Medicare and Medicaid Services.** 2008. *Medicare Program Integrity Manual*. Centers for Medicare and Medicaid Services, Baltimore, MD. http://www.cms.hhs.gov/manuals/iom/ItemDetail.asp?ItemID=CMS019033.
2. **Institute of Medicine.** 2000. *Medicare Laboratory Payment Policy: Now and in the Future*. National Academy Press, Washington, DC.
3. **Office of the Inspector General.** 1998. Compliance program guidance for clinical laboratories. *Fed. Regist.* **63:**45076–45087.

APPENDIX 1.1–1

Glossary of Reimbursement and Compliance Terminology and Acronyms

Terminology

Terminology	Definition
Abuse	Systematically accepting improper payment without knowledge of illegality (*see* Fraud)
Add on	A test added after the original date of service
Bundling	Placing codes together in a panel
Carrier	CMS contractor for part B claims
Carve out	Exclude from a capitated contract and bill fee-for-service
Code jamming	Inserting ICD-9 codes to pass claims review
Code stacking	The use of two or more procedural codes to denote a single reportable test result
Code steering	Encouraging ICD-9 code use to satisfy medical necessity
Comparative effectiveness	Assessment of clinical comparability of procedures that may result in reimbursement of a new technology at the rate of an older technology
Composite	The use of two or more test codes simultaneously in accordance with standard of care and not otherwise described as a panel
Contractor	Generic term for Medicare claims administrator
Covered lives	Population insured by a managed-care contract
Crosswalked	Deemed equivalent (*see* Mapped)
Custom panel	User-defined composite of test codes performed simultaneously which is not a standard of care
Downcoding	Using a lower paying code to encourage utilization
E codes	Codes for external sources of injury
Fair market value	A reasonable payment amount for a specified service
Fraud	Knowingly or willingly accepting improper payments with knowledge of illegality (*see* Abuse)
Frequency limits	Number of times a service may be reimbursed
Gap fill	Temporary assignment of reimbursement
Inducement	Service or item with monetary value given to encourage utilization by a purchaser of laboratory services
Kickback	Acceptance of an inducement
Mapped	Deemed comparable (*see* Crosswalked)
Medical necessity	Determination of ICD-9-CM codes for which a CPT code will be reimbursed as reasonable and necessary
Modifiers	Two-character (numeric for CPT and alpha for HCPCS) units appended to a CPT code to indicate a condition affecting payment
Neg Reg	Negotiated Rulemaking Committee on diagnostic clinical laboratory tests
Not medically necessary	Determination that an ICD-9 CM does not justify payment for a CPT code
Panel	CMS-approved test composite encompassed in a single CPT code
Program integrity	Process by which CMS monitors for fraud and abuse
Reasonable charge methodology	Based on inherent reasonableness, authority to arbitrarily increase or decrease payment
Reflex	Second related test automatically performed when an initial test result is positive or abnormal
Remittance advice codes	Provides explanation of any adjustment made to expected payment for a service; includes claim adjustment reason codes and remittance advice remark codes
Sink testing	Fraudulently reporting results for tests not performed
Standing orders	Preapproved set of test codes performed on a regular defined basis
Unbundling	Coding individual tests rather than a single defined panel
Upcoding	Using a higher-paying code to maximize reimbursement
V codes	Generally, health screening codes
Zero tolerance	Absolute institutional policy that no fraudulent practice will be allowed

APPENDIX 1.1–1 *(continued)*

Acronyms

Acronym	Definition
ABN	Advanced beneficiary notice
ALJ	Administrative law judge
AMA	American Medical Association
APC	Ambulatory payment classification in the OPPS
ASR	Analyte-specific rule
BBA '97	Balanced Budget Act of 1997
CAC	Carrier Advisory Committee
CAP	College of American Pathologists
CCI	Correct Coding Initiative (also known as the National Correct Coding Initiative, NCCI)
CDC	Centers for Disease Control and Prevention
CERT	Comprehensive Error Rate Testing Program
CLFS	Clinical laboratory fee schedule (*see* NLA)
CLIA '67	Clinical Laboratory Improvement Act of 1967
CLIA '88	Clinical Laboratory Improvement Amendments of 1988
CLIAC	Clinical Laboratory Improvement Advisory Committee
CMD	Contractor medical director
CMS	Centers for Medicare and Medicaid Services
CPT	Current Procedural Terminology
DRG	Diagnosis-related group in the IPPS
EOMB	Explanation of medical benefits (also explanation of benefits, EOB)
ESRD	End-stage renal disease (dialysis center)
FBI	Federal Bureau of Investigation
FDA	Food and Drug Administration
FI	Fiscal intermediary
FTE	Full-time equivalent
HCFA	Health Care Financing Administration (now CMS)
HCPAC	Health Care Professionals Advisory Committee
HCPCS	HCFA Common Procedure Coding System
HIPAA	Health Insurance Portability and Accountability Act
ICD-9-CM	International Classification of Diseases, 9th ed., Clinical Modification
ICD-10	International Classification of Diseases, 10th ed.
IDE	Investigational device exemption
IOM	Institute of Medicine
IPPS	Inpatient prospective payment system (*see* DRG)
LCD	Local coverage decision (previously known as LMRP)
LMIP	Laboratory Management Index Program
LMRP	Local medical review policy (now LCD)
MAC	Medicare administrative contractor (for A and B claims)
MedPAC	Medicare Payment Advisory Council
MIPPA	Medicare Improvement for Patients and Providers Act of 2008
MMA '03	Medicare Modernization Act of 2003
MUE	Medically unlikely edit for frequency in the CCI edits
NCD	National coverage decision (previously known as NCP)
NCP	National coverage policy (now NCD)
NLA	National limitation amount in the CLFS
NPI	National provider identifier
OCE	Outpatient code editor (for OPPS)
OIG	Office of the Inspector General
OPPS	Outpatient prospective payment system
PCC	Pathology coding caucus
PFS	Physician fee schedule
PPAC	Practicing Physician Advisory Council
RAC	Recovery audit contractor
SBT	Standardized billable test
SNF	Skilled-nursing facility (nursing home)

APPENDIX 1.1–2

Websites and Guidance Documents

I. WEBSITES

A. All contractor LCDs, http://www.cms.hhs.gov/mcd/overview.asp
B. CMS website, http://www.cms.hhs.gov (to subscribe to regular updates, go to http://www.cms.hhs.gov/AboutWebsite/EmailUpdates/list.asp)
C. General Accounting Office, http://www.gao.gov (search "Medicare" for recent reports)
D. FDA, http://www.fda.gov (to subscribe to regular updates, go to http://www.fda.gov/AboutFDA/ContactFDA/StayInformed/GetEmailUpdates/default.htm)
E. Health Care Compliance Association, http://www.hcca-info.org
F. IOM study on Medicare Laboratory Payment Policy (http://www.iom.edu/iom/iomhome/nsf/pages/clinlab + home + page)
G. Medicare Clinical laboratory fee schedules, http://www.cms.hhs.gov/ClinicalLabFeeSched/02_clinlab.asp#TopOfPage
H. Medicare fee schedules: general information, http://www.cms.hhs.gov/FeeScheduleGenInfo/
I. Medicare manuals, http://www.cms.hhs.gov/Manuals/01_Overview.asp
J. Medicare physician fee schedule, http://www.cms.hhs.gov/PhysicianFeeSched/
K. Medicare Learning Network, http://www.cms.hhs.gov/MLNGenInfo/
L. Medicare Payment Advisory Commission, http://www.medpac.gov
M. National Center for Health Statistics for ICD-9-CM updates, http://www.cdc.gov/nchs/icd.htm
N. National Correct Coding Initiative, http://www.cms.hhs.gov/NationalCorrectCodInitEd/
O. National Technical Information Service for published NCCI edits, http://www.ntis.gov
P. OIG website for Compliance Program, Fraud Alerts, Advisory Opinions, Red Book, Work Plan, http://oig.hhs.gov (to subscribe to regular updates, go to http://oig.hhs.gov/mailinglist.asp)
Q. Strategic Management (previously The American Compliance Institute), http://www.strategicm.com/

II. OTHER GUIDANCE DOCUMENTS

A. ABN current format and instructions, http://www.cms.hhs.gov/BNI/02_ABN.asp
B. Guidance for industry and FDA staff—commercially distributed analyte-specific reagents, frequently asked questions, http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm078423.htm
C. Medical devices: classification/reclassification, restricted devices, analyte-specific reagents, *Fed. Regist.* **62:**62243–62260 (1997)
D. Medicare: HCFA faces challenges to control improper payments, T-HEHS-00-74, 9 March 2000, http://www.gao.gov
E. Medicare Claims Review Program: NCCI edits, MUEs, CERT, and RAC, Medicare Learning Network Publication (October 2008), http://www.cms.hhs.gov/MLNProducts/downloads/MCRP_Booklet.pdf
F. Medicare clinical trials policies, http://www.cms.hhs.gov/ClinicalTrialPolicies/
G. Medicare Fraud & Abuse, Medicare Learning Network Publication (January 2009), http://www.cms.hhs.gov/MLNProducts/downloads/110107_Medicare_Fraud_and_Abuse_brochure.pdf
H. Medicare program: application of inherent reasonableness to all Medicare part B services (other than physician services), *Fed. Regist.* **67:**76684–76697 (2002)
I. Medicare Program: criteria and procedures for extending coverage to certain devices and related services, *Fed. Regist.* **60:**48417–48425 (1995)
J. Medicare program: establishment of procedures that permit public consultation under the existing process for making coding and payment determinations for clinical laboratory tests and new durable medical equipment, *Fed. Regist.* **66:**58743–58745 (2001)
K. Medicare program: negotiated rulemaking: coverage and administrative policies for clinical diagnostic laboratory services; final rule, *Fed. Regist.* **66:**58787–58836 (2001)

APPENDIX 1.1–2 *(continued)*

L. Medicare program: procedures for making national coverage decisions, *Fed. Regist.* **64:**22619–22625 (1999)
M. National Provider Identifier Standard, http://www.cms.hhs.gov/NationalProvIdentStand/01_Overview.asp#TopOfPage
N. Physician fee schedules, http://www.cms.hhs.gov/PhysicianFeeSched/01_overview.asp

1.2 Procedure Coding, Reimbursement, and Billing Compliance

I. PURPOSE

The Office of the Inspector General (OIG) of the U.S. Department of Health and Human Services issued compliance program guidance for clinical laboratories in August 1998 (2). This anti-fraud and abuse document addresses Medicare and Medicaid program integrity, with emphasis on issues such as coding and billing; medical necessity; sales and marketing; arrangements with outside providers, suppliers, and vendors; and auditing and monitoring. Every clinical laboratory should be committed to doing business with any client, governmental or private, in an honest and trustworthy manner. While this document covers principles laid out in the federal compliance program guidance, the issues of integrity and the prevention of wrongdoing must be customized for each clinical laboratory to be compatible with the overall institutional compliance program. Specific details pertaining to elements of the Medicare program are detailed in *Medicare Laboratory Payment Policy* from the Institute of Medicine (1).

II. ELEMENTS OF A COMPREHENSIVE COMPLIANCE PROGRAM

A. The seven essential elements

At a minimum, a comprehensive compliance program for a clinical laboratory should include seven key elements.

1. Development of written standards of conduct (detailed in item III.A below)
2. Designation of a CCO and a Compliance Committee
   A committee composed of the laboratory director, the laboratory manager, clinical consultants, the materials manager, the business office manager, and the technical supervisors of each laboratory section will assist the chief executive officer and the chief compliance officer (CCO) in the surveillance of the institutional compliance program. Each committee member will be responsible for day-to-day observation that no fraudulent activities are occurring. However, all employees are responsible for reporting any problems to the CCO immediately. A zero-tolerance policy should be adopted for dealing with any individual demonstrated to have engaged in fraudulent activity.
3. Development of regular, effective training and education programs (detailed in item V below)
4. Development of a process to receive complaints
   A dedicated hotline should be established for the reporting of potentially fraudulent activities. Anonymity should be absolutely assured. No employee should ever be disciplined for reporting a problem even if the miscreant is part of upper management.
5. Development of a system to respond to allegations of potentially fraudulent activities
   A systematic investigation of all reports to the compliance officer should occur, and a report should be made to the Compliance Committee.
6. Establishment of an ongoing system of audits and monitors for compliance (detailed in item VIII below)

II. ELEMENTS OF A COMPREHENSIVE COMPLIANCE PROGRAM *(continued)*

7. Development of a system for investigation and remediation of systemic problems and for dealing with business associates who are sanctioned
It is the responsibility of the employer to ensure that problems have been corrected, repayments have been made, and all employees and business associates are not sanctioned by the Medicare program for previous illegal activities.

B. Written procedures and policies
All procedures and policies should be developed under the direction of the CCO, with formal review by the Compliance Committee. All documents should be in written form and readily available to employees to whom the policies apply. Additions and revisions should be clearly indicated and expediently communicated to employees, with documentation maintained.

III. WRITTEN PROCEDURES AND POLICIES APPLICABLE TO MICROBIOLOGY

A. Standards of conduct
All laboratory employees are expected to be honest in all their endeavors and in compliance with all applicable regulatory requirements. Practices such as sink testing (sending out results but not performing the work), issuing results when controls are out of range (in conflict with the Clinical Laboratory Improvement Amendment [CLIA '88]), and billing for work which is not ordered or necessary are major areas of inappropriate practice which should not be tolerated. In addition, no laboratory professional will induce anyone to send laboratory work by offering kickbacks or inducements of any kind. This includes practices such as the provision of free work to clients or their families or friends. No laboratory employee will induce any client to order a more expensive test if a less expensive one will suffice to make the clinical diagnosis, nor will they encourage anyone to order a reflex or composite test(s) if not medically necessary. No employee will suggest Current Procedural Terminology (CPT) coding that systematically results in higher reimbursement, or International Classification of Diseases, Clinical Modification (ICD-9-CM), coding that will guarantee reimbursement if not medically appropriate. In general, if any employee has to reflect on whether some action is legal or ethical, it is probably best not to undertake that action. Referral of questionable issues to the CCO or Compliance Committee is encouraged. It should be emphasized that a dishonest or unethical practice renders an individual subject to immediate sanctions. Sanctions may include oral or written warning, disciplinary probation, suspension, demotion, dismissal from employment, or revocation of medical staff privileges.

B. Medical-necessity issues
Medical necessity as defined by the Centers for Medicare and Medicaid Services (CMS) is an assessment of whether the clinician's reason for ordering a test is covered (i.e., reimbursable) for the diagnosis or treatment of a specific illness or injury. Examinations or diagnostic procedures performed in the absence of signs or symptoms (often performed based on a patient's age and/or family history) are considered screening tests by the CMS and, with a few statutory exceptions, are not reimbursed. The physician must provide a narrative diagnosis or diagnosis code (ICD-9-CM) so that Medicare or Medicaid and other contractors can assess the claim for medical necessity as defined by national coverage decisions (NCDs), local coverage decisions (LCDs), or other published policies. Any ICD-9-CM code beginning with a "V" designates a screening procedure and will therefore generally not be reimbursed.

1. Requisition design
a. The requisition should have orderable tests indicated by descriptor or mnemonic consistent with the menu described in the current laboratory

III. WRITTEN PROCEDURES AND POLICIES APPLICABLE TO MICROBIOLOGY *(continued)*

service manual. The orderable tests should be only CMS defined (i.e., CPT codeable) specific individual tests or CMS-approved panels unless one of the conditions below applies. The provision of CPT codes on the requisition is optional if they are readily available elsewhere.

b. The requisition should ensure that the physician has the ability to make an independent decision with regard to each billed test, e.g., must be able to order a culture with or without a susceptibility test or direct stain.

c. The requisition should identify reflex situations and offer the option not to reflex. A reflex test is a second related test performed automatically when initial results are positive or abnormal, e.g., a quantitative titer on a cryptococcal antigen if the qualitative screen is positive. A list of some microbiology reflex tests is shown in Appendix 1.2–1.

d. The requisition should identify situations in which the standard of care is to provide services which are a composite of two or more CPT codes and offer the opportunity to order individual tests. A list of common composites is shown in Appendix 1.2–1.

e. The requisition should require an ICD-9-CM code (preferably) or narrative diagnosis.

f. The requisition should indicate noncovered services (screening tests).

g. The requisition should identify limited-coverage tests (i.e., those having NCDs or LCDs).

h. The requisition should include or allow for an advanced beneficiary notice (ABN). An ABN is typically obtained at the time of specimen collection for a test which the provider believes will be denied as not medically necessary. It constitutes a waiver of financial liability in which a Medicare or Medicaid beneficiary acknowledges that he or she will pay for the service since it does not fulfill the criteria for medical necessity.

i. The date of service on the requisition is the date of specimen collection.

j. All test requests must be in writing, including requests for reflex, composite, or add-on procedures. An add-on is defined as a test requested on an existing specimen. However, if the sample has been archived for an extended period, the date of service is the date of retrieval rather than collection.

k. The requisition should meet all other requirements of licensure (e.g., by CLIA), voluntary accreditation (e.g., by the CAP), and other regulations, particularly those relating to privacy and confidentiality (e.g., the Health Insurance Portability and Accountability Act).

l. The requisition should be accompanied by or supplemented with thorough instructions for accurate completion (e.g., in a compliance section of a service manual).

2. Notices to physician clients

Annual notices to physician clients should include all of the following.

a. Each institution should determine if there are any active infectious-disease LCDs or NCDs. These policies indicate the ICD-9-CM codes which justify the medical necessity of specific CPT codes. These limited-coverage policies should be shared with clients if they are not altered in any manner which might encourage inappropriate utilization or coding.

b. Reflex test protocols and composite test groups should be clearly defined.

(1) For noninstitutional clients, a letter should be sent which clearly identifies laboratory policies.

(2) For institutional clients, annual medical staff approval will suffice for documentation.

c. Only CMS-approved panels should be offered unless a composite has been established as a recognized standard of care.

III. WRITTEN PROCEDURES AND POLICIES APPLICABLE TO MICROBIOLOGY *(continued)*

d. Physician-requested custom panels require a signed physician acknowledgment of the financial and compliance implications of routinely ordering the custom panel.

e. The Medicare fee schedule should be provided, along with a statement that the Medicaid fee schedule will be equal to or less than the Medicare amounts. Fee schedules should include applicable CPT codes for each test.

f. The name of the individual who serves as the CLIA Clinical Consultant in the specialty of microbiology should be publicized, and a phone number should be provided.

g. A list of standing orders for applicable clients should be reviewed and renewed at least annually.

3. Physician acknowledgments

a. Specific written acknowledgment of receipt of an annual notice is not required. However, the laboratory should maintain records of the documentation and the date sent.

b. Specific written acknowledgment of special request protocols (custom panels, reflexes, and standing orders) must be obtained and renewed on a regular basis, at least annually.

4. Use of ABNs

a. ABNs will be used whenever there is a likelihood that an ordered test will not be covered. Only the format and language currently recommended by CMS will be used.

b. The laboratory should identify tests which require FDA clearance or approval but which do not have such. These tests are generally considered not reasonable and necessary by Medicare and require an ABN. "Home brew" tests using analyte-specific reagents (ASRs) as defined by the FDA may be covered unless excluded by a specific coverage policy. Coverage conditions for other payers must be determined on a case-by-case basis.

c. ABNs must specify the specific test and the specific date of service as well as the anticipated reason for denial and estimated cost to the beneficiary.

d. Because microbiology specimens are usually collected at a location remote from the laboratory by nonlaboratory personnel, it is essential to verify that an appropriate ABN has been obtained.

e. If an ABN has not been obtained, the ordering provider should be contacted to obtain one. It is essential to educate physicians regarding the importance of this process.

f. Modifiers should be added to claims to indicate the status of the ABN (i.e., whether on file or not).

5. Test utilization monitoring

a. The laboratory should monitor yearly the utilization rates for the top 30 tests performed annually. In microbiology, this may include urine cultures, bacterial identification or susceptibility tests, and tests for *Neisseria gonorrhoeae* or *Chlamydia trachomatis.*

b. Any increase of more than 10% should be evaluated to ascertain the cause and rule out inappropriate utilization.

c. Any other aberrancy noted by the laboratory which might be related to inappropriate utilization should similarly be investigated.

C. Coding and billing issues

1. CPT selection

a. The laboratory should have access to the American Medical Association annual updates of the CPT code book. Medicare/Medicaid payment is based on assigning the most correct code(s) for the work actually per-

III. WRITTEN PROCEDURES AND POLICIES APPLICABLE TO MICROBIOLOGY *(continued)*

formed. A hierarchy of analyte, specific method, generic method, and unlisted should be followed in that order when assigning a code. Reflex coding and composite coding should also be considered. Technical input by a microbiologist is absolutely essential to correct coding.

- **b.** CPT codes should be reviewed annually beginning with the new edition published each October, with review complete by the end of the year, as use for Medicare is mandatory beginning January 1.
- **c.** Quarterly review of the Correct Coding Initiative edits should be performed to ensure that mutually exclusive and column 1/column 2 code edits will not result in denial and to determine code pairs where use of modifier −59 to override edits may be appropriate.
- **d.** A careful cost analysis and charge review should accompany the CPT review.
- **e.** Revenue projection adjustments should be made based on the national limitation amount for each test.
- **f.** All documentation pertaining to the annual CPT review should be approved by the Compliance Committee.
- **g.** Any changes in coding should be communicated to clients in the annual notice, or anytime they are made on an interim basis.

2. ICD-9-CM selection

- **a.** The ICD-9-CM is used to document the reason the physician ordered the test.
- **b.** Test requests should be accompanied by ICD-9-CM code(s).
- **c.** A narrative diagnosis is acceptable and may be translated into a code by a trained coder.
- **d.** If the clinician does *not* supply an ICD-9-CM code, or if a narrative cannot be accurately translated, the laboratory must obtain a diagnosis.
- **e.** The ICD-9-CM code must be specific for a test and date of service. "Code jamming" (arbitrarily inserting an ICD-9-CM code not meeting this condition) is not acceptable.
- **f.** Avoid "code steering" (the practice of suggesting use of a specific code[s] that guarantees reimbursement or allows reimbursement for a screening test).

3. Tests covered by claims

- **a.** The laboratory should ensure that the client understands the specific test by CPT code that is being ordered, performed, and billed (e.g., *Chlamydia* direct fluorescent antibody, EIA, direct probe, amplified probe, or culture). The requisition and service manual should make this clear.
- **b.** In the case of an ambiguous order (e.g., specimen-test mismatch or insufficient information to assign a test and CPT code[s]), the laboratory must contact the ordering provider to clarify the request.
- **c.** In the case of a specimen and valid request received but not reported due to technical issues, the laboratory must not submit a claim for service.
- **d.** Modifiers must be used where appropriate to indicate special claims processing conditions. In particular, modifier −59 is used in microbiology when replicates of the same CPT code are used on the same date of service on unique specimens from different sources or to override Correct Coding Initiative edits if allowed and testing is medically necessary.

4. Billing for calculations

Any calculations performed during the course of performing or determining results of a test are not separately billable.

5. Reflex testing

- **a.** Reflex testing is defined as testing that automatically occurs when an initial test is outside normal parameters and indicates that a second related test is medically appropriate and is considered a standard of care.

III. WRITTEN PROCEDURES AND POLICIES APPLICABLE TO MICROBIOLOGY *(continued)*

b. Reflex tests must be clearly described as such on the requisition and in other laboratory test information resources. The client must have a means by which the reflex may or may not be performed.

c. Reflex protocols must be reviewed and approved by the Compliance Committee annually and included in the annual physician notice or approved annually by an institutional medical staff committee.

d. Composites (a grouping of two or more codes in accordance with standard-of-care and accreditation or licensure requirements) and confirmations (a second test done to validate an initial positive) should be dealt with in a manner similar to reflex testing.

D. Standing orders

1. Standing orders are acceptable in connection with situations involving extended treatment but must have a fixed term of validity and be renewed in writing with the ordering provider at term. The term should be no more than 1 year.

2. Environments in which standing orders may be applicable include nursing homes and end-stage renal disease (ESRD) centers. However, microbiology testing is generally performed in cases of specific signs or symptoms of an infection and is not commonly a component of standing orders.

E. Compliance with fraud alerts

1. Through the CCO, the laboratory should have access to review all applicable OIG- and CMS-published documents outlining fraud risk areas.

2. If applicable to microbiology practice, the laboratory should review the alert and take steps to alter any current practices that are relevant. A full report of the review and the corrective action plan should be given to the Compliance Committee.

F. Marketing

1. Any marketing information provided for microbiology testing should adhere to the same principles of clear, correct, nondeceptive, and fully informative guidance affecting any other section.

2. No free services should be marketed which may be construed as inducement for the submission of Medicare work. For example, antibiograms that are provided to external clients (e.g., nursing homes) must be billed at a fair market rate.

G. Prices charged to physicians and other providers

1. Laboratories should establish fair market value fee schedules that will not be viewed as inducement for referral of federally reimbursed laboratory work.

2. In general, a federal charge substantially in excess of a charge to any other third-party payer cannot be established. Although only the contractor fee schedule amount for Medicare will be reimbursed, these charges are used in the calculation of the prevailing charges.

3. Laboratories should review and justify charges if 50% or more of non-Medicare work is heavily discounted.

4. Discounts below costs, particularly if done to match competitor pricing, may be viewed as inducement.

5. The laboratory should review test cost information on at least an annual basis to ensure that the above conditions are met.

H. Retention of records

All records should be maintained as required by applicable statutes or regulations for use if needed in the investigation of potential fraud. There may also be additional requirements based on voluntary accreditation standards. Federal statutes include the following.

III. WRITTEN PROCEDURES AND POLICIES APPLICABLE TO MICROBIOLOGY *(continued)*

1. 42 CFR 482.24(b)(1)
Condition of participation for hospitals; standard form and retention of records; specifies 5 years.

2. 42 CFR 488.5(a)
Discussion of accreditation standards deemed to meet Medicare conditions of participation; variable times, but at least 5 years.

3. 42 CFR 493.1105
CLIA '88; 2 years for test requisitions.

4. 42 CFR 493.1107
CLIA '88; 2 years for test records.

5. 42 CFR 493.1107 and 1109
CLIA '88; transfusion medicine records; minimum of 5 years.

6. 42 CFR 493.1257(g)
CLIA '88; cytology slides for 5 years.

7. 42 CFR 1003.132
Related to civil actions (False Claims Act); may be initiated up to 6 years after the date of claim presentation.

I. Compliance as an element of a performance plan

Compliance training (initial and annual retraining) should be incorporated into an employee's annual performance evaluation and competency assessment. Any policies specific to microbiology should also be discussed in detail with employees and incorporated into the competency assessment.

IV. THE CCO AND COMPLIANCE COMMITTEE

A. The CCO should be known to all members of the laboratory. Any issues specific to microbiology should be brought to the attention of the CCO or the supervisor or reported through the hotline.

B. The Compliance Committee for the laboratory should have representation from all areas of the laboratory, including microbiology. The microbiologist will have responsibility for providing expertise in the evaluation of any protocols, audits and monitors, or issues pertaining to the diagnosis of infectious diseases.

V. EDUCATION AND TRAINING

Compliance training should be conducted during orientation and at least annually thereafter. Training should emphasize those areas that each individual will deal with under normal circumstances; e.g., microbiology personnel should receive training in assisting clients in proper microbiology test ordering. CMS and Medicare contractor fraud alerts, compliance newsletters, minutes of Compliance Committee meetings, and results of audits and monitors of relevance to microbiology should be used for continuing education at monthly laboratory meetings.

VI. EFFECTIVE LINES OF COMMUNICATION

A. Lines of communication for discussion and reporting of potential compliance issues between employees, the CCO, and other administrative staff should be open, convenient, and anonymous. Microbiology personnel must be made aware of the routes of communication. In no case should any person reporting a violation be ignored or ostracized. Anonymity should be protected, and there should be no retaliation for coming forward.

B. Communication routes should include an anonymous hotline which is posted in the laboratory and the inclusion of compliance topics in regular departmental meeting agendas.

VII. ENFORCING STANDARDS THROUGH DISCIPLINARY GUIDELINES

A. Zero tolerance for error applies to all aspects of adherence to compliance policies.

B. Disciplinary action against an employee found committing fraud should be commensurate with the violation. Employees who make an honest mistake should be counseled and retrained. Employees showing a consistent pattern of unsavory practices should be terminated. All violations must be investigated by the Compliance Committee, which should make a recommendation as to the appropriate disposition of each incident.

C. Hiring of new employees must include a background check to ensure that the individuals have not previously been restricted from providing service in a federal health care program.

VIII. AUDITING AND MONITORING

A. Audits should be conducted on a regular basis whenever necessary to address specific issues brought forward by in-house individuals, or by knowledgeable individuals from outside laboratories and/or consulting firms, to ensure adherence to all written compliance policies. Results should be reviewed by the Compliance Committee and reported to all relevant laboratory sections.

B. Examples of audits applicable to microbiology include the following.

1. List the top 30 codes in the laboratory annually, and evaluate any microbiology codes with a >10% increase.

◩ **NOTE:** The laboratory may also choose to list top codes in microbiology only and look for trends.

2. Select requisitions and review the entire test process for accuracy through ordering, testing, determining test results, and billing. Requisitions may be pulled randomly or in a targeted fashion, but ensure that microbiology CPT codes are evaluated in the process.

3. Establish a method for systematic review of denials for microbiology testing and for evaluation of the root cause(s) for the denials.

4. Establish a tracking system for supplies provided for collection of microbiologic test samples. Ensure that specimens are received on an acceptable percentage basis. An acceptable percentage should be set by each laboratory on the basis of historical data.

C. Establish an action plan for responding to any discrepancies noted in audits, and ensure the audit results and plans are reviewed by the Compliance Committee.

IX. RESPONDING TO COMPLIANCE ISSUES

A. Be familiar with the laboratory plan for investigating and reporting any finding representing overpayment or possible violation.

B. Take each and every issue regarding microbiology practice seriously, and respond in accordance with the institutional overall policies.

X. SUMMARY OF AREAS REQUIRING COMPLIANCE POLICY DEVELOPMENT

- **A. ABN use**
- **B. Ambiguous test orders**
- **C. Anonymity and nonretribution**
- **D. Billing for calculations**
- **E. Claims submission and postsubmission review of explanations of medical benefits and denials**
- **F. Claims submission and presubmission review**
- **G. CLIA regulations**
  - **1.** Client contracts
  - **2.** Provision of and monitoring of client supplies
- **H. Confidentiality of medical information**
- **I. Contracts with third-party billing companies**
- **J. Cost reporting; laboratory component**
- **K. Courier service**
- **L. CPT coding**
- **M. Custom panels and physician acknowledgment**
- **N. Data summaries as a free service**
- **O. Diagnosis information; translating to ICD-9-CM codes**
- **P. Discounts and special prices**
- **Q. Education and training for customers as inducement**
- **R. Employees, including phlebotomists in client offices**
- **S. ESRD arrangements**
- **T. Excused charges and adjustments**
- **U. Fraud alerts; review and compliance**
- **V. Gifts, including contributions and entertainment**
- **W. Health fairs as inducement**
- **X. Home health service client arrangements**
- **Y. ICD-9-CM codes; obtaining and using**
- **Z. Indigent care services**
- **AA. Medical-necessity policy review**
- **BB. Monitoring utilization of laboratory services by clients**
- **CC. Nonemployment of sanctioned individuals**
- **DD. Notices to physicians**
- **EE. Nursing home client arrangements**
- **FF. OSHA regulations**
- **GG. Placement of equipment or products in client offices**
- **HH. Professional courtesy**
- **II. Record retention**
- **JJ. Reflex and composite testing**
- **KK. Release of test results by phone, fax, and/or other nonroutine methods**
- **LL. Removal of hazardous waste for clients as inducement**
- **MM. Rental or lease of space from health care providers**
- **NN. Reporting compliance issues and open-door policy**
- **OO. Requisition design**
- **PP. Sales and marketing**
- **QQ. Sales proposals**
- **RR. Standing orders**
- **SS. Test not ordered and/or not performed**
- **TT. Test ordering by authorized individuals**
- **UU. Test orders, requisitions, and electronic order review**
- **VV. Verbal and add-on test orders**

REFERENCES

1. **Institute of Medicine.** 2000. *Medicare Laboratory Payment Policy: Now and in the Future*. National Academy Press, Washington, DC.
2. **Office of the Inspector General.** 1998. Compliance program guidance for clinical laboratories. *Fed. Regist.* **63:**45076–45087.

APPENDIX 1.2–1

Reflex and Composite Scenarios in Microbiology

I. BACTERIOLOGY

Culture (separate charges for Gram stain, presumptive identification for urine isolates, definitive identification, and antimicrobial susceptibility added if appropriate for source or a potential pathogen is recovered) and ability to note if any of the following applies

A. Gram stain not desired
B. Identification not desired
C. Susceptibility not desired

II. MYCOLOGY

Culture (separate charges for fungal stains, identification, and susceptibility added if appropriate for source or a potential pathogen is recovered) and ability to note if any of the following applies

A. Fungal stain not desired
B. Identification not desired
C. Susceptibility not desired

III. CRYPTOCOCCUS ANTIGEN

Includes additional charge for quantitation if qualitative screen is positive) and ability to note if quantitation is not desired

IV. MYCOBACTERIOLOGY

Culture (separate charges for acid-fast stain, identification, and susceptibility added if appropriate for source or a potential pathogen is recovered) and ability to note if any of the following applies

A. Acid-fast stain not desired
B. Identification not desired
C. Susceptibility not desired

V. VIROLOGY

Culture by tube and shell vial reflex to specific identification, or molecular detection reflex to typing (separate charges for each component) and ability to note if any of the following applies

A. Isolate typing not desired
B. Genotyping or sequencing not desired

VI. PARASITOLOGY

Microscopic analysis includes direct wet mount with concentration and permanently stained smear (e.g., Trichrome). Additional specific requests are appropriate for:

A. *Giardia* direct fluorescent antibody or enzyme immunoassay
B. *Cryptosporidium* direct fluorescent antibody or enzyme immunoassay
C. Other special procedures required to detect unique, relevant pathogens (e.g., microsporidia)

SECTION 2

Specimen Collection, Transport, and Acceptability

SECTION EDITOR: *Andrea J. Linscott*

2.1. Collection, Transport, and Manipulation of Clinical Specimens and Initial Laboratory Concerns

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2.1 Collection, Transport, and Manipulation of Clinical Specimens and Initial Laboratory Concerns

The tables in this section address the initial events that lead to the accurate, rapid identification of microorganisms and viruses. Remember that results can only be as good as the original specimen. All the long-established precautions must be observed, keeping in mind that many specimens are obtained from anatomic sites that encourage specimen contamination with indigenous microbiota and results must be evaluated accordingly (10).

Table 2.1–1 summarizes the microorganisms, including viruses, involved in disease production at various anatomic sites. The term "clinical impression" is used to indicate the most likely diagnosis for the patient condition listed. The corresponding organisms are those most frequently isolated and that demonstrate some involvement with the disease manifestations in the patients (3, 6, 9). This table should be useful in counseling individuals preparing to collect specimens and in alerting laboratory personnel to choose the most appropriate means to ensure the isolation of the most likely etiological agents.

Table 2.1–2 summarizes instructions for the collection of specimens for transport to the laboratory and for bacteriological analyses. These instructions are intended to help health care personnel charged with the responsibility of collecting specimens and to help laboratory personnel in their effort to ensure that only specimens properly obtained, placed in appropriate containers, and of adequate volume are analyzed. Specimens that do not meet collection and transport criteria should be rejected (*see* Table 2.1–7). The excuse most often proffered for inadequate or mishandled specimens is the inability to secure a correct specimen for repeat examination. Decisions about proceeding with analyses under these circumstances are the laboratory director's responsibility and should be referred to these individuals whenever possible. A policy reflecting the laboratory director's opinion on such claims and the consequent action to be taken should be made part of the instruction manual of the laboratory and that provided to the clinical staff. In Table 2.1–2, the column headed "Helpful clinical information" is intended to heighten the awareness of all personnel involved that pertinent clinical information and the patient's history are indispensable in the interpretation of microbiological results. Similarly, the "Comments" column provides additional information and caveats where needed.

Table 2.1–3 addresses the collection of specimens to detect infrequently encountered organisms (10, 11). The column "Organism(s)" lists some of the more commonly requested organisms that are infrequently encountered. Appropriate specimen types are listed in the "Specimen type" column. The "Transport issues and comments" column lists proper transport and storage conditions, possible laboratory safety hazards, and preferred diagnostic test as indicated. This table should guide the clinician and laboratorian for the best possible specimen type and transport conditions for the recovery of these organisms that are not routinely ordered.

Table 2.1–4 provides guidelines for the collection of fungal specimens. Pityriasis versicolor usually is not cultured—microscopic demonstration in scrapings suffices; therefore, scrapings are best submitted in sterile tubes or small sterile containers for direct examination in the laboratory.

Table 2.1–5 lists the requirements for collecting and transporting clinical specimens for parasite examinations. Blood smears for the detection of plasmodia are frequently submitted to the hematology laboratory. Arrangements between the two laboratories are advisable, enabling both to provide input concerning the presence and identity of these protozoa. For blood parasites other than the etiological agents of malaria and babesiosis, note Table 2.1–5 footnote *b* and the appropriate references provided.

A great variety of skin preparations for obtaining blood, CSF, and other specimens have been advocated. Remember, the purpose of such preparations is the elimination of contaminants which can make interpretation of results difficult for the laboratorian and yield misleading results for the clinician.

Table 2.1–6 is an abbreviated listing of commercially available transport media that should be used to ensure the integrity of the specimen from patient to laboratory. Most of these preparations do not require refrigeration if the specimens can be delivered to the laboratory within 24 h (or, in some cases, 48 h). Plastic meshes (polyurethane) rather than cotton on collection swabs provide an effective and nontoxic means for adsorbing, rather than absorbing, microorganisms from clinical material and can be used for the isolation of anaerobic bacteria in casual specimens. For sinus tracts, biopsy specimens, deep surgical or trauma wounds, and body fluids, a variety of excellent transport media are available. Microbiologists who still manufacture their own transport media can consult appropriate sections of this handbook for details. While this reference also addresses the requirements for transporting parasites and ova, even more detailed advice is provided by Garcia (4, 5). Detailed instruction concerning the transport of viral specimens and the requirements for shipping specimens to reference laboratories can be found in this handbook. Likewise, detailed instructions for the collection and transport of clinical specimens needed for molecular analyses can be found in this handbook.

Once a specimen arrives in the microbiology laboratory, personnel must ascertain that all pertinent information has been provided, that the specimen has been collected in the proper transport device, and that all other conditions for an acceptable specimen have been met. The previously mentioned need for a policy of rejecting specimens that do not meet these requirements is expanded in Table 2.1–7 in an effort to provide a sample policy that should be modified by each individual laboratory to address its particular problems. Footnote *b* is a special reminder to all laboratory personnel to be acutely aware that a record of any and all actions concerning the lack of acceptibility of a clinical specimen, including the name and location of the person notified by telephone, must be documented and maintained. Similarly, footnote *c* may seem redundant but is meant to emphasize that only appropriate specimens are suitable for anaerobic cultures. To attempt to use unsuitable specimens for this purpose is a waste of material and labor and will likely provide meaningless results.

Table 2.1–8 outlines an example of how laboratory personnel can proceed to process specimens. Accessioning varies greatly from institution to institution, but the essential steps enumerated in section I of Table 2.1–8 must be followed. Note that each specimen receives an identification number for that particular specimen alone. Specimens obtained from the same patient but of different anatomic origins must be designated separately. Table 2.1–8 does not mention that smears should be prepared for many of the specimens. While this step is not needed universally, smears remain one of the best and most cost-effective means of providing important information almost immediately. The smear also serves as a control for culture results and permits an informed guess as to the cellular responses of immunocompetent patients. The suggestions for media to be used are just that: suggestions. Many laboratory workers prefer to use the media they encountered when first introduced to microbiology. It does not matter which particular medium is used as long as the microorganisms presumed to be present as pathogens or members of the normal microbiota can be separated from the clinical specimen.

The procedure for streaking agar plates is intended to make the experienced laboratorian pause to consider whether shortcuts have been introduced into this procedure with time and to guide the neophyte microbiologist to the easiest way to dilute an inoculum to obtain isolated colonies. Urine is singled out, since some quantitation of the microbial bioload is desirable. Laboratories that use dipstick technology as a prescreen for culture of urine must remember that immunocompromised patients may not meet a microbial challenge with increased segmented neutrophils and thus will not react positively for leukocyte esterase, while many of the microorganisms other than members of the family *Enterobacteriaceae* do not reduce nitrate to nitrite. Microbiologists should keep in mind that a Gram stain of well-mixed, uncentrifuged urine showing two or more bacteria per oil immersion field reflects approximately 100,000 CFU/ml.

Time and again, health care personnel and even an occasional administrator will inquire about "normal values" for microbiology. Confusion and disbelief usually meet the microbiologist's assertion that "normal" in microbiology can be defined only by the isolation or demonstration of an "abnormal" representation of the microbial world, that is, an organism that should not be found in a particular specimen. However, all clinical microbiology personnel must be aware of the urgent need to report the observations and isolations listed in Table 2.1–9. This list may be expanded, shortened, or modified in accordance with existing conditions and the emergence or reemergence of potentially pathogenic microorganisms. Examples of emerging resistance, such as vancomycin-resistant enterococci, vancomycin-intermediate and -resistant staphylococci, penicillin-tolerant pneumococci, and gram-negative rods producing carbapenemases or extended-spectrum beta lactamases, deserve special attention and reporting. In addition, the organisms listed in Table 2.1–10 might be missed unless the laboratory staff is alerted to the clinician's suspicion. This "alert request" should be part of the manual that provides health care personnel with the requirements of the clinical pathology service.

Table 2.1–11 lists the serodiagnostic tests presently available to be performed in-house or to be sent to reference laboratories. Unless a laboratory performs a test routinely (at least once a week on several specimens), it is advisable to enlist the services of an established reference laboratory. It is important that the reference laboratory provide not only reports of expeditiously performed tests but also the values obtained with appropriate controls. These reports should be made part of the patient's record, with clear indications that the test was not performed in the institutional laboratory. Copies of reference laboratory reports should be kept in the microbiology laboratory files. Table 2.1–11 also lists normal values that represent the cutoff points generally accepted for these examinations. However, these interpretations are subject to the instructions on the package insert, the variations reported by the reference laboratory, or the results obtained when the population in a particular geographic locale is studied. It should also be pointed out that commercially available reagents for serologies, particularly those for parasitic diseases, are difficult to interpret and subject to technical error. Instructions provided by reference laboratories concerning the collection and transport for these examinations must be followed to the letter. For some tests, refrigerated storage space for acute- and convalescent-stage specimens should be available unless the reference laboratory indicates that the acute-stage specimen will be held in its facility. If the tests are performed in-house, it is advisable to store serum rather than clotted blood.

Acknowledgments. The tables in this section have been gleaned from the laboratory manuals used by several of the editors and authors who participated in preparing this volume. I am indebted to each and every one of them for their generosity and assistance.

Table 2.1–1 "Rule-out" clinical impressions and potential etiological agents

Clinical impression	Potential etiological agent
Head and neck infections	
Gingivitis	Spirochetes, *Prevotella intermedia, Prevotella oralis*
Periodontitis	*Prevotella melaninogenica,* spirochetes
Juvenile	*Actinobacillus actinomycetemcomitans*
Early onset	Spirochetes, *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans*
Adult	Spirochetes, *Prevotella melaninogenica, Actinobacillus actinomycetemcomitans*
Progressive	*Bacteroides forsythus, Campylobacter rectus*
Peritonsillar abscess (Quinsy)	Polymicrobic: *Streptococcus pyogenes,* aerobic bacteria, plus normal microbiota of the oral cavity
Ludwig's angina	Viridans streptococci, peptostreptococci, *Prevotella melaninogenica, Fusobacterium nucleatum*
Pterygopalatine, infratemporal	Anaerobes, aerobic and facultatively anaerobic gram-negative rods
Para- and retropharyngeal abscesses, temporal fossa infections	Polymicrobic with anaerobes, streptococci, staphylococci, *Enterobacteriaceae*
Septic jugular thrombophlebitis (postanginal sepsis)	Anaerobic streptococci, *Prevotella* spp., *Porphyromonas* spp., viridans streptococci, *Streptococcus pyogenes, Streptococcus pneumoniae, Eikenella corrodens*
Suppurative parotitis	*Staphylococcus aureus,* anaerobes, *Enterobacteriaceae, Pseudomonas aeruginosa,* oral microbiota, *Eikenella corrodens*
Parotitis (viral)	Mumps, coxsackievirus, influenza virus, parainfluenza virus types 1 and 3, lymphocytic choriomeningitis virus, cytomegalovirus
Sinusitis	*Streptococcus pneumoniae, Haemophilus influenzae, Prevotella* spp., *Porphyromonas* spp., *Fusobacterium* spp., *Peptostreptococcus* spp., *Staphylococcus aureus, Veillonella* spp., *Streptococcus pyogenes, Moraxella catarrhalis,* aerobic and facultatively anaerobic gram-negative rods, microsporidia, free-living amebae, rhinovirus, influenza virus, parainfluenza virus, adenovirus (children)
Cranial epidural abscess	*Peptostreptococcus* spp., viridans streptococci, *Streptococcus milleri* group, *Bacteroides* spp. (*Prevotella* spp.), *Enterobacteriaceae, Pseudomonas aeruginosa, Staphylococcus aureus*
Otitis media	*Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Streptococcus pyogenes, Staphylococcus aureus, Pseudomonas aeruginosa*
Chronic suppurative	As above plus *Staphylococcus epidermidis, Candida* spp., *Corynebacterium* spp., *Bacteroides* spp., *Peptostreptococcus* spp.
Pharyngitis	*Streptococcus pyogenes,* groups C and G beta-hemolytic streptococci (rare—*Corynebacterium diphtheriae, Corynebacterium ulcerans, Arcanobacterium haemolyticum, Neisseria gonorrhoeae,* mixed anaerobes [Vincent's angina], *Yersinia enterocolitica, Treponema pallidum*), rhinovirus, coronavirus, adenovirus, influenza viruses A and B, parainfluenza viruses (less common herpes simplex virus [types 1 and 2], coxsackie A virus [types 2, 4, 5, 6, 8, and 10], Epstein-Barr virus, cytomegalovirus, human immunodeficiency virus [HIV])
Laryngitis	*Moraxella catarrhalis, Bordetella pertussis, Bordetella parapertussis, Haemophilus influenzae* (may be secondary invaders), *Mycobacterium tuberculosis, Corynebacterium diphtheriae* (rare—also *Histoplasma capsulatum, Coccidioides immitis, Blastomyces dermatitidis, Candida* spp., *Treponema pallidum,* herpes simplex virus, varicella virus), influenza virus, parainfluenza virus, rhinovirus, adenovirus
Epiglottitis	*Haemophilus influenzae* serogroup b, *Haemophilus parainfluenzae, Streptococcus pneumoniae, Staphylococcus aureus, Streptococcus* spp., non-b *Haemophilus influenzae, Pasteurella multocida*
Tracheitis	*Staphylococcus aureus, Streptococcus pyogenes, Haemophilus influenzae* b
Thyroiditis	
Acute suppurative	Oral microbiota, *Staphylococcus* spp., *Streptococcus pneumoniae,* anaerobes (needle aspiration); in immunocompromised patients, *Pseudoallescheria boydii, Candida* spp., *Aspergillus* spp., *Coccidioides immitis, Actinomyces* spp.
Subacute granulomatous	Mumps virus, rubeola virus, influenza virus, adenovirus, Epstein-Barr virus, coxsackievirus, cytomegalovirus, *Yersinia* spp.
Common cold	>200 different viruses, including different serotypes of adenoviruses, coronaviruses, influenza virus, parainfluenza virus, respiratory syncytial virus, rhinovirus, and enterovirus
Pleuropulmonary infections	
Acute community-acquired pneumonia (adults)	*Streptococcus pneumoniae, Haemophilus influenzae, Staphylococcus aureus, Legionella pneumophila, Legionella* spp., oral anaerobes (aspiration), *Neisseria meningitidis, Moraxella catarrhalis, Mycoplasma pneumoniae* (very rare—*Yersinia pestis, Bacillus anthracis, Francisella tularensis, Pseudomonas pseudomallei*), *Histoplasma capsulatum, Blastomyces dermatitidis, Coccidioides immitis, Actinomyces* spp., *Cryptococcus neoformans, Chlamydophila pneumoniae, Chlamydophila psittaci,* rubeola virus, varicella virus, influenza virus (A, B, C), adenovirus, *Nocardia asteroides, Sporothrix schenckii, Penicillium marneffei, Geotrichum* spp., *Histoplasma duboisii* (rare)

(continued)

Table 2.1–1 "Rule-out" clinical impressions and potential etiological agents *(continued)*

Clinical impression	Potential etiological agent
Pneumonia in immunocompromised host	Members of *Enterobacteriaceae, Pseudomonas* spp., and related obligately aerobic gram-negative rod-shaped bacteria; *Staphylococcus aureus, Streptococcus pneumoniae, Nocardia* spp., *Legionella* spp., *Mycobacterium tuberculosis, Mycobacterium avium-intracellulare, Mycobacterium haemophilum, Aspergillus* spp., zygomycetes, *Cryptococcus neoformans, Curvularia lunata, Fusarium* spp., *Paecilomyces varioti, Candida* spp., *Trichosporon* spp., *Candida glabrata, Pneumocystis jiroveci, Toxoplasma gondii, Strongyloides stercoralis, Cryptosporidium* spp., microsporidia, cytomegalovirus, varicella-zoster virus, herpes simplex virus
Bronchitis	*Haemophilus influenzae, Haemophilus parainfluenzae, Streptococcus pneumoniae, Moraxella catarrhalis, Neisseria* spp., *Klebsiella* spp., *Pseudomonas* spp., obligately aerobic gram-negative rod-shaped bacteria
Aspiration pneumonia (caused by microorganisms)	*Peptostreptococcus* spp., *Fusobacterium nucleatum, Porphyromonas asaccharolytica, Prevotella melaninogenica* (*Enterobacteriaceae, Pseudomonas* spp., and related bacteria in nosocomial aspiration pneumonia)
Lung abscess	*Peptostreptococcus* spp., *Fusobacterium nucleatum, Prevotella melaninogenica, Bacteroides fragilis* group, *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Streptococcus pneumoniae*
Granulomas, cavities, etc.	*Mycobacterium tuberculosis, Nocardia* spp., *Burkholderia pseudomallei, Paracoccidioides brasiliensis, Paragonimus* spp., *Chlamydia trachomatis*
Miscellaneous	*Dirofilaria immitis,* Hantavirus (especially Sin Nombre)
Cardiovascular infections	
Bloodstream infections	*Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus agalactiae, Enterococcus* spp. (especially *E. faecalis* and *E. faecium*), *Streptococcus bovis, Streptococcus* serogroup G, viridans streptococci, *Staphylococcus aureus, Staphylococcus epidermidis,* other *Staphylococcus* spp., *Corynebacterium jeikeium, Listeria monocytogenes, Enterobacteriaceae, Pseudomonas* spp., *Stenotrophomonas maltophilia, Burkholderia cepacia, Acinetobacter* spp., *Flavobacterium* spp., *Haemophilus* spp., *Neisseria* spp., *Moraxella catarrhalis, Bacteroides* spp., *Fusobacterium* spp., *Prevotella* spp., *Porphyromonas* spp., *Peptostreptococcus* spp., other anaerobic bacteria, *Mycobacterium* spp., *Candida* spp., *Hansenula* spp., *Malasezzia furfur* and *Plasmodium* spp., *Leptospira* spp., *Babesia* spp., *Trypanosoma* spp. (rare), *Leishmania donovani* (rare), *Toxoplasma gondii* (rare), *Wuchereria bancrofti* (rare), *Brugia malayi* (rare), *Brugia timori* (rare), *Loa loa* (rare)
Septic shock	*Lactococcus* spp., *Vagococcus* spp. (rare), *Globicatella sanguinis* (rare), *Leuconostoc* spp. (rare), *Aerococcus* spp. (rare), most gram-negative bacteria, *Neisseria meningitidis, Haemophilus influenzae, Salmonella* spp., other *Enterobacteriaceae,* including *Yersinia* spp., *Vibrio* spp., *Pseudomonas* spp., *Streptococcus pneumoniae, Mycobacterium* spp. (steroids; T-cell abnormalities), zygomycetes (diabetes, neutropenia), *Aspergillus* spp., *Candida* spp., *Listeria* spp. (T-cell abnormality), herpes simplex virus (T-cell abnormalities), cytomegalovirus (T-cell abnormalities), varicella-zoster virus (T-cell abnormalities)
Endocarditis	
Native valve	Viridans streptococci (*Streptococcus sanguinis, Streptococcus salivarius, Streptococcus mutans, Streptococcus mitis,* etc.), *Enterococcus faecalis, Enterococcus faecium, Enterococcus durans, Streptococcus bovis* (caveat: colon malignancy), *Streptococcus equinus, Streptococcus pyogenes, Streptococcus agalactiae, Staphylococcus aureus, Staphylococcus lugdunensis,* coagulase-negative staphylococci, *Streptococcus pneumoniae, Neisseria gonorrhoeae, Haemophilus* spp., *Pseudomonas* spp., *Listeria* spp., *Corynebacterium* spp. (*Candida* spp., *Candida glabrata,* and *Aspergillus* spp. can produce native valve endocarditis in severe underlying disease, corticosteroid therapy, prolonged antibiotic use, or cytotoxic therapy)
Intravenous drug abuser	*Staphylococcus aureus, Streptococcus* spp., *Enterococcus* spp., gram-negative rod-shaped bacteria (mostly *Pseudomonas* spp. and *Serratia* spp.), *Candida* spp., *Plasmodium* spp., *Leishmania* spp., *Haemophilus* spp., *Neisseria* spp., anaerobic oral bacteria
Prosthetic valve	
Early	*Staphylococcus epidermidis, Staphylococcus aureus,* aerobic gram-negative rods, fungi (usually *Candida* spp. and/or *Aspergillus* spp.), *Streptococcus* spp., *Enterococcus* spp., *Corynebacterium* spp.
Late	Viridans streptococci, *Staphylococcus* spp., *Enterococcus* spp. (staphylococci, gram-negative rods, fungi, and corynebacteria are isolated in earlier infections occurring at <18 months)
Vascular graft infections	*Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus* spp., *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa,* anaerobic bacteria, *Candida* spp.

Table 2.1–1 *(continued)*

Clinical impression	Potential etiological agent
Pericarditis, myocarditis	*Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogenes, Escherichia coli, Proteus* spp., *Pseudomonas aeruginosa, Salmonella* spp., *Shigella* spp., *Neisseria meningitidis, Histoplasma capsulatum, Candida* spp., *Aspergillus* spp., *Trypanosoma cruzi,* coxsackievirus (especially B), echoviruses, *Mycobacterium tuberculosis, Echinococcus granulosum, Entamoeba histolytica, Toxoplasma gondii, Trichinella spiralis, Mycoplasma pneumoniae*
Fevers (viral)	Lassa virus
Transfusion-associated sepsis	*Babesia microti, Bacillus cereus, Borrelia* spp., *Campylobacter jejuni,* Colorado tick fever viruses, cytomegalovirus, Epstein-Barr virus, hepatitis viruses, HIV, *Leishmania* spp., parvovirus B19, *Plasmodium* spp., *Pseudomonas fluorescens/putida, Salmonella* spp., *Serratia marcescens,* coagulase-negative staphylococci, *Toxoplasma gondii, Treponema pallidum, Trypanosoma cruzi, Yersinia enterocolitica*
Intestinal tract infections	
Diarrhea	*Shigella* spp., *Salmonella* spp., *Vibrio cholerae, Escherichia coli, Giardia lamblia, Campylobacter* spp., *Entamoeba histolytica, Dientamoeba fragilis,* rotavirus, norovirus, toxins of *Clostridium difficile, Staphylococcus aureus, Bacillus cereus, Clostridium perfringens, Yersinia enterocolitica, Plesiomonas shigelloides, Cryptosporidium* spp., *Isospora belli, Sarcocystis hominis, Cyclospora cayetanensis, Encephalitozoon* spp., *Nosema* spp., *Enterocytozoon* spp., *Pleistophora* spp., *Microsporidium* spp., *Balantidium coli, Vibrio parahaemolyticus, Blastocystis hominis*
Salmonellosis	
Gastroenteritis	*Salmonella enterica* serovar Typhi and *S. enterica* subsp. *enterica*
Enteric fever	*Salmonella enterica* serovars Typhi, Paratyphi A, other *Salmonella* spp., *Burkholderia pseudomallei*
Bacteremia	*Salmonella enterica* serovars Typhimurium, Cholavasuis, Heidelberg, other *Salmonella* serovars
Carrier state	*Salmonella* serovars
Escherichia coli diarrheagenic infections	Enterotoxigenic *E. coli,* common O serogroups (6, 8, 15, 20, 25, 27, 63, 78, 80, 114, 115, 128ac, 148, 153, 159, 167); enteropathogenic *E. coli,* common O serogroups (55, 86, 111, 119, 125, 126, 127, 128ab, 142); enteroadherent *E. coli,* significance not determined; enteroinvasive *E. coli,* common O serogroups (28ac, 29, 112, 124, 136, 143, 144, 152, 164, 167); enterohemorrhagic *E. coli,* common O serogroups (157:H7; also implicated, 26:H11, 111:H8, 103:H2, 113:H21, 104:H21); enteroaggregative *E. coli,* common O serogroups (3:H2, 44:H18) (NOTE: flagellar antigen designation provided only when required for pathogenic manifestation)
Intestinal campylobacteriosis (diarrhea)	*Campylobacter jejuni, Campylobacter coli, Campylobacter lari, Campylobacter upsaliensis, Campylobacter fetus*
Gastric/duodenal ulcers	*Helicobacter pylori*
Viral gastroenteritis	Rotavirus, norovirus, adenovirus (enteric), astrovirus, calicivirus, norovirus-like viruses
Food poisoning (food as vehicle and/or contains toxins)	*Bacillus cereus, Campylobacter* spp., *Clostridium perfringens, Salmonella* spp., *Shigella* spp., *Staphylococcus aureus, Clostridium botulinum, Escherichia coli, Vibrio parahaemolyticus, Yersinia enterocolitica, Listeria monocytogenes, Giardia lamblia,* microsporidia, *Entamoeba histolytica, Anisakis* spp. (raw saltwater fish), *Taenia saginata, Taenia solium, Diphyllobothrium latum,* lung and liver trematodes, *Trichinella spiralis,* hepatitis A virus, norovirus
Intra-abdominal infections	*Escherichia coli, Klebsiella* spp., *Proteus* spp., *Enterobacter* spp., *Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus* spp., *Bacteroides* spp., *Fusobacterium* spp., *Veillonella* spp., *Peptostreptococcus* spp., *Propionibacterium* spp., *Staphylococcus* spp.
Intra-abdominal abscess, including appendicitis, diverticulitis	*Escherichia coli, Bacteroides fragilis, Enterococcus* spp., *Peptostreptococcus* spp., *Clostridium* spp., *Proteus* spp., *Fusobacterium* spp., *Klebsiella* spp., *Pseudomonas* spp., aerobic gram-negative rod-shaped bacteria, *Staphylococcus* spp., *Eubacterium* spp., *Streptococcus pyogenes, Streptococcus* spp., *Angiostrongylus costaricensis*
Liver abscess/infections	*Streptococcus* spp., *Escherichia coli, Proteus* spp., *Peptostreptococcus* spp., *Fusobacterium* spp., *Bacteroides* spp., *Entamoeba histolytica, Leishmania donovani,* microsporidia, hepatitis viruses (A–E)
Liver granulomata	*Mycobacterium tuberculosis, Mycobacterium* spp., *Brucella* spp., *Francisella tularensis, Histoplasma capsulatum, Coccidioides immitis, Coxiella burnetii, Treponema pallidum* (secondary syphilis), *Echinococcus* spp., *Schistosoma* spp., cytomegalovirus, Epstein-Barr virus
Bile duct infections	*Clonorchis sinensis, Opisthorchis felineus, Clostridium* spp., *Cryptosporidium* spp.
Pancreatic infections	*Escherichia coli, Enterococcus* spp., *Staphylococcus* spp., *Klebsiella* spp., *Proteus* spp., *Candida* spp., *Pseudomonas* spp., *Streptococcus* spp., *Candida glabrata, Haemophilus* spp., *Corynebacterium* spp., *Serratia marcescens*

(continued)

Table 2.1–1 "Rule-out" clinical impressions and potential etiological agents *(continued)*

Clinical impression	Potential etiological agent
Splenic abscess	*Staphylococcus* spp., *Salmonella* spp., *Escherichia coli, Enterococcus* spp., *Streptococcus* spp., *Klebsiella* spp., *Enterobacter* spp., *Proteus* spp., *Pseudomonas* spp., *Corynebacterium* spp., *Shigella* spp., *Bacteroides* spp., *Propionibacterium* spp., *Clostridium* spp., *Fusobacterium* spp., *Candida* spp., *Aspergillus* spp., *Leishmania donovani,* microsporidia, *Blastomyces dermatitidis*
Intestinal/abdominal parasites	*Enterobius vermicularis, Fasciola hepatica* (liver), *Fasciola gigantica* (liver), *Fasciolopsis buski, Necatur americanus, Ancylostoma duodenale, Ancylostoma caninum* (rare), *Hymenolepis nana, Hymenolepis diminuta, Dypilidium caninum*
Genitourinary tract infections	
Urinary tract infections	*Escherichia coli, Staphylococcus saprophyticus, Proteus* spp., *Klebsiella* spp., *Enterococcus* spp., *Pseudomonas* spp., *Candida* spp., *Staphylococcus* spp.
Urethritis	
Male	*Neisseria gonorrhoeae, Chlamydia trachomatis, Ureaplasma urealyticum*
Female	*Escherichia coli, Staphylococcus saprophyticus, Chlamydia trachomatis, Ureaplasma urealyticum*
Prostatitis	*Escherichia coli,* other *Enterobacteriaceae, Pseudomonas* spp., *Staphylococcus aureus, Enterococcus* spp., *Trichomonas vaginalis*
Epididymitis	*Chlamydia trachomatis, Neisseria gonorrhoeae, Enterobacteriaceae, Pseudomonas* spp.
Renal abscess	
Cortical	*Staphylococcus aureus*
Corticomedullary	*Escherichia coli, Klebsiella* spp., *Proteus* spp.
Perinephric	*Staphylococcus aureus, Escherichia coli, Proteus* spp., *Klebsiella* spp., *Pseudomonas* spp., *Streptococcus* spp., *Enterococcus* spp., *Mycobacterium* spp., *Bacteroides* spp., *Candida* spp.
Sexually transmitted diseases (STD)	
Acute pelvic inflammatory disease	*Neisseria gonorrhoeae, Chlamydia trachomatis, Mycoplasma hominis, Peptostreptococcus* spp., *Bacteroides* spp., *Enterobacteriaceae*
Neonatal/perinatal complications of STD	*Neisseria gonorrhoeae, Chlamydia trachomatis,* cytomegalovirus, herpes simplex virus, *Ureaplasma urealyticum, Mycoplasma hominis*
Potential factors in neoplasia	Human papillomavirus, hepatitis B virus, HIV, herpes simplex virus
Mucopurulent cervicitis	*Chlamydia trachomatis, Neisseria gonorrhoeae,* herpes simplex virus
Vaginitis, vulvovaginitis	*Trichomonas vaginalis, Candida* spp., *Bacteroides* spp., *Prevotella bivia, Prevotella disiens, Prevotella* spp., *Actinomyces* spp., *Peptostreptococcus* spp., *Eubacterium nodatum, Mobiluncus* spp. (*Corynebacterium diphtheriae*—rare)
Vaginosis	*Gardnerella vaginalis, Mycoplasma hominis, Mobiluncus* spp.
Genital ulcers with lymphadenopathy	*Treponema pallidum, Haemophilus ducreyi, Chlamydia trachomatis* (lymphogranuloma venereum strains), *Calymmatobacterium granulomatis,* herpes simplex virus
Endometritis	*Chlamydia trachomatis, Neisseria gonorrhoeae* (*Streptococcus agalactiae, Mycoplasma hominis* isolated from cervix)
Amniotic fluid infections	*Ureaplasma urealyticum, Mycoplasma hominis, Bacteroides* spp., *Gardnerella vaginalis, Streptococcus agalactiae, Peptostreptococcus* spp., *Escherichia coli, Enterococcus* spp., *Fusobacterium* spp., *Bacteroides* spp.
Skin and soft tissue infections	
Impetigo	*Streptococcus pyogenes, Staphylococcus aureus* (*Corynebacterium diphtheriae*—rare)
Echthyma	*Streptococcus pyogenes, Staphylococcus aureus*
Blistering distal dactylitis	*Streptococcus pyogenes, Staphylococcus aureus*
Foliculitis	*Staphylococcus aureus*
Euruncles and carbuncles	*Staphylococcus aureus*
Erythema migrans	*Borrelia burgdorferi*
Erysipelas	*Streptococcus pyogenes* (serogroup G, C, and B streptococcus—rare)
Cellulitis, acute	*Streptococcus pyogenes, Staphylococcus aureus* (*Haemophilus influenzae, Enterobacteriaceae, Clostridium* spp., *Bacillus anthracis, Pasteurella multocida, Erysipelothrix* spp., *Aeromonas hydrophila, Vibrio vulnificus, Mycobacterium* spp.—rare), *Vibrio alginolyticus,Vibrio damsela, Helcococcus* spp.
Fasciitis, necrotizing	*Streptococcus pyogenes* or synergistic infection by facultative and anaerobic bacteria, *Apophysomyces elegans*
Nodules, papules, subcutaneous, tissue involvement and sinus, tracts, other skin manifestations	*Mycobacterium leprae, Loa loa, Wuchereria bancrofti, Onchocerca volvulus, Mansonella perstans, Mansonella streptocerca,* molluscipox virus, *Nocardia brasiliensis, Nocardia asteroides,* other *Nocardia* spp., *Actinomadura* spp., *Nocardiopsis dassonvillei, Streptomyces somaliensis, Madurella* spp., *Pseudoallescheria boydii,* other fungi, *Treponema carateum, Yersinia pestis, Ancylostoma brasielensis, Ancylostoma caninum, Gnathostoma spiniserum*

Table 2.1–1 *(continued)*

Clinical impression	Potential etiological agent
Fournier's gangrene	*Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Enterococcus* spp., *Staphylococcus* spp., *Peptostreptococcus* spp., *Bacteroides* spp.
Abscess, cutaneous	
Axilla, paronychia, breast, hands, head, neck, and trunk	*Staphylococcus aureus, Staphylococcus epidermidis, Propionibacterium* spp., *Peptostreptococcus* spp., *Leishmania tropica* complex, *Leishmania braziliense* complex
Perineal, vulvovaginal, scrotal, perianal, and buttocks	*Staphylococcus aureus, Staphylococcus epidermidis, Propionibacterium* spp., *Peptostreptococcus* spp., *Streptococcus* spp.
Myositis	
Bacterial	*Staphylococcus aureus, Streptococcus* spp., influenza virus, coxsackievirus, Epstein-Barr virus, herpes simplex virus type 2, parainfluenza virus type 3, adenovirus type 21, echovirus type 9
Protozoan	*Toxoplasma gondii, Trypanosoma* spp., microsporidia
Tinea favosa	*Trichophyton schoenleinii* (*Trichophyton mentagrophytes, Trichophyton verrucosum, Microsporum canis*—rare)
Piedra	
White	*Trichosporon beigelii* (Indonesia, South America, Far East)
Black	*Piedraia hortae* (tropical South America, Africa, Pacific Islands, Far East)
Tinea corporis (ringworm)	*Trichophyton* spp., *Microsporum* spp., *Epidermophyton* spp.
Tinea imbricata	*Trichophyton rubrum, Trichophyton mentagrophytes, Epidermophyton floccosum*
Onychomycosis	
Dermatophytic	*Trichophyton rubrum, Trichophyton mentagrophytes* (*Epidermophyton* spp., *Microsporum* spp., *Scytalidium* spp.—rare)
Nondermatophytic	*Candida albicans, Geotrichum candidium, Scopulariopsis brevicaulis, Cephalosparium* spp., *Acremonium* spp.
Chromomycosis (dornatoblastomycosis, dermatitis verrucosa)	*Phialophora verrucosa, Fonsecaea pedrosii, Fonsecaea computa, Cladiosporium carrionii, Rhinocladiella aquaspersa, Botryomyces caespiosus, Exophiala spinifera, Exophiala jeanselmei*
Viral skin infections	Rubeola rubella virus, varicella virus, herpes simplex virus (types 1 and 2), herpes zoster virus, papillomavirus (warts), parvovirus (erythema infectiosum), human herpesvirus 6-B (roseola); enteroviral exanthems—hand-foot-mouth syndrome, coxsackievirus A (select types); other types of rashes—echovirus and coxsackievirus, maculopapular; coxsackievirus and echovirus, petechial (caveat: hard to differentiate from meningococcemia rash) (maculopapular rash—Marburg virus, Ebola virus, parapoxviruses [very rare])
Fungal and parasitic soft tissue/skin infections	*Dracunculus medinensis, Dirofilaria tenui, Dirofilaria ursi, Dirofilaria repens, Brugia* spp. (lymph nodes), *Onchocerca volvulus, Madurella* spp., *Pseudoallescheria boydii, Scedosporium apiospernum, Exophiala jeanselmei, Acremonium* spp., *Paracoccidioides brasiliensis, Acanthamoeba* spp.
Animal bites	*Acinetobacter* spp., *Actinobacillus actinomycetemcomitans, Bacteroides* spp., *Capnocytophaga* spp., *Corynebacterium* spp., *Eikenella corrodens, Enterococcus* spp., *Fusobacterium* spp., *Haemophilus aphrophilus, Micrococcus luteus, Moraxella* spp., *Neisseria canis, Neisseria weaveri, Pasteurella* spp., *Peptostreptococcus* spp., *Porphyromonas* spp., *Prevotella* spp., *Staphylococcus aureus, Staphylococcus intermedius, Staphylococcus epidermidis, Streptococcus* spp., *Veillonella parvula*
Burn wounds	*Acinetobacter baumannii, Enterobacter cloacae, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis*
Trauma-associated wounds	Anaerobes, *Aspergillus* spp., *Enterobacteriaceae, Fusarium* spp., *Pseudomonas* spp., *Staphylococcus* spp., *Streptococcus* spp., zygomycetes, *Acanthamoeba* spp.
Bone and joint infections	
Osteomyelitis	
Hematogenous	*Staphylococcus aureus, Staphylococcus* spp., *Streptococcus agalactiae, Candida* spp., *Pseudomonas aeruginosa, Salmonella* serovars, *Burkholderia pseudomallei, Echinococcus* spp.
Trauma associated	*Streptococcus* spp., *Propionibacterium* spp., *Enterobacteriaceae, Pseudomonas* spp., *Staphylococcus* spp., anaerobic bacteria
Vascular insufficiency	*Enterobacteriaceae,* anaerobic bacteria
Septic arthritis, nongonococcal arthritis	*Neisseria gonorrhoeae, Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumoniae, Streptococcus* spp., *Enterococcus* spp., *Enterobacteriaceae,* obligately aerobic gram-negative rod-shaped bacteria

(continued)

Table 2.1–1 "Rule-out" clinical impressions and potential etiological agents *(continued)*

Clinical impression	Potential etiological agent
Rare arthritides	*Brucella* spp., *Salmonella* spp., rubella virus, mumps virus, coxsackievirus, echovirus (transient), parvovirus B19, hepatitis B virus (immune response), *Mycobacterium* spp., *Legionella* spp., *Fusobacterium necrophorum*
Infections of the eye	
Eyelid	*Staphylococcus aureus*, herpes simplex virus, varicella virus, papillomavirus, *Trichophyton* spp., *Microsporum* spp., *Trichosporon* spp.
Styes	*Staphylococcus aureus*
Chalazion	*Staphylococcus aureus, Moraxella lacunata*
Conjunctivitis	
Purulent	*Neisseria gonorrhoeae, Neisseria meningitidis* (newborn); *Chlamydia trachomatis* serovars D–K, *Staphylococcus aureus, Streptococcus pyogenes, Streptococcus pneumoniae, Haemophilus influenzae* (*Haemophilus aegyptius*), *Pseudomonas aeruginosa, Escherichia coli, Moraxella* spp., *Corynebacterium diphtheriae,* feline strains of *Chlamydophila psittaci*
Chronic	*Moraxella lacunata, Staphylococcus* spp.
Parinaud's oculoglandular	*Bartonella henselae* (or possibly *Afipia felis* [both possibly agents of cat scratch disease]), *Lymphogranuloma venereum, Mycobacterium tuberculosis, Treponema pallidum, Haemophilus ducreyi, Francisella tularensis,* Epstein-Barr virus, mumps virus
Viral	Adenovirus type 8, 19, or 37, usually causing keratoconjunctivitis severe disease types 8, 5, and 19; serotypes 3, 7, and 4—pharyngoconjunctival fever; herpes simplex viruses, varicella-zoster virus, Epstein-Barr virus, cytomegalovirus, rubeola virus, mumps virus, influenza virus, Newcastle disease virus (paramyxovirus)
Corneal infections	
Bacterial keratitis	*Staphylococcus aureus, Staphylococcus* spp., *Streptococcus pneumoniae, Streptococcus* spp., *Pseudomonas aeruginosa, Bacillus cereus, Enterobacteriaceae, Neisseria* spp., *Moraxella lacunata, Mycobacterium fortuitum, Mycobacterium chelonei,* anaerobes
Fungal keratosis	*Fusarium solani, Candida albicans, Aspergillus fumigatus, Alternaria* spp., *Curvularia* spp., *Acremonium* spp.
Viral keratosis	Herpes simplex virus, varicella-zoster virus
Protozoan keratitis	*Acanthamoeba* spp.
Lacrimal system infections	
Dacryoadenitis	*Staphylococcus aureus, Streptococcus* spp., *Neisseria gonorrhoeae, Mycobacterium tuberculosis, Treponema pallidum,* mumps virus, Epstein-Barr virus
Canaliculitis	*Actinomyces israelii, Streptococcus* spp., *Candida* spp., *Aspergillus* spp., herpes simplex virus, varicella-zoster virus
Dacryocystitis	*Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae, Pseudomonas aeruginosa, Proteus mirabilis, Candida albicans, Aspergillus* spp.
Retina and choroid infections	Cytomegalovirus, *Toxoplasma gondii*
Endophthalmitis	
Exogenous (surgical or nonsurgical trauma)	*Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus* spp., *Bacillus* spp., *Pseudomonas* spp., *Enterobacteriaceae, Haemophilus influenzae, Propionibacterium* spp.
Endogenous	*Staphylococcus aureus, Neisseria meningitidis, Streptococcus* spp., *Bacillus cereus, Enterobacteriaceae, Pseudomonas aeruginosa, Nocardia asteroides*
Fungal (endogenous)	*Candida albicans, Aspergillus fumigatus, Aspergillus flavus*
Fungal (exogenous)	*Candida* spp., *Aspergillus* spp., *Cephalosporium* spp., *Penicillium* spp., *Curvularia* spp.
Orbital infections	*Staphylococcus aureus, Streptococcus* spp., *Peptostreptococcus* spp., *Pseudomonas aeruginosa, Haemophilus influenzae, Mycobacterium* spp., zygomycetes, *Aspergillus* spp., *Echinococcus* spp., *Taenia solium*
Nervous system infections	
Acute bacterial meningitis	*Neisseria meningitidis, Streptococcus pneumoniae, Streptococcus agalactiae, Listeria monocytogenes, Escherichia coli, Enterobacteriaceae, Leptospira* spp., *Haemophilus influenzae, Staphylococcus aureus;* less commonly, *Peptostreptococcus* spp., *Fusobacterium necrophorum, Prevotella melaninogenica, Bacteroides fragilis, Clostridium perfringens;* rarely (zoonotic) *Brucella* spp., *Francisella tularensis, Streptococcus suis, Yersinia pestis*
Acute viral meningitis	Coxsackievirus, echovirus, poliovirus, herpes simplex viruses 1 and 2, varicella-zoster virus, flaviviruses (St. Louis encephalitis), mumps virus, bunyaviruses (California group, La Crosse), rubeola virus, lymphocytic choriomeningitis virus, adenoviruses

Table 2.1–1 *(continued)*

Clinical impression	Potential etiological agent
Chronic meningitis	*Mycobacterium tuberculosis, Brucella* spp., *Francisella tularensis, Listeria monocytogenes* (rare), *Neisseria meningitidis* (rare), *Tropheryma whipplei* (rare), *Borrelia burgdorferi, Leptospira* spp. (rare), *Treponema pallidum, Actinomyces* spp., *Nocardia* spp., *Cryptococcus neoformans, Coccidioides immitis* (rare), *Histoplasma capsulatum* (rare), *Candida* spp., *Aspergillus* spp. (rare), zygomycetes (rare), *Parastrongyloides cantonensis*, lymphocytic choriomeningitis virus, mumps virus, herpes simplex virus, varicella-zoster virus, arbovirus, flavivirus, echovirus, parasites (rare), *Acanthamoeba* spp.
Brain abscess	*Streptococcus* spp., *Peptostreptococcus* spp., *Porphyromonas* spp., *Bacteroides* spp., *Prevotella* spp., *Fusobacterium* spp., *Staphylococcus aureus, Enterobacteriaceae, Burkholderia cepacia, Streptococcus pneumoniae, Neisseria meningitidis, Haemophilus influenzae, Listeria monocytogenes, Haemophilus aphrophilus, Actinomyces* spp., *Nocardia* spp., zygomycetes, *Mycobacterium* spp., *Naegleria* spp. (primary meningoencephalitis), *Acanthamoeba* spp., *Balamuthia mandrillaris* (granulomatous encephalitis), *Taenia solium*
Spinal cord, peripheral and cranial nerves	Poliomyelitis virus, herpesvirus simiae, human immunodeficiency virus type 1, human T-lymphotrophic virus type 1; myelitis associated—cytomegalovirus, herpes simplex virus; following infection with rubeola virus, varicella-zoster virus, influenza virus, mumps virus; *Borrelia burgdorferi, Borrelia recurrentis, Chlamydophila* spp.
Epidural abscess	*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Streptococcus* spp., *Peptostreptococcus* spp., *Salmonella* serovars, *Staphylococcus* spp., *Nocardia* spp., *Actinomyces* spp., *Fusobacterium* spp., *Mycobacterium* spp., *Aspergillus* spp., *Brucella* spp., *Treponema pallidum*
Shunt-associated infections	*Enterobacteriaceae, Enterococcus* spp., *Neisseria meningitidis, Propionibacterium acnes, Pseudomonas* spp., *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus warneri, Streptococcus* spp.
Ear infections	
Otitis externa	*Staphylococcus aureus, Propionibacterium acnes, Pseudomonas aeruginosa*
Otitis media	*Streptococcus pneumoniae, Haemophilus influenzae, Streptococcus pyogenes, Staphylococcus aureus, Moraxella catarrhalis, Pseudomonas aeruginosa, Alloiococcus otitidis*, respiratory syncytial virus, influenza virus, enteroviruses, rhinoviruses, *Chlamydia trachomatis* (infants <6 months old) (*Corynebacterium diphtheriae, Mycobacterium tuberculosis, Mycobacterium chelonei, Clostridium tetani, Ascaris lumbricoides*—rare)
Encephalitides (7)	
Viral	
Mosquito borne	Japanese encephalitis (JE), western equine encephalitis (WEE), eastern equine encephalitis (EEE), St. Louis encephalitis (SLE), Murray Valley (MV) encephalitis (Australia), La Crosse, California, Rocio encephalitis, Jamestown Canyon, snowshoe hare viruses
Tick borne	Far-Eastern (Russian spring/summer), central European, louping ill, powassan (member of *Flaviviridae*) viruses
	EEE and WEE (*Togaviridae*), JE, Kunjin, MV encephalitis, SLE, and Rocio encephalitis viruses (*Flaviviridae*), La Crosse, California, Jamestown Canyon, snowshoe hare viruses (*Bunyaviridae*)
Spongiform	Creutzfeldt-Jakob, Creutzfeldt-Jakob variant bovine spongiform encephalitis—prions; *Paramyxoviridae* (Hendra and Nipah viruses)
Meningoencephalitis	*Naegleria fowleri*
Granulomatous encephalitis	*Acanthamoeba* spp., *Balamuthia mandrillaris*
Infections caused by rather recently recognized etiological agents that may or may not be cultured in routine microbiology laboratories	
Bartonellosis	*Bartonella bacilliformis*
Chromobacterium bacteremia	*Chromobacterium violaceum*
Strawberry foot rot (exudative, scabbing dermatitis)	*Dermatophilus congolensis*
Bacterial vaginosis	*Gardnerella vaginalis, Mobiluncus* spp., *Mycoplasma* spp.
Legionellosis and related diseases	*Legionella* spp.
Chronic nodular skin lesions (joint infections, wound infections)	*Prototheca* spp.
Occasional endocarditis, dialysis	*Stomatococcus mucilaginosus*
Rat bite fever (Haverhill fever)	*Streptobacillus moniliformis*
Rat bite fever (Sodoku)	*Spirillum minus* (Wright stain/dark field)
Cat scratch fever	*Bartonella henselae* (*Afipia felis*)
Rhinosporidiosis	*Rhinosporidium seeberi* (epithelial cell tissue culture)
Lobomycosis	*Loboa loboi* (histological diagnosis)
Whipple's disease	*Tropheryma whipplei*
Erythema infectiosum (fifth disease)	Parvovirus B19
Hemorrhagic fever	Hantavirus (pulmonary and renal [outside northeast Asia])

Table 2.1–2 Collection of specimens for bacteriological analysis[a,b]

Specimen	Preparation	No., type, volume	Container	Helpful clinical information	Comments
Anaerobic cultures					
Actinomycosis	Decontaminate skin.	Aspirated pus	Anaerobic TM	History of "lumpy jaw"	Fistulating chronic infection often in the neck, jaw, and upper chest area.
Body fluids					
Secretions, pus	Decontaminate skin.	>1 ml[c]	Anaerobic TM	Foul-smelling discharge, abdominal surgery, abscess aspirate	Do not refrigerate; immediately transport to laboratory.
Respiratory tract	Transtracheal aspirate, pleural or empyema fluid only	>1 ml	Anaerobic TM	Foul-smelling sputum, history of aspiration	Do not submit sputum.
Tissues	Surgery	1 cm if possible	Anaerobic TM	Clinical impression	Do not add fluid. Larger specimens (>1 cm) tolerate short exposure to air.
Autopsy material					
Blood	Best collected before body is handled too much or opened. Decontaminate skin or sear surface of heart or other organ before inserting needle or cutting out tissue block.	10 ml of right-heart blood	Sterile tube, vacutainer with anticoagulant, lysis centrifugation tube (Isolator)	Clinical diagnosis, postmortem interval, autopsy impression, previous positive cultures, suspected infection	Autopsy cultures are often contaminated with bacteria from the water faucet and with enteric bacteria.
Tissue	Same as above	6 cm^3 (if possible) with one serosal or other surface; this large size is preferred because aseptic collection of smaller fragments is difficult.	Sterile container		A block of spleen tissue may be submitted in lieu of a blood culture. (*Note:* Coccidiomycosis and tuberculosis are often discovered at autopsy only.[d,e,f,g])
Blood					
Peripheral	Skin decontamination with 70% alcohol followed by povidone iodine or chlorhexidine	10 ml per bottle (adults and older child); 1–2 ml (infants); 3 samples per 24 h or 4–6 for FUO	Culture bottle for direct inoculation; vacutainer with SPS; Isolator tube; designated containers for automated instruments		Clinical diagnosis, antimicrobics and chemotherapy, immune status. Inoculate and incubate as soon as possible.
Bone marrow	Same as above	≥1 ml	Same as above		Direct smears should be made. Recommended by many authorities for diagnosis of systemic histoplasmosis and for other fungus infections, also for diagnosis of miliary TB, brucellosis.

Table 2.1–2 *(continued)*

Specimen	Preparation	No., type, volume	Container	Helpful clinical information	Comments
Body fluids (other than blood, urine, CSF)					Body fluids may be injected directly into blood culture bottles or can be used in addition to plated media if the blood culture bottles have been validated for use.
Bile	Surgery or decontaminate skin area before aspiration	Several ml (first ml from post-op drain site often contains contaminants)	Sterile container, TM	Consider viruses, fungi, and parasites for analysis.	Sample may contain gallstones, which should be examined; duodenal aspirates sometimes submitted for special tests.
Hematomas	Skin decontamination	Several ml	Sterile tube, vacutainer, TM	Suspected abscess	May clot; when in doubt, use anticoagulant (SPS).
Joint fluid	Same as above	Several ml	Same as above	History of trauma, previous surgery, or infection; consider GC chlamydia, etc., in analysis.	Often proteinaceous; may clot. Do not add acetic acid or other fluid which may precipitate protein; this makes cell evaluation impossible. Distilled sterile water is acceptable.
Pericardial fluid	Same as above	Several ml	Sterile jar, tube, TM	History of TB or previous surgery	Consider viral etiology, especially coxsackievirus.
Peritoneal fluid	Same as above	Several ml or more	Sterile jar, tube, TM	History of TB, surgery, or cancer	Same as for joint fluids; consider also GC; specimen may be peritoneal dialysis fluid.
Pleural fluid	Same as above	Several ml or more	Sterile tube, jar, TM	Same as above	
Breast milk	Skin decontamination of nipple	Several ml; first few may be contaminated.	Sterile tube, jar, TM	Suspected abscess	Often submitted for presence of *Staphylococcus aureus* and/or hemolytic streptococci.
Catheter tips					
Foley catheter		Not recommended			Foley catheters or tips should not be cultured except unused, as sterility check.
Vascular cannulae, venous access devices, arterial lines	Skin decontamination; careful aseptic removal mandatory	Segment near skin and tip end should be used; use sterile scissors to cut.	Sterile jar or tube	History of local infection, signs and symptoms	Peripheral blood cultures help with interpretation.
Central nervous system, brain biopsy	Surgery	See tissues (under "Autopsy material" above).	As for aerobic and anerobic cultures	Suspected abscess, cryptococcosis, toxoplasmosis, etc.	Needs coordination with Pathology. Include viruses in diagnostic analysis.

(continued)

Table 2.1–2 Collection of specimens for bacteriological analysis[a,b] *(continued)*

Specimen	Preparation	No., type, volume	Container	Helpful clinical information	Comments
CSF	Skin decontamination	Several ml if possible	Sterile, clean, screw-cap tube	Tentative clinical diagnosis and/or suspicion	Since culture yields more from a larger volume, an alternate method is to aseptically pool all tubes collected (after cell count). The supernatant goes to Chemistry and Serology, with aliquot reserved for antigen detection when indicated. Obtain smears from precipitate.
Eye					
Internal	Surgery	Volume of specimen often suboptimal	Sterile tube	History of trauma or postoperative infection	Since specimen is usually small and obtained under great difficulty, speed in transport and care in handling are very important.
External	Cleanse skin around eye with mild antiseptic. Gently remove makeup and ointment with sterile cotton and saline.	For most cases, moistened swabs are used. For diagnosis of viral or chlamydial infections and for cytology, conjunctival and/or corneal scrapings are necessary. Make two slides per lesion.	Moist sterile swabs in sterile tubes with a small amount of nutrient broth; alcohol-cleaned glass slides for scrapings; sterile tube for scrapings to be cultured (AFB, fungi)	History and suspected agent, e.g., bacterial, fungal, AFB, inclusion bodies (viral or chlamydial), GC only, allergic	Handle carefully; inoculate at bedside (or office) as culture for *Acanthamoeba* on special media. Transport to laboratory immediately. Often only a few microorganisms present. Scraping should be done by ophthalmologist. Consult with physician about use of terms or the handling of the specimen (CD-RIGHT EYE, OS-LEFT EYE). Giemsa and Gram stains are frequently requested.
Genital tract–female					
Amniotic fluid		Uncontaminated fluid	Sterile tube	Premature rupture of membranes >24 h	Treat as any other normally sterile body fluid; may contain *Neisseria gonorrhoeae.*
Cervix (endocervix)	Wipe cervix clean of vaginal secretion and mucus. Use speculum and no lubricant.	Uncontaminated endocervical secretions; take two swabs.	Sterile container with TM; appropriate collection containers for demonstration of specific organisms, such as *N. gonorrhoeae, Chlamydia,* HSV, etc.	Venereal disease, postpartum infection	Viability of *N. gonorrhoeae* held in Amies or modified Stuart TM decreases substantially after several h.
Cul de sac (culdocentesis)	Surgical procedure	Fluid, secretions	See "Anaerobic cultures," STD diagnosis (*see* Table 2.1–1).	Venereal disease; pelvic inflammatory disease	Pelvic inflammatory disease

Table 2.1–2 *(continued)*

Specimen	Preparation	No., type, volume	Container	Helpful clinical information	Comments
Endometrium	As for cervix	Curettings or aspiration	Sterile container, anaerobic TM	Postpartum fever, venereal disease	Likelihood of external contamination is high for cultures obtained through the vagina.
Intrauterine device	Surgical	Entire device plus secretion, pus	Sterile container	History of bleeding	Unusual organisms may be isolated, e.g., *Actinomyces, Torulopsis,* and other yeasts.
Lymph nodes (inguinal)	Skin decontamination	Biopsy or needle aspirate	Sterile container	History of venereal disease	May require sending to reference laboratory.
Products of conception (fetal tissue, placenta, membranes, lochia)	Surgical	Tissue or aspirates	Sterile container		Occasionally, this type of specimen is expelled into toilet and is grossly contaminated.
Urethra	Wipe clean with sterile gauze or swab.	Swab with urethral secretion or free discharge	TM	History of discharge	Discharge may be stimulated by gently stripping and massaging the urethra against public symphysis through the vagina.
Tubes, ovaries	Surgical	Tissue, aspirates, or swabs	Sterile container; see "Anaerobic cultures."	Salpingo-oophoritis	Consider venereal, fungal, anaerobic, and AFB infection.
Vagina	Use of speculum without lubricant	Aspirate or swab, Gram stains, and wet mounts	Swab with TM, Transgrow	History of discharge	Ulcerations should be checked for syphilis, soft chancre, or genital herpes. Yeast common. For GC, cervical specimen is preferred. Wet mount is for yeast and *Trichomonas,* "clue cells," *Gardnerella,* and organisms of vaginosis.
Vaginal cuff		Aspirate of abscess	See "Anaerobic cultures."	Postsurgery	
Vulva (including labia, Bartholin glands)	Do not use alcohol for mucous membranes. Skin prep for regular skin sites.	Swab or aspirate (Bartholin gland abscess)	Swab with TM. See "Anaerobic cultures" for aspirate.	Discharge	Same as above
Lesion (dark field, for *Treponema pallidum*)	1- to 2-h soaking with sterile saline on gauze	Several slide preparations or aspirate fluid into capillary tube	Slide and coverslip or capillary tube		Characteristic motility is seen only on warm material. Seal coverslip or capillary tube with lanolin or petrolatum. Specimen should be examined within 15 min of collection because the organism loses motility as the temperature decreases.
Genital tract–male					
Lymph nodes		Same as for female genital tract			

(continued)

Table 2.1–2 Collection of specimens for bacteriological analysis[a,b] *(continued)*

Specimen	Preparation	No., type, volume	Container	Helpful clinical information	Comments
Penile lesion	Skin prep for regular skin sites.			Duration of lesion, pain, discomfort	Special culture/ microscopic techniques are required for chancroid and granuloma inguinale.
Culture		Swab	Swab with TM if pus		
Dark field (for *T. pallidum*)			Same as for female genital tract		
Prostatic fluid		Secretion for smear and culture	Sterile tube or swab with TM	History of chronic UTI	Not recommended for GC cultures but helpful in some chronic UTI, *Trichomonas* spp.
Urethra		Same as for female	Secretion, slide, and/or swab	History and duration of painful discharge	In males, diagnosis of gonorrhea can often be made by microscopic examination of a Gram-stained smear.
Pus/abscess			See "Anaerobic cultures" and "Skin (deep suppurative lesion)."		May be labeled "incision and drainage" (I & D)
Intestinal					
Duodenal contents	Through tube	Several ml	Sterile tube	Travel, food	Examine for bacterial overgrowth, *Salmonella enterica* serovar Typhi, and parasites.
Feces		At 1 g, 3 consecutive specimens	Stool preservative, Culturette for bacteria, special transport for parasites and ova	Travel, food suspected etiology	See section 9 (Parasitology) for further information.
Rectal swab		3 consecutive specimens	Swab with TM; GN broth	Same as above	Not useful for the detection of carriers
Gastric aspirate, neonate		Enough for smear and culture	Sterile container	History of ruptured membranes	May visualize and isolate causative agent of septicemia before blood cultures become positive
Respiratory tract					
Throat/pharynx		Swab	Swab with TM; commercial kits for *Streptococcus*	Agent suspected (e.g., group A streptococci, *N. gonorrhoeae*)	Do not touch oral mucosa or tongue with swab.
Epiglottis	Swab		TM	Suspected bacteria include *Haemophilus influenzae*	Do not swab throat in cases of acute epiglottitis unless prepared for tracheostomy.
Nasal sinuses			See "Anaerobic cultures (Body fluids)."		
Nasopharynx		Swab	Thin wire or flexible swab with TM	Agent suspected (e.g., *Bordetella pertussis*)	Transport to laboratory immediately or inoculate at bedside. Prepare smear for DFA.
Nose			Swab with TM	Mainly for staphylococcal carriers	

Table 2.1–2 *(continued)*

Specimen	Preparation	No., type, volume	Container	Helpful clinical information	Comments
Oral cavity					
Mucosal surface of gums or teeth	Rinse mouth.	Scraping, swab	Swab, tongue depressor, or slide in sterile container	Duration, agent suspected	Culture for yeast, smear for yeast or organisms of Vincent's angina.
Dental abscess, root abscess	Rinse mouth; prep with dry sterile gauze.	Exudate	See "Anaerobic cultures."		Predominant pathogens are anaerobes, including *Actinomyces* and various streptococci and gram-negative rods.
Bronchoscopy		Brushings, transbronchial biopsies, bronchial secretions	Sterile container	Agent(s) suspected on clinical impression	Gram stains may help direct culture effort. Patient's history important. May require culture for mycobacteria, legionellae in addition to DFA, stains for *Pneumocystis*, culture for fungi, viruses, and parasites.
Expectorated sputum	May require ultrasonic nebulization, hydration, physiotherapy, or postural drainage.	Sputum, not saliva	Sterile container	Pneumonia	Culture processing must be preceded by Gram stain demonstrating >25 PMNs and <10 epithelial cells/lpf. See section 7 on acid-fast organisms if submitted for mycobacteria. May be refrigerated overnight.
Trachael aspirate		Sputum	Sterile container	Pneumonia	Cellular composition may be misleading due to inflammatory reaction caused by endotracheal tube. May be refrigerated.
Transtracheal aspirate (infralaryngeal aspirate)	Skin is cleansed, anesthetized, and decontaminated.		Sterile container. See "Anaerobic cultures"	Pneumonia, aspiration, TB	Process immediately. See "Bronchoscopy."
Skin					
Superficial wound	Clean wound surface with 70% alcohol.	Pus, biopsy	Aspirate or swab with transport medium	Animal bite or trauma, duration, travel	
Extensive burns,[a] decubitus ulcer	Clean wound surface with 70% alcohol.	For quantitative culture, 3- to 4-mm dermal punch	Sterile container		Consider quantitative culture. See section 3. Decubitus ulcers should not be cultured casually; appropriate debridement and punch biopsy are required for quantitative evaluation of decubitus.
Deep suppurative lesion, closed abscess	Clean and decontaminate.	Pus, >1 ml if possible	Syringe or anaerobic container	Duration, location	See "Anaerobic cultures."

(continued)

Table 2.1–2 Collection of specimens for bacteriological analysis[a,b] *(continued)*

Specimen	Preparation	No., type, volume	Container	Helpful clinical information	Comments
Fistula, sinus tract	Clean surface; decontaminate.	Pus, >1 ml if possible	Syringe or swab with TM	Duration, location	Examine stained smear for guidance on isolation media.
Rash	Clean surface with 70% alcohol.	Pus, fluid	Syringe		
Tissue, surgical, or biopsy	Surgical	5 to 10 mm^3 or aspirate	Sterile container with TM		See "Anaerobic cultures." Do not discard leftover tissue. Freeze in sterile broth until culture and pathology are completed.

[a] NOTE: Standard precautions must be observed at all times. When the "Container" column indicates the specimen may be submitted in a syringe, the caveats of standard precautions must be followed, i.e., the syringe must be capped with a sterile closure; syringes with needles in place are unacceptable. Many specimens may contain important yeasts, moulds, or viruses. Follow the instructions of the director of the laboratory in concert with Infectious Disease Advisory Committee which designate such examinations as routine procedures with select specimens or follow the request of the physician of record based on the patient's history and clinical impression. Perform smears whenever possible.

[b] Abbreviations: TM, transport medium; TB, tuberculosis; SPS, sodium polyanethole sulfonate; GC, gonorrhea; AFB, acid-fast bacillus; STD, sexually transmitted disease; UTI, urinary tract infection; GN, gram negative; DFA, direct fluorescent-antibody assay; Fuo, fever of unknown origin; HSV, herpes simplex virus; lpf, low-power field.

[c] If copious amounts are available, fill sterile tube.

[d] Preparation of smears for Gram (and other) stains at time of autopsy is helpful.

[e] Culture for viruses based on clinical impression; inoculate virus transport vial if intended for reference laboratory.

[f] Culture for yeasts and moulds if suspected from clinical history or premortem findings.

[g] Cultures for mycobacteria and fungus require special attention. See the appropriate sections.

Table 2.1–3 Collection of specimens to detect infrequently encountered organisms[a]

Organism(s)	Specimen type	Transport issues and comments
Afipia spp., *Bartonella* spp. (cat scratch fever)	Blood Tissue Lymph node aspirate	1 wk at 4°C; indefinitely at −70°C Organisms may be seen in or on erythrocytes using Giemsa stain. Tissue should be stained with Warthin-Starry silver stain.
Anaplasma spp. (human granulocytic ehrlichiosis), *Ehrlichia* spp. (human monocytic ehrlichiosis)	Blood smear Skin biopsy specimen Blood (heparin or EDTA) CSF Serum	Transport specimen on ice, keeping tissue sterile and moist. Hold specimen at 4–20°C until tested or at −70°C for shipment. Serological diagnosis preferred. For PCR test, transport on ice or frozen.
Borrelia burgdorferi (Lyme disease)	Skin biopsy sample from lesion periphery Blood CSF	Keep tissue moist and sterile. Hand carry specimen to lab if possible. As culture yield is low, serology should be considered in addition.
Borrelia spp. (relapsing fever)	Blood smear	Hand carry specimen to lab if possible. Laboratory safety hazard.
Brucella spp.	Blood Bone marrow	Transport at room temp. Pediatric lysis-centrifugation tube is helpful. Blood culture bottles are useful if held 30 days. Serological diagnosis is preferred. Laboratory safety hazard.
Coxiella spp. (Q fever), *Rickettsia* spp. (spotted fevers, typhus)	Serum Tissue Blood	Serological diagnosis is preferred. Laboratory safety hazard. Send to reference lab.
Francisella spp. (tularemia)	Lymph node aspirate Scrapings Lesion biopsy sample Blood Sputum	Serological diagnosis is preferred. Laboratory safety hazard. Send to reference lab. Rapid transport to lab or freeze. Ship on dry ice.
Leptospira spp.	Serum Blood (heparin or sodium oxalate) CSF (first week of illness) Urine (after first week of illness)	Serological diagnosis is preferred. Blood <1 h Urine <1 h or dilute 1:10 in 1% bovine serum albumin or neutralize with sodium bicarbonate. Store at 4–20°C.
Streptobacillus spp. (rat bite fever, Haverhill fever)	Blood Joint fluid aspirate	High-volume bottle preferred. Supplement with blood serum or ascitic fluid. Do not refrigerate.

[a] Adapted from references 10 and 11.

Table 2.1–4 Laboratory approaches to suspected fungal infections

Disease	Specimen type	Culture medium[a]
Superficial mycoses		
Pityriasis versicolor	Scrapings	Not necessary
Tinea nigra	Skin scrapings	SAB-SPEC
Piedra	Cut hair	SAB
Cutaneous mycoses		
Tinea capitis	Epilated hair	SAB-SPEC, DTM
Tinea corporis	Skin scrapings	SAB-SPEC
Onychomycosis	Nail scrapings	SAB-SPEC C
Candidiasis	Skin, nail scrapings, mucocutaneous scrapings, vaginal swab	SAB-SPEC
Subcutaneous mycoses		
Chromoblastomycoses	Scrapings, crust exudate from lesions	SAB-SPEC
Mycetoma	Pus from draining sinuses, aspirated fluids, biopsy	SAB, BHI, BHIA with blood
Phaeohyphomycosis	Sputum, BW,[b] e.g., body fluids, pus, corneal scrapings	SAB-SPEC C
Sporotrichosis	Pus from lesions, aspirated fluids	SAB-SPEC
Systemic mycoses		
Yeastlike fungi		
Candidiasis	Sputum, BW, biopsy, CSF, urine, stool, blood	SAB-SPEC C, BHIA, blood culture (isolation)
Cryptococcosis	CSF, sputum, blood, bone marrow, urine, scrapings from skin lesions, pus from abscesses and sinus tracts	SAB-SPEC C, birdseed agar
Geotrichosis	Sputum, BW, stools	SAB-SPEC C
Dimorphic fungi		
Blastomycosis	Scrapings from edge of lesions	SAB-SPEC
	Pus from abscesses and sinus tracts, urine, sputum, BW	BHIA, yeast extract phosphate medium
Paracoccidioidomycosis	Scrapings from edges of lesions, mucous membranes, biopsied lymph nodes, sputum, BW	SAB-SPEC, BHIA yeast extract, phosphate agar
Coccidioidomycosis	Sputum, BW, CSF, urine scrapings from lesions, pus from sinuses, abscesses	SAB-SPEC, yeast extract, phosphate agar
Histoplasmosis	Blood, bone marrow, sputum, BW, CSF, pus from sinus tract or ulcer, scrapings from lesion	Yeast extract, phosphate agar, blood agar
Miscellaneous mycoses		
Aspergillosis	Sputum, BW	SAB-SPEC C
Zygomycosis	Sputum, BW, biopsy	SAB-SPEC C
Hyalohyphomycosis	Sputum, BW, nail scrapings, blood, body fluids, pus, wound scrapings	SAB-SPEC C
External otitis	Epithelial scales and detritus	SAB-SPEC C

[a] SAB, Sabouraud's agar; SAB-SPEC, Sabouraud's agar with chloramphenicol and cycloheximide; SAB-SPEC C, Sabouraud's agar with chloramphenicol only; DTM, dermatophyte test medium (presumptive); BHIA, BHI agar; blood culture, any approach extant in laboratory is acceptable—however, lysis-centrifugation (Isolator-Wampole) is preferred. All media required are available commercially (see Appendix A at the end of this handbook). For selected commercial suppliers, see the mycology section.

[b] BW, bronchial wash.

Table 2.1–5 Collection of specimens to detect parasites[a]

Site	Specimen options	Collection method
Blood	Smears of whole blood	A minimum of four each (separate slides) of thick and thin blood films
	Anticoagulated blood[b,c]	Anticoagulant EDTA vacutainer tube (purple top)
Bone marrow	Aspirate[b,c]	Sterile tube
Central nervous system	Spinal fluid[b,c]	Sterile tube
Cutaneous	Aspirates from below surface	Sterile tube plus air-dried smears
Ulcers	Biopsy	Sterile tube, nonsterile to Histopathology (formalin acceptable)
Eye	Biopsy	Sterile tube (in saline), nonsterile tube to Histopathology
	Scrapings	Sterile tube (in saline)
	Contact lens	Sterile tube (in saline)
	Lens solution	Sterile tube
Intestinal tract	Feces[d]	PVA (polyvinyl alcohol or zinc), 5 or 10% buffered formalin. SAF (sodium acetate, acetic acid, formalin), single-vial collection system (TOTAL-FIX, Unifix, ECOFIX)
	Sigmoidoscopy	Fresh, PVA, or Schaudinn's smears
	Duodenal contents	Entero-test or aspirates
	Anal impression smear	Cellulose tape (pinworm examination)
	Adult worm/worm segments	Saline, 70% alcohol
Liver, spleen	Aspirates	Sterile tube, collected in four separate aliquots (liver)
	Biopsy	Sterile tube
Lung	Sputum	True sputum (not saliva)
	Induced sputum	No preservative (10% formalin if time delay)
	BAL	Sterile tube (container)
	Transbronchial aspirate[b,c]	Air-dried smears
	Tracheobronchial aspirate[b,c]	Same as above
	Brush biopsy[b,c]	Same as above
	Open lung biopsy[b,c]	Same as above
	Aspirate	Sterile
Muscle	Biopsy	Fresh, squash preparation, nonsterile, to Histopathology (formalin acceptable)
Skin	Scrapings	Aseptic, smear or vial
	Skin snip	No preservative
	Biopsy	Sterile (in saline)
Urogenital system	Vaginal discharge	Saline swab, transport swab (no charcoal), culture medium, plastic envelope culture, air-dried smear for FA
	Urethral discharge	Same as above
	Prostatic secretions	Same as above
	Urine	Single unpreserved specimen, 24-h unpreserved specimen, early morning

[a] Abbreviations: FA, fluorescent antibody; BAL, bronchoalveolar lavage.

[b] Immediate delivery to laboratory desirable; if request is for organisms other than *Plasmodium* spp., request it be indicated on label and on requisition for parasites such as filariae (2, 4).

[c] Immediate delivery to laboratory desirable; requires prompt processing.

[d] Examinations of fresh stool specimens require special attention. Liquid stool specimens for protozoan trophozoites must be examined within 30 min of passage (not 30 min after arriving at laboratory); soft stools should be examined within 1 h of being passed, although protozoan cysts survive and can be detected in firm stools within 24 h of being passed. The preserved stool specimens are suitable for examination for *Cryptosporidium* spp., *Isospora* spp., and related coccidia. NOTE: Stool examinations for ova, parasites, and enteropathogenic bacteria should not be requested if patients have been hospitalized for 3 days or more.

Table 2.1–6 Commercially available transport media[a]

Medium	Purpose
Stuart's medium (13)	Most aerobic and facultatively anaerobic bacteria
Amies medium (1)	Same as above
Amies medium with charcoal	Use for transport of gonococci.
Carey-Blair medium (2)	Transport of pathogenic stool bacteria, e.g., *Salmonella, Shigella, Vibrio, Campylobacter, Yersinia* spp.
Buffered glycerol	Transport of potentially pathogenic stool bacteria
Anaerobic transport medium	Numerous variations are available; objective is to preserve appropriate atmosphere. Use of swabs should be discouraged; aspirates or tissue in proper transport devices is preferable.
Synthetic mesh	Recently made available on plastic shaft in a protective tube; mesh entraps organisms and preserves them without buffer or medium; can be used for recovery of aerobic and most anaerobic organisms. Suitable for smear preparation and/or antigen detection when two shafts are provided.
Z-PVA (4)	For transport of intestinal parasites; suitable for preparation of slides to be stained; specimens may be concentrated.
SAF (4)	Sodium acetate-acetic acid-formalin as PVA substitute
Single-vial collection system for parasites	For transport of intestinal parasites; suitable for concentration and permanent staining; may be suitable for use with enzyme immunoassays (TOTAL-FIX, Unifix, ECOFIX).
Buffered formalin (7)	Especially for ova and larvae and for concentrations
Viral transport	Contains salt solutions to stabilize viral agents

[a] Various commercial suppliers have these transport vials, tubes, and devices available, often packaged with polyester or flocked nylon fiber tips and plastic shanks (to avoid toxic effects of cotton and wood). Consult section 9 for names of suppliers. Z-PVA, zinc-polyvinyl alcohol.

Table 2.1–7 Rejection criteria for microbiological specimens[a,b]

Criterion[c]	Procedure
Clerical errors	
Discrepancy between patient identification on requisition and specimen container label	Notify physician or nurse in charge. Request new specimen or have physician or nurse correct error in person in the laboratory.
No identification on container	Notify physician or charge nurse. Proper label must be brought to laboratory and specimen identity verified.
Specimen source or type not noted	Call physician or charge nurse to ascertain missing information.
Test not indicated on requisition	Call physician or charge nurse to ascertain missing information.
General microbiology	
Specimen received in fixative (formalin); exception, stool for parasites and ova	Notify physician or charge nurse and request new specimen; indicate "received in fixative" on requisition and return.
Foley catheter tip	Notify physician or charge nurse that specimen is not suitable for microbiological analysis. Note rejection on requisition and return.
Containers	
Unpreserved urine held in refrigerator for >24 h	Notify physician or nurse in charge and request new specimen. If contact insists specimen be processed, refer to supervisory personnel. Return requisition with appropriate comment.
Improper or nonsterile container	
Leaking container	
Dry swab	Notify physician or charge nurse and request new specimen, properly submitted in appropriate transport device. If physician insists dry swab be cultured, note on laboratory record and report with caveat: "microorganisms recovered may not reflect actual microbiota."
More than one specimen of urine, stool, sputum, wound, or routine throat specimens submitted on the same day from the same source	Notify physician or charge nurse that as stated in laboratory manual only one specimen will be processed per day.
Only one swab submitted with multiple requests for various organisms (bacteria, AFB, fungi, virus, ureoplasmas, etc.)	Notify physician or charge nurse and request additional material. If additional material cannot be procured, ask physician to prioritize.
Anaerobes	
Specimen for anaerobes not received in appropriate container	Notify physician or charge nurse and request properly handled specimen. If physician insists specimen be processed, refer to supervisory personnel or comment in laboratory record and in report form that inappropriate transport may have influenced recovery of significant anaerobic bacteria.
Anaerobic cultures requested on autopsy material; bronch wash; decubitus ulcer material (not punch biopsy of tissue beneath eschar); drain; drain site; environment; exudate; feces; gastric washing (other than newborn); midstream or catheterized urine; mouth, nose, or prostatic secretions; sputum material on swabs from ileostomy or colostomy; fistual or intestinal contents; throat or vaginal secretions	Inform physician or charge nurse that, as detailed in laboratory manual, these specimens are not cultured for anaerobic bacteria, since these anatomic sites harbor anaerobes normally and usually. If physician insists, refer him/her to supervisory personnel.
Aerobic bacteriology	
Gram stain for *Neisseria gonorrhoeae* on specimens from cervix, vagina, and anal crypts	Notify physician or charge nurse that these smears are not examined for GC, since these anatomic loci may harbor nongonococcal neisseriae.
Specimens for GC and/or *Chlamydia* culture received in Gen Probe transport medium	Notify physician or charge nurse that the specimens are in a fixative that kills bacteria; only molecular-probe results may be offered with this fixed specimen.
Respiratory culture requested on throat specimens of patients older than 10 yr	Notify physician or charge nurse that a screening culture for group A streptococci only will be performed. Exceptions are transplant, cystic fibrosis, and head and neck clinic patients.
Sputum specimen with <25 WBC, >10 epithelial cells/lpf	Inform physician or charge nurse that specimen is mostly saliva and is not appropriate for culture. Request a repeat specimen.
Sputum with or without WBC and numerous and varying tinctorial bacterial morphotypes for *Legionella* DFA	Notify physician or charge nurse that specimen is unsuitable for DFA.

(continued)

Table 2.1–7 Rejection criteria for microbiological specimens[a,b] *(continued)*

Criterion[c]	Procedure
Stool specimens submitted for culture for inpatients after third hospital day	Notify physician or charge nurse that specimens submitted after third day of hospitalization are not acceptable without prior consultation.
More than 2 stool specimens/patient submitted for culture	Notify physician or charge nurse that >2 stool specimens submitted for culture are not acceptable without prior consultation.
Mycobacteriology/mycology	
24-h collection of urine or sputum for AFB or fungus culture	Inform physician or nurse that according to laboratory manual, three separate first morning specimens of sputum or of urine are the best samples for analysis; reject 24-h specimens.
Sputum swabs for AFB or fungi	Notify physician or charge nurse that specimen is inadequate in quantity for the isolation of these microorganisms. Request properly collected specimens.
Parasitology	
Ova and parasites for examination received in only formalin or only PVA	Inform physician or charge nurse that stool in both transport media required for adequate parasitological analysis; request repeat with both transport media used.
Stool for *Cryptosporidium* and/or *Isospora* spp. received in PVA only	Notify physician or charge nurse that both PVA and formalin are required for accurate performance of test; request repeat submission with both transport media.
Excess barium or oil noted in stool submitted for parasitological examination	Notify physician or charge nurse that the barium and/or oil will obscure the examination and that a specimen should be resubmitted in 10 days.
Stool for *Entamoeba histolytica/E. dispar* differentiation by EIA sent in PVA or formalin	Notify physician or charge nurse that a fresh stool sample is required; request fresh stool sample to be sent
Virology	
Stool specimens received in preservative for viral culture for detection of *C. difficile* toxins, rotavirus, or adenovirus 40/41	Notify physician or charge nurse to request new proper specimen; record on requisition "specimen unsatisfactory; received in preservative."
Clotted blood for viral culture (serum acceptable when enterovirus suspected)	Inform physician or charge nurse that blood has clotted; request new specimen and admonish to mix blood well to prevent clotting.

[a] Abbreviations: GC, gonococcus; AFB, acid-fast bacillus; lpf, low-power field; DFA, direct fluorescent antibody assay; PVA, polyvinyl alcohol.

[b] A record for all rejections or discrepancies must be maintained (book, card file, or computer). Note patient demographic information; physician of record; date and time specimen received; type and source of specimen; examination requested; reason for rejection or discrepancy noted; person contacted by phone; date, time, and manner of contact (phone, computer, or FAX); and final disposition or resolution. Records should be reviewed by supervisory personnel at regular intervals.

[c] Specimens unsuitable for routine anaerobic cultures: throat swabs; nasopharyngeal swabs; gingival and other internal mouth surface swabs; expectorated sputum; sputum obtained by nasotracheal or orotracheal suction; bronchial washings or other specimens obtained with bronchoscopy unless procured with a protected double-lumen catheter or by properly executed bronchoalveolar lavage; gastric and small-bowel contents; large-bowel contents except for *Clostridium difficile*, *Clostridium botulinum*, *Anaerobiospirillum succiniciproducens*, and other specific causative agents; ileostomy and colostomy effluents; feces, except for large-bowel contents (as directed above); voided or catheterized urine; vaginal or cervical swabs; female genital tract cultures collected via vagina, except for suction curettings or other specimens collected with a double-lumen catheter; surface swabs from decubitus ulcers, perirectal abscesses, foot ulcers, exposed wounds, eschars, or pilonidal and other sinus tracts; any material adjacent to a mucous membrane that has not been adequately decontaminated.

Table 2.1–8 Procedure for processing clinical specimens in microbiology[a]

I. Evaluation of the specimen for adequacy
   A. The specimen must be properly labeled.
   B. The transmittal must be submitted with the specimen, and the information on the transmittal must match the information on the specimen label.
   C. The specimen must be submitted in the proper transport container.
   D. The specimen volume must be adequate to perform all tests requested.
   E. The specimen sent must be appropriate for the test ordered.

   NOTE: If any of these requirements are not met, refer to list of rejection criteria in Table 2.1–7.

II. Medium selection and labeling
   A. Select appropriate media for the tests ordered.
   B. Examine all media for expiration date and contamination before the media are inoculated.
   C. Individually label all media with an accession number and date. Do not obscure the names and expiration dates of the media.
   D. All media made in Microbiology must be properly labeled with the name of the medium and the expiration date.

III. Order of inoculation media. Inoculate the least selective medium first. This prevents any carryover of an inhibitory substance to another medium. Arrange labeled plates in order from least to most selective.
   A. Routine aerobic culture
      1. BAP
      2. CHOC
      3. MS
      4. MAC
      5. SEA
      6. Nutrient broth
   B. Genital culture
      1. BAP
      2. CHOC
      3. MS
      4. TM
      5. MAC or EMB
   C. Stool culture
      1. PEA
      2. CAMPY
      3. MAC or EMB
      4. HE
      5. GN broth
   D. Fungal culture
      1. SAB-SEL
      2. BHI blood agar with gentamicin and chloramphenicol
      3. SAB
      4. Dermatophyte test media for hair, skin, and nails
   E. Anaerobe culture
      1. BRU
      2. PEA
      3. BBE/LKV (optional)
      4. Chopped-meat broth or THIO
   F. Urine culture[b]
      1. BAP
      2. MAC or EMB
      3. SEA

IV. Procedure for streaking plates for primary isolation
   A. Sterilize the inoculating loop in the incinerator for 5 to 10 s. Allow the loop to cool thoroughly before streaking medium. A sterile plastic loop can be used in place of the sterile inoculating loop.
   B. Pass the cooled loop back and forth through the inoculum in the first quadrant several times.
   C. Do not flame the loop between the quadrants unless necessary.

(continued)

Table 2.1–8 Procedure for processing clinical specimens in microbiology[a] *(continued)*

D. Turn the plate a quarter turn, and pass the loop through the edge of the first quadrant approximately four times while streaking into the second quadrant. Continue streaking in the second quadrant without going back to the first quadrant, approximately four times.
E. Turn the plate another quarter turn, and pass the loop through the edge of the second quadrant approximately four times while streaking into the third quadrant. Continue streaking in the third quadrant without going back into the second quadrant, approximately four times.
F. Flame the loop between plates to prevent carrying over a possible contaminant from the previous plate.
G. Continue streaking the rest of the culture media in the same manner.
H. Inoculate suitable nutrient broth with 1 to 2 loopfuls of specimen or swab.

[a] Abbreviations: MS, mitis salivarius agar; SEA, selective enterococcus agar; TM, transport medium; PEA, phenyl ethanol agar; CAMPY, campylobacter agar; HE, Hektoen enteric agar; GN, gram-negative; SABSEL, Sabouraud's selective agar; BRU, brucella agar; BBE/LKV, bacteroides bile esculin agar/laked blood-kanamycin-vancomycin agar.

[b] Special instructions for processing urine specimens:

1. Do not centrifuge urine before culturing.
2. Mix urine well before culturing.
3. Use 0.001-ml calibrated loop for inoculating media for routine urine.
4. Transfer 1 loopful of urine to BAP.
5. Pull the loop down the surface of the agar to form a single streak in the center of the first quadrant.
6. Spread the inoculum over the first quadrant by streaking the loop back and forth.
7. Streak for isolation in the other quadrants.
8. Transfer 1 loopful of urine to MAC or EMB plate. Repeat procedure using SEA or colistin-nalidixic acid (CNA) agar.
9. Pull loop down surface of agar to form a single streak that crosses the center of the plate.
10. Cross streak through the initial inoculum streak by moving the loop back and forth at perpendicular angles to initial streak.
11. To inoculate media for special-collection urines, i.e., suprapubic tap, high and low counts, or nephrostomy, inoculate two sets of plates:
    a. 0.001 loop: BAP and MAC (EMB) and SEA (CNA) agars.
    b. 0.01 loop: BAP and MAC (EMB) and SEA (CNA) agars.
    c. Use the same inoculation procedure outlined above for both the 0.001 loop and the 0.01 loop.
12. If a urine Gram stain is ordered, use a ringed microscope slide.

NOTE: Biplates, commercially available, consisting of MAC and CNA or MAC and SEA may be used instead of single-agar plates of each.

NOTE: bioMérieux-Vitek AMS has urine cards that can be substituted for culture analysis.

Table 2.1–9 Panic values in microbiology[a]

Organisms seen in CSF
Organisms seen in joint fluids
Positive cryptococcal antigen detection
Positive CSF antigen detection for pneumococci, *Streptococcus agalactiae*, *Neisseria meningitidis*, and *Haemophilus influenzae* type b (now rare)
Positive AFB smear
Positive blood cultures (not contaminated)
Positive CSF cultures
Positive blood films for *Plasmodium* spp.
Positive eye cultures growing *Pseudomonas aeruginosa* or *Bacillus* spp.
Isolation of *Mycobacterium tuberculosis*
Isolation of *Escherichia coli* O157:H7
Isolation of pathogenic neisseriae
Isolation of *Streptococcus agalactiae* from pregnant women (cultures taken at 35–37 wks' gestation)
Isolation of the reportable etiological agents of any of the following:
Lymphogranuloma venereum
Malaria
Meningitis
Aseptic
Haemophilus spp.
Meningococcal
Other (specify type)
Meningococcemia
Mumps
Pertussis
Plague
Poliomyelitis
Psittacosis
Rabies
Rocky Mountain spotted fever
Rubella
Salmonellosis
Shigellosis
Syphilis (specify stage)
Tetanus
Toxic shock syndrome
Trichinosis
Tuberculosis
Tularemia
Typhoid
Typhus
Varicella
Yellow fever
Yersiniosis
Examples of communicable diseases (requiring immediate notification of referring physician and, for some, governmental agencies)
Amebiasis
Anthrax
Babesiosis
Botulism
Brucellosis
Campylobacteriosis
Chancroid
Cholera
Cryptosporidiosis
Diphtheria
Encephalitis
Escherichia coli O157:H7 infections
Giardiasis
Gonococcal infection
Granuloma inguinale
Hemolytic-uremic syndrome
Haemophilus influenzae infection (invasive disease)
Hepatitis (A, B, C)
Histoplasmosis
Legionellosis
Leprosy
Leptospirosis
Listeriosis
Lyme disease

[a] Panic values in microbiology encompass the detection of clinically important microorganisms and viruses that require notification, immediate action by the physician of record or his/her designate, action by hospital personnel and visitors, and notification of governmental agencies (this may differ from state to state). AFB, acid-fast bacterium.

Table 2.1–10 Alert request

Request all physicians to notify the Microbiology Laboratory if they suspect the following etiological agents in specimens to be sent to the laboratory:	
Bacillus anthracis	*Yersinia pestis*
Bordetella pertussis	*Blastomyces dermatitidis*
Brucella spp.	*Coccidioides immitis*
Francisella tularensis	*Histoplasma capsulatum*
Burkholderia pseudomallei	*Paracoccidioides brasiliensis*

Table 2.1–11 Serodiagnostic tests[a]

Test name	Method(s)[b]	Normal value(s)	Specimen requirement(s)[c]
Adenovirus antibody (IgG)	IFA	<1:40	Blood (5 ml)
Amoeba antibody	IHA	<1:64	Blood (5 ml)
Amoeba antibody panel	IHA, ID	<1:64 negative	Blood (5 ml)
Antideoxyribonuclease B	EN	<1:170	Blood (5 ml)
Antihyaluronidase titer	EN	<1:32	Blood (5 ml)
Antistreptolysin-O titer	LPA	<200 Todd units	Blood (5 ml)
Arbovirus antibody panel (western equine encephalitis, eastern equine encephalitis, St. Louis encephalitis, California encephalitis)	IFA	<1:16 (IgG) <1:10 (IgM)	Blood (5 ml) CSF (0.5 ml)
Aspergillus antibody (*A. fumigatus, A. flavus, A. niger*)	ID	Negative	Blood (5 ml)
Bacterial antigen	LPA	Negative	CSF (2 ml)
Detection panel (group B streptococci, *Streptococcus pneumoniae, Neisseria meningitidis, Haemophilus influenzae* type b)			
Blastomyces antibody	CF	<1:8 (serum) <1:2 (CSF)	Blood (5 ml)
Blastomyces antibody panel	ID CF	Negative <1:8	Blood (5 ml)
Bordetella pertussis antibody	MAT	<1:2	Blood (5 ml)
Borrelia (relapsing fever)	IFA	<1:16	Blood (5 ml)
Borrelia burgdorferi antibody	(*see* "Lyme disease antibody")		
Brucella antibody	SA	<1:20	Blood (5 ml)
C-reactive protein	LPA	<6 mg/liter	Blood (5 ml)
California encephalitis antibody	IFA	<1:16 (IgG) <1:10 (IgM)	Blood (5 ml) CSF (0.5 ml)
Candida antibody	ID	Negative	Blood (5 ml)
Candida antigen	LPA	Negative	Blood (5 ml)
Chagas' disease antibody panel (*Trypanosoma cruzi*)	IFA	<1:16	Blood (5 ml)
Chlamydia/Chlamydophila antibody (*C. psittaci, C. pneumoniae, C. trachomatis*)	IFA	<1:64	Blood (5 ml)
Coccidioides antibody	CF	<1:2	Blood (5 ml) or CSF (0.5 ml)
Coccidioides antibody panel			
Latex agglutination	LPA (IDTP)	Negative	CSF (0.5 ml)
Immunodiffusion	IDCF	Negative	
Complement fixation	CF	<1:2	
Cold agglutinin titer	TA	<1:4	Blood (5 ml) (Do not refrigerate)
Coxsackie A antibody panel (includes types 7, 9, 10, 21)	CF	<1:8 (serum) <1:2 (CSF)	Blood (5 ml) CSF (0.5 ml)
Coxsackie B antibody panel (includes types 1 through 6)	CF	<1:8 (serum) <1:2 (CSF)	Blood (5 ml) CSF (0.5 ml)
Cryptococcus antigen	LPA	Negative	Blood (5 ml) or CSF (1 ml)
Cysticercosis antibody	EIA	<1:10 (serum) <1:2 (CSF)	Blood (5 ml) CSF (1 ml)
CMV antibody (IgG)	IFA	<1:10	Blood (5 ml)
CMV antibody panel (includes IgG and IgM)	IFA	<1:10 (IgG)	Blood (7 ml)
Diphtheria antitoxin antibody	EIA	>0.01 IU/ml	Blood (5 ml)
Eastern equine encephalitis antibody	IFA	<1:16 (IgG) <1:10 (IgM)	Blood (5 ml) or CSF (0.5 ml)
Echinococcus antibody	IHA	<1:10	Blood (5 ml)
Echovirus antibody panel (includes types 4, 9, 11, 16)	CF	<1:8 (serum) <1:2 (CSF)	Blood (7 ml) CSF (0.5 ml)

Table 2.1–11 *(continued)*

Test name	Method(s)[b]	Normal value(s)	Specimen requirement(s)[c]
Epstein-Barr virus antibody panel			
Viral capsid IgG	IFA	>1:10	Blood (10 ml)
Viral capsid IgM	IFA	<1:10	
EBNA IgG	ACIF	>1:2	
Early antigen IgG	IFA	<1:40	
Febrile agglutinin panel (*Salmonella enterica* serovar Typhi, *S. enterica* serovar Paratyphi A/B, *Brucella*, *Proteus* (OX19)	SA, TA	<1:20	Blood (5 ml)
Fluorescent treponemal antibody absorption	IFA	Nonreactive	Blood (5 ml)
Filariasis antibody	EIA	<1:160	Blood (5 ml)
Francisella tularensis antibody	EIA	<1:20	Blood (5 ml)
Fungal disease antibody panel (*Aspergillus*, *Blastomyces*, *Coccidioides*, *Cryptococcus*, *Histoplasma*)	LPA, ID, CF	By report	Blood (10 ml) or CSF (2 ml)
Haemophilus influenzae type b vaccine response (polyribose phosphate antibody)	EIA	By report	Blood (5 ml)
Herpes simplex virus antibody type 1 (IgG)	IFA	>1:10	Blood (5 ml)
Herpes simplex virus antibody (antibody panel includes types 1 and 2, IgG and IgM)	IFA	>1:10 (IgG) <1:10 (IgM)	Blood (7 ml)
Heterophile antibody screen	LPA	Negative	Blood (5 ml)
Histoplasma antibody by CF	CF	<1:8 (serum) <1:2 (CSF)	Blood (5 ml) CSF (0.5 ml)
Histoplasma antibody panel	ID, CF	Negative <1:0 (serum) <1:2 (CSF)	 Blood (5 ml) CSF (0.5 ml)
HIV-1 antibody screen	EIA	Negative	Blood (5 ml)
HIV-1 antibody by Western blotting	IB	No bands detected (IB)	Blood (5 ml)
HIV-1 antigen assay	EIA	Negative	Blood (5 ml)
HTLV-1 antibody	EIA	Negative	Blood (5 ml)
Hypersensitivity pneumonitis (antibody panel includes 9 antigens: 5 thermophilic *Actinomyces*, 3 *Aspergillus*, and avian serum proteins)	ID	None detected	Blood (7 ml)
Influenza virus A and B IgG antibody panel	IFA	<1:40	Blood (5 ml)
Influenza virus A and B antibody	IFA	<1:40 (IgG)	Blood (5 ml)
Legionella pneumophila antibody	IFA	<1:64	Blood (5 ml)
Leishmaniasis (visceral) antibody (includes *L. donovani*, *L. mexicana*, *L. tropica*, *L. braziliensis*)	IFA	<1:16	Blood (5 ml)
Leptospira antibody	IHA	<1:50	Blood (5 ml)
Lyme disease antibody	IFA	<1:16	Blood (5 ml)
Lymphocytic choriomeningitis antibody	CF	<1:8 (serum) <1:2 (CSF)	Blood (5 ml) CSF (0.5 ml)
LGV antibody	IFA	<1:64	Blood (5 ml)
Measles (rubeola) IgG antibody	IFA	>1:10	Blood (5 ml)
Measles (rubeola) IgG and IgM antibody panel	IFA	>1:10 (IgG)	Blood (5 ml)
Melioidosis (*Pseudomonas pseudomallei*)	IFA	<1:16	Blood (5 ml)
Mumps virus IgG antibody	IFA	>1:10	Blood (5 ml)
Mumps virus IgG and IgM antibody panel	IFA	>1:10 (IgG) <1:10 (IgM)	Blood (5 ml)
Mycoplasma IgG antibody	IFA	<1:40	Blood (5 ml)
Mycoplasma IgG and IgM antibody panel	IFA	<1:40 (IgG) <1:20 (IgM)	Blood (5 ml)
Paragonimus antibody	EIA	None detected	Blood (5 ml)
Parainfluenza virus IgG antibody panel (includes types 1, 2, 3)	IFA	<1:40	Blood (5 ml)

(continued)

Table 2.1–11 Serodiagnostic tests[a] *(continued)*

Test name	Method(s)[b]	Normal value(s)	Specimen requirement(s)[c]
Parainfluenza virus IgG and IgM antibody panel (includes types 1, 2, 3)	IFA	<1:40 (IgG) <1:10 (IgM)	Blood (5 ml)
Poliovirus antibody by neutralization	NT	By report	Blood (5 ml)
Poliovirus antibody panel (1, 2, 3)	CF	<1:8	Blood (5 ml)
Premarital testing, male	RPR	Nonreactive	Blood (5 ml)
Premarital testing, female			
RPR	RPR	Nonreactive	Blood (5 ml)
Rubella	LPA	Immune	
Psittacosis antibody	IFA	<1:64	Blood (5 ml)
Q fever (*Coxiella burnetii*) total antibody	IFA	<1:16	Blood (5 ml)
Q fever (*Coxiella burnetii*) IgG and IgM antibody panel	IFA	<1:16 (IgG) <1:10 (IgM)	Blood (5 ml)
Respiratory virus IgG and IgM antibody panel, adult influenza A and B, mycoplasma	IFA	<1:40 (IgG) <1:10 (IgM)	Blood (7 ml)
Respiratory syncytial virus IgG antibody	IFA	<1:40	Blood (3 ml)
Rickettsial disease total antibody (Rocky Mountain spotted fever, typhus, Q fever)	IFA	<1:16	Blood (5 ml)
Rickettsial disease IgG and IgM antibody panel (Rocky Mountain spotted fever, typhus, Q fever)	IFA	<1:16 (IgG) <1:10 (IgM)	Blood (5 ml)
Rocky Mountain spotted fever total antibody	IFA	<1:10	Blood (5 ml)
Rocky Mountain spotted fever IgG and IgM antibody panel	IFA	<1:16 (IgG) <1:10 (IgM)	Blood (5 ml)
Rotavirus antigen detection	EIA	None detected	Stool (1 g)
Rubella virus IgG antibody	LPA	>1:10	Blood (5 ml)
Rubella virus IgG and IgM antibody panel	LPA, EIA	>1:10 (IgG) Negative (IgM)	Blood (5 ml)
Streptococcal antibody panel			
Antistreptolysin-O	LPA	<1:200	Blood (7 ml), Peds: blood (3 ml)
Antideoxyribonuclease B	EN	<1:170	
Syphilis serology (nontreponemal antibody, RPR)	RPR	Nonreactive	Blood (5 ml)
Syphilis serology (nontreponemal antibody, VDRL on CSF)	VDRL	Nonreactive	CSF (1 ml)
Syphilis serology (treponemal antibody, MHA-TP)	IHA	Nonreactive	Blood (5 ml)
Syphilis serology (fluorescent treponemal antibody absorption)	IFA	Nonreactive	Blood (5 ml)
Tetanus antitoxin antibody (referred to outside lab)	EIA	0.01–0.05 IU/ml	Blood (5 ml)
TORCH IgG antibody panel			
Toxoplasma	IFA	<1:16	Peds: blood (3 ml)
Rubella virus	LPA	<1:10	
CMV	IFA	<1:10	
Herpes simplex virus	IFA	<1:10	
TORCH IgG and IgM antibody panel			
Toxoplasma	IFA	<1:16 (IgG) <1:10 (IgM)	Peds: blood (3 ml)
Rubella virus	LPA, EIA	<1:10 (IgG) Negative (IgM)	
CMV	IFA	<1:10 (IgG) <1:10 (IgM)	
Herpes simplex virus	IFA	<1:10 (IgG) <1:10 (IgM)	
Toxocara antibody	EIA	<1:4	Blood (5 ml)
Toxoplasma total antibody	IFA	<1:16	Blood (5 ml)
Toxoplasma IgG antibody	IFA	<1:16	Blood (5 ml)
Toxoplasma IgG and IgM antibody panel	IFA	<1:16 (IgG) <1:10 (IgM)	Blood (5 ml)

Table 2.1–11 *(continued)*

Test name	Method(s)[b]	Normal value(s)	Specimen requirement(s)[c]
Trichinosis antibody	LPA	<1:5	Blood (5 ml)
Typhus (epidemic, endemic) total antibody	IFA	<1:16	Blood (5 ml)
Typhus (epidemic and endemic) IgG and IgM antibody panel	IFA	<1:16 (IgG) <1:10 (IgM)	Blood (5 ml)
Varicella-zoster virus IgG antibody (immune status)	EIA	Seropositive Index >1.0	Blood (5 ml)
Varicella-zoster virus IgG antibody titer	IFA	>1:10	Blood (5 ml)
Varicella-zoster IgG and IgM antibody panel	IFA	>1:10 (IgG) <1:10 (IgM)	Blood (5 ml)
Western equine encephalitis antibody	IFA	<1:16 (IgG)	Blood (5 ml)
Yersinia antibody	MAT	<1:20	Blood (5 ml)

[a] Tests can be performed in the laboratory or sent to a reference laboratory. Abbreviations: IgG, immunoglobulin G; LGV, lymphogranuloma venereum; MHA-TP, microhemagglutination; CMV, cytomegalovirus; HIV-1, human immunodeficiency virus type 1; HTLV-1, human T-cell leukemia virus type 1.

[b] ACIF, anticomplement immunofluorescence; CF, complement fixation; DA, direct agglutination; IB, immunoblot; EIA, enzyme immunoassay; EN, enzyme neutralization; ID, immunodiffusion; IFA, indirect fluorescent antibody; IHA, indirect hemagglutination; LPA, latex particle agglutination; MAT, microagglutination titer; MIF, micro-indirect fluorescence; MC, mucin clot technique; NT, neutralization; PHA, passive hemagglutination; RPR, rapid plasma reagin test; SA, slide agglutination; VDRL, venereal disease research laboratory; IDTP, immunodiffusion tube precipitin test; IDCF, immunodiffusion complement-fixing antigen F test; TA, tube agglutinin.

[c] Draw blood in red-top tube except when otherwise indicated.

REFERENCES

1. **Amies, C. R.** 1967. A modified formula for the preparation of Stuart's transport medium. *Am. J. Public Health* **58:**296–299.
2. **Carey, S. G., and E. B. Blair.** 1964. New transport medium for shipment of clinical specimens. I. Fecal specimens. *J. Bacteriol.* **88:**96–98.
3. **Forbes, B. A., D. F. Sahm, and A. S. Weissfeld.** 2007. *Bailey and Scott's Diagnostic Microbiology*, 12th ed. Mosby, Inc., St. Louis, MO.
4. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
5. **Garcia, L. S.** 2009. *Practical Guide to Diagnostic Parasitology*, 2nd ed. ASM Press, Washington, DC.
6. **Gorbach, S. L., J. G. Barlett, and N. R. Blacklow.** 1992. *Infectious Diseases.* The W. B. Saunders Co., Philadelphia, PA.
7. **Heymann, D. L.** 2008. *Control of Communicable Diseases Manual*, 19th ed. American Public Health Association, Washington, DC.
8. **Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Barron, H. W. Wexler, and S. M. Finegold.** 2002. *Wadsworth—KTL Anaerobic Bacteriology Manual*, 6th ed. Star Publishing Co., Belmont, CA.
9. **Mandell, G. L., J. E. Bennett, and R. Dolin.** 2005. *Principles and Practices of Infectious Diseases*, 6th ed. Elsevier Inc., Philadelphia, PA.
10. **Miller, J. M.** 1999. *A Guide to Specimen Management in Clinical Microbiology*, 2nd ed. ASM Press, Washington, DC.
11. **Murray, P. R., and T. R. Shea.** 2004. *Pocket Guide to Clinical Microbiology*, 3rd ed. ASM Press, Washington, DC.
12. **Stuart, R. D., S. R. Tosach, and T. M. Patsula.** 1954. The problem of transport for gonococci. *Am. J. Public Health* **45:**73–77.

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I gratefully acknowledge the following clinical microbiologists for their expertise in providing comments, helpful additions, and review of this section of the handbook: Merrie Bass, Holly d'Souza, Manju Mudambi-best, and Nancy Troup of Stanford Hospitals and Clinics, Stanford, CA; Nancy Bennett of Northridge Hospital Medical Center, Northridge, CA; Michele Holloway of Glendale Memorial Hospital and Health Center, Glendale, CA; Rohan Nadarajah of University of California, San Francisco Hospitals and Clinics, San Francisco; Sharon Abbott of Department of Health Services, Richmond, CA; and Caroline I. Mohr of CDC, Atlanta, GA.

3.1 Introduction to the Section

The Aerobic Bacteriology section of the handbook has been reorganized to place each part of the procedure together, including collection, specimen processing, supplies, QC, and the actual step-by-step testing. This will allow the user to see an overview of the entire procedure together. When several different methods of testing are acceptable, each option is presented. The users should not reproduce the procedural text of this handbook in its entirety but rather should choose among the various options presented to produce practical procedures applicable to their laboratory.

Procedures are first organized by anatomic site. The user may wish to separate each section of these procedures, as recommended in NCCLS document GP2-A4 (NCCLS is now known as the Clinical and Laboratory Standards Institute [CLSI]) (9). For example, the information in Specimen Collection can be used to provide a separate nursing manual, the information in Quality Control can be used for QC procedure, the information in Materials can be used for an inventory for reagent preparation and procurement of supplies, and the information in the beginning of each Procedure and many of the tables can be used for a specimen inoculation manual and a teaching manual for new employees, etc. Flowcharts and tables within the Procedure can be used to prepare technical bench manuals. References are provided to allow the reader further information for use in decision making when different options are being considered for test methods. Every attempt was made to provide significant original reviewed articles to support the recommended procedures. For procedures 3.3.1 and 3.3.2, general textbooks are listed, which can be purchased for reference material.

Often the laboratory is requested to examine a specimen for only one microorganism. To avoid duplication, when a procedure is presented for a specific organism, the procedure is listed following the general procedure for the most common anatomic site of isolation of the organism. For example, the detection of *Neisseria gonorrhoeae* is listed following the genital culture procedure, although *N. gonorrhoeae* may be sought in throat cultures. The procedure for *Brucella* is found following the general blood culture procedure, yet the organism can be found in a variety of other sterile specimens, such as joint and spinal fluid.

Following the procedures by anatomic site are procedures for biochemical testing in alphabetical order. The tests that are listed are the generally accepted tests that laboratories should be able to perform to identify the clinically important microorganisms encountered in the laboratory. Smaller laboratories may choose to perform fewer tests and refer cultures when less common microorganisms are found in culture. Procedures for automated methods and multitest kits are not presented because the list is extensive and manufacturers provide updated package inserts with the details for the performance of their products and preparation of laboratory procedure manuals for their kits. However, tables comparing these kits are presented to allow the user to have information for decision making in the purchase of such kits (also see references 8 and 10). The biochemical tests selected for inclusion in this handbook emphasize those that are rapidly performed. Consequently, the X and V factor procedure is not listed, because laboratories are encouraged to perform the more rapid δ-aminolevulinic acid test in combination with growth on CHOC or the satellite test for *Haemophilus influenzae*. If some media or biochemical tests are now thought to be less sensitive than other tests, the less sensitive media or tests are *not* listed. For example, V agar is reported to be less sensitive than human blood Tween bilayer media (procedure 3.9.1) for growth of *Gardnerella vaginalis* and *Burkholderia cepacia* selective agar is more sensitive than *Pseudomonas cepacia* agar for *B. cepacia* (procedure 3.11.3). Thus, V agar and *P. cepacia* agar are not listed as choices in the procedures.

Lastly, flowcharts are listed for common and important organisms. These flowcharts are different from any you will encounter, because they emphasize different levels of identification for different anatomic sites and rely on the reported sensitivities and specificities of each test for decision on the need to confirm the results. The flowcharts are designed to rapidly detect clinically important microorganisms with very few tests, but do not yield a species level identification unless it would be clinically relevant. The tables that follow the flowcharts should help further in the identification of both *common organisms and those that are of great clinical importance.* Because partial DNA sequencing in combination with phenotypic methods is increasingly being used to identify common pathogens with unusual biochemical reactions or fastidious or unusual pathogens, organism flowcharts and tables have been updated in procedure 3.18 to include reference to the recently published CLSI guideline MM18-1A (2). For more extensive identifications in cases of repeated isolation of organisms that do not usually initiate disease and for information on unusual organisms, the reader is referred to other reference material (3–8, 11, 12).

I would like to thank the original authors of the first edition for their phenomenal work from which the updated handbook was modeled and often duplicated in

this edition. Without their procedures and the expert editor of the first edition, Marie Pezzlo, this edition would not have been possible. I am grateful to the many contributors to the first edition and appreciate their thoughtful presentation of the material, especially Frank Citron for the many illustrations and Yvonne Ramsay Shea, Paula J. Malloy (deceased), and Joanne J. Bradna for the extensive information on specimen collection and processing, which is used throughout the procedures. The contribution of Patricia Kruezak-Filipov and Roxanne G. Shively to the Gram stain procedure will be a resource to laboratorians for many years. Many of the tables and figures that they developed have been retained in this version of the handbook.

Companies that supply media, reagents, and other products tend to change rapidly. Thus, the products listed from these suppliers may not always be available as they are described. Listing is limited to companies that sell products that are available in the United States. Rather than repeat the details of these companies, a list of common vendors that supply laboratory media and reagents is presented in Appendix 3.1–1. Other vendors' addresses are listed in the specific procedures when they provide a product used for that procedure alone.

REFERENCES

1. **Forbes, B. A., D. F. Sahm, and A. S. Weissfeld.** 2007. *Bailey and Scott's Diagnostic Microbiology,* 12th ed. Mosby, St. Louis, MO.
2. **Clinical and Laboratory Standards Institute.** 2008. *Interpretive Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing.* Approved guideline MM18-1A. CLSI, Wayne, PA.
3. **Garrity, G. (ed.).** 2001. *Bergey's Manual of Systematic Bacteriology,* vol. 1. Williams & Wilkins, Baltimore, MD.
4. **Garrity, G. (ed.).** 2005. *Bergey's Manual of Systematic Bacteriology,* vol. 2. Williams & Wilkins, Baltimore, MD.
5. **Garrity, G. (ed.).** 2009. *Bergey's Manual of Systematic Bacteriology,* vol. 4. Williams & Wilkins, Baltimore, MD.
6. **Garrity, G. (ed.).** 2009. *Bergey's Manual of Systematic Bacteriology,* vol. 5. Williams & Wilkins, Baltimore, MD.
7. **Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams.** 1994. *Bergey's Manual of Determinative Bacteriology,* 9th ed. Williams & Wilkins, Baltimore, MD.
8. **Murray, P. R., E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.).** 2007. *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
9. **NCCLS/CLSI.** 2002. *Clinical Laboratory Technical Procedure Manuals,* 4th ed. Approved guideline GP2-A4. NCCLS/CLSI, Wayne, PA.
10. **Truant, A. L. (ed.).** 2002. *Manual of Commercial Methods in Microbiology.* ASM Press, Washington, DC.
11. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1996. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria,* 2nd ed. Williams & Wilkins, Baltimore, MD.
12. **Winn, W. C., Jr., S. D. Allen, W. Janda, E. Koneman, G. Procop, P. Schreckenberger, and G. Woods (ed.).** 2005. *Koneman's Color Atlas and Textbook of Diagnostic Microbiology,* 6th ed. J.B. Lippincott, Philadelphia, PA.

APPENDIX 3.1–1

Vendors for Microbiology Supplies and Equipment

Abbott Diagnostics
100 Abbott Park Rd.
Abbott Park, IL 60064
(847) 937-6100
http://www.abbottdiagnostics.com/

AdvanDx, Inc.
10A Roessier Rd.
Woburn, MA 01801
(781) 376-0009
http://www.advandx.com

BD Diagnostic Systems
7 Loveton Circle
Sparks, MD 21152
(800) 638-8663
http://www.bd.com

Biolog, Inc.
21124 Cabot Blvd.
Hayward, CA 94545
(510) 785-2564
http://www.biolog.com

bioMérieux, Inc.
100 Rodolphe St.
Durham, NC 27712
(800) 682-2666
http://www.biomerieux-vitek.com

Biowhittaker/BMA
New name: Cambrex Bio Science
Walkersville, Inc.
8830 Biggs Ford Rd.
Walkersville, MD 21793
(800) 638-8174
http://www.cambrex.com

Cepheid
904 Caribbean Dr.
Sunneyvale, CA 94089
(408) 541-4191
http://www.cepheid.com

EY Laboratories
107-127 N. Amphlett Blvd.
San Mateo, CA 94401
(800) 821-0044
http://www.eylabs.com

APPENDIX 3.1–1 *(continued)*

Focus Diagnostics, Inc.
5785 Corporate Ave.
Cypress, CA 90630
(800) 445-0185
http://www.focusdx.com

Gen-Probe Incorporated
10210 Genetic Center Dr.
San Diego, CA 92121
(800) 523-5001
http://www.gen-probe.com

Hardy Diagnostics
1430 W McCoy Ln.
Santa Maria, CA 93455
(800) 266-2222
http://www.hardydiagnostics.com

Inverness Medical Professional Diagnostics
2 Research Way
Princeton, NJ 08540
(800) 257-9525
http://www.invernessmedicalpd.com

Invitrogen Corporation
5791 Van Allen Way
Carlsbad, CA 92008
(760) 603-7200
http://www.invitrogen.com

Key Scientific Products
1113 E. Reynolds St.
Stamford, TX 79553
(800) 843-1539
http://www.keyscientific.com

Meridian Bioscience, Inc.
3481 River Hills Dr.
Cincinnati, OH 45244
(513) 271-3700
http://www.meridianbioscience.com

MIDI, Inc.
125 Sandy Dr.
Newark, DE 19713
(800) 276-8068
http://www.midi-inc.com

Oxoid Inc. (part of Thermo Fisher Scientific)
Wade Road
Basingstoke
Hampshire
RG24-8PW, UK
44(0) 1256 841144
http://www.oxoid.com

PML Microbiologicals, Inc. (subsidiary of bioMérieux, Inc.)
27120 S.W. 95th Ave.
Wilsonville, OR 97070
(800) 547-0659
http://www.pmlmicro.com

Pro-Lab Diagnostics
9701 Dessau Rd., Suite 802
Austin, TX 78754-3941
(800) 522-7740
http://www.pro-lab.com

Remel Inc. (part of Thermo Fisher Scientific)
12076 Santa Fe Dr.
Lenexa, KS 66215
(800) 255-6730
http://www.remel.com
E-mail: remel@remel.com

Roche Molecular Systems Inc.
4300 Hacienda Dr.
Pleasanton, CA 94588-2722
(925) 730-8000
http://www.molecular.roche.com

Roche Molecular Systems Inc.
Building 500
1080 U.S. Highway 202
Branchburg, NJ 08876
(908) 253-7200
http://molecular.roche.com

Siemens Healthcare Diagnostics
(Dade Behring, Inc.)
1717 Deerfield Rd.
Deerfield, IL 60015-0778
(847) 267-5300
http://www.diagnostics.siemens.com

Sigma-Aldrich
P.O. Box 14508
St. Louis, MO 63178
(800) 325-3010
http://www.sigmaaldrich.com/united-states.html

TECHLAB, Inc.
2001 Kraft Dr.
Blacksburg, VA 24060-6358
(800) TechLab (832-4522)
http://www.techlabinc.com
E-mail: techlab@techlab.com

Thermo Fisher Scientific
81 Wyman St.
Waltham, MA 02454
(781) 622-1000
http://www.thermofisher.com

Trek Diagnostic Systems, Inc.
982 Keynote Circle, Suite 6
Cleveland, OH 44131
(800) 871-8909
http://www.trekds.com

3.2.1 Gram Stain

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The Gram stain is used to classify bacteria on the basis of their forms, sizes, cellular morphologies, and Gram reactions; it is additionally a critical test for the presumptive diagnosis of infectious agents and serves to assess the quality of clinical specimens (13, 20, 22). The test was originally developed by Christian Gram in 1884. The modification currently used for general bacteriology was developed by Hucker in 1921; it provides greater reagent stability and better differentiation of organisms. Other modifications have been specifically developed for staining anaerobes (Kopeloff's modification) and for weakly staining gram-negative organisms (*Legionella* spp., *Campylobacter* spp., *Brucella* spp., etc.) by using a carbol fuchsin or basic fuchsin counterstain (7, 23). In fact, many laboratories use these counterstains routinely, especially for direct smears of clinical material.

Bacteria stain either gram positive or gram negative on the basis of differences in their cell wall compositions and architectures. Gram-positive species have a thick peptidoglycan layer and large amounts of teichoic acids; they are unaffected by alcohol decolorization and retain the initial stain, appearing deep violet if their cell walls are undamaged by age, antimicrobial agents, or other factors. Gram-negative species have a single peptidoglycan layer attached to an asymmetric lipopolysaccharide-phospholipid bilayer outer membrane interspersed with protein; the outer membrane is damaged by the alcohol decolorizer, allowing the crystal violet-iodine complex to leak out and be replaced by the counterstain (2, 7).

Interpretation of Gram-stained smears involves consideration of staining characteristics and cell size, shape, and arrangement (20). These characteristics may be influenced by many factors, including culture age, medium, incubation atmosphere, staining methods (1), and presence of inhibitory substances. Similar considerations apply to the interpretation of smears from clinical specimens, but additional factors include the presence of particular host cell types and phagocytosis.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimens

1. Clinical specimens, generally excluding throat swabs, nasal swabs, sputum from cystic fibrosis patients, fecal material, and prosthetic devices. Direct smears are particularly useful for wounds, eye lesions, sterile fluids, body tissues, and certain discharges.
2. Broth and blood cultures to determine growth, Gram reaction, or morphology of bacteria
3. Colonies growing on solid medium

◩ **NOTE:** Young cultures (<24 h old) from noninhibitory media and fresh clinical specimens yield the most favorable results. When morphology is important (e.g., streptococci and gram-positive rods), broth cultures are preferred.

B. Specimen collection

1. Refer to procedures 3.4 to 3.13 for specimen collection by anatomic type.
2. Generally the smear is made in the laboratory; however, when there is a concern that transport will be delayed or that the preservative for culture will alter the specimen, prepare smears and submit slides to the laboratory.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

C. Rejection criteria

1. Gram stains are of little value for direct smears of stool, throat samples, and urine and for sputum from cystic fibrosis patients. Gram stains are also not routinely performed on blood cultures due to the low organism density in most samples. However, a direct blood smear may allow rapid detection of the bacteremia type in patients with fulminant sepsis where the organism load is high (e.g., suspected meningococcemia or asplenic patients with suspected pneumococcemia).
2. Stains are not part of standard protocols for evaluation of catheter tip specimens (procedure 3.6).

III. MATERIALS

A. Reagents

1. Methanol, absolute
   Store in brown bottles or plastic containers.
2. Crystal violet
   - **a.** Hucker's modification (1, 6, 7)
   - **b.** Kopeloff's crystal violet
3. Iodine
   - **a.** Gram's reagent
   - **b.** Kopeloff's iodine
4. Decolorizers
   - **a.** Slowest: ethanol, 95%
   - **b.** Intermediate: acetone-alcohol
     (1) Mix 50:50.
     (2) Combine in brown glass bottle, label with 1-year expiration date, and store at room temperature.
   - **c.** Fastest: acetone (reagent grade)

   Caution: *Ethanol and acetone are flammable.*
5. Counterstain
   - **a.** Safranin
   - **b.** Carbol fuchsin
   - **c.** Basic fuchsin (0.8, 0.1, or 0.2%)
   - **d.** Kopeloff's safranin
6. Reagents can be purchased or prepared in-house.
   - **a.** Refer to Appendix 3.2.1–1 for instructions on preparation of reagents.
   - **b.** Generally prepare large volumes and prepare working solutions as needed (this is efficient and ensures lot uniformity). Decant reagents into smaller bottles for daily use; however, replace the smaller bottles of the crystal violet and counterstain monthly to avoid the formation of precipitate on the slides.

◩ Indicate the reagent name, preparation date, "in use" date, lot number, expiration date, and storage conditions on the bottles and in the work record. It is not necessary to label the working bottles with this information other than the name of the reagent, if the "in use" date is clearly on the stock bottles and in the control records.

B. Other supplies

1. Wax pencil
2. Precleaned glass slides (25 by 75 mm), frosted ends desirable. Slides with etched rings are an alternative for liquid specimens.
3. Sterile 0.85% NaCl (saline), water, or broth
4. Sterile Pasteur pipettes, wood applicator sticks, inoculating loops, or needles
5. Supplies for disposal of biological waste, including sharps
6. Sterile tubes with caps
7. Sterile scissors, scalpels, and forceps
8. Immersion oil

C. Equipment, depending on specimen source or laboratory protocol

1. Bacticinerator or flame burner with automatic shutoff
2. Electric slide warmer, 45 to 60°C
3. Cytospin centrifuge
4. Vortex mixer
5. Device for collection of toxic stains for chemical hazard disposal

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Check appearance of reagents daily.
- **1.** If crystal violet has precipitate or crystal sediment, refilter before use.
- **2.** Change working solutions regularly if not depleted with normal use. Evaporation may alter effectiveness of reagents.
- **3.** Limit reuse of working stain containers by discarding at least monthly.

◩ **NOTE:** Stains can become contaminated. When contamination is suspected, use a new lot of stain.

B. Test laboratory staining procedure prior to use of new lots of each staining and decolorizing reagent and at least weekly thereafter, using a gram-positive and gram-negative microorganism. For laboratory staff that perform Gram stains infrequently, it may be appropriate to have them test a positive and negative control daily or even with each patient specimen tested.
- **1.** Prepare a faintly turbid broth culture of *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923).
- **2.** Make slides using 2 drops per slide spread in the size of a dime.
- **3.** Fix in methanol and store at −20°C.
- **4.** Stain by laboratory Gram stain method.
- **5.** Expected results
  - **a.** Gram-negative rods, pink
  - **b.** Gram-positive cocci, deep violet
- **6.** Alternatively, with a broken applicator stick or toothpick, procure material from between teeth and apply to the end of slide used for the specimen, separating this area of the slide with a marker. This method provides a built-in control with gram-positive and gram-negative representatives.

C. Take corrective action when stained smear preparations show evidence of poor quality, stains are difficult to interpret, or interpretations are inaccurate. Poor staining characteristics (e.g., faintly staining gram-positive organisms, retention of crystal violet by gram-negative organisms, staining only of the edges of a smear, precipitate on slide, etc.) may be due to specimen preparation, reagents, or staining procedure. The following are some common causes of poor Gram stain results.
- **1.** Use of glass slides that have not been precleaned or degreased
- **2.** Smear preparations that are too thick
- **3.** Overheating of smears when heat fixation is used
- **4.** Excessive rinsing during the staining procedure, especially if smear is not properly fixed
- **5.** Precipitate in reagents

D. Additionally, to ensure accuracy of interpretation, establish a system for reviewing Gram stain reports.
- **1.** Review of selected Gram stains by supervisory personnel to determine training needs and aid in correlating relevant clinical information
- **2.** Compare final culture results with Gram stain reports to check for recovery of morphologies noted in the Gram stain but not recovered in the culture. Similarly, review both the smear and the culture when organisms in 3 to 4+ quantities are recovered in culture but not observed on the Gram stain.

  ◩ **NOTE:** An appreciable number of organisms discerned on a smear can be cultivated. Discrepancies should be investigated for errors in smear evaluation or for indications for further culturing methods (e.g., anaerobic, fungal, or acid-fast bacillus [AFB] culture).
- **3.** Maintain a set of reference slides for competency training.

V. PROCEDURE

Observe standard precautions.

The Gram stain is initially used to determine the presence of purulence and bacterial morphotypes in a variety of clinical specimens. Bacterial culture results should also be correlated to the Gram smear results from the original clinical specimen. Organisms that fail to grow in culture but are seen on the Gram stain are typically fastidious or anaerobic bacterial species that require specific media and growth conditions to be viable.

A. Slide preparation

1. General considerations

a. Place frosted-end glass slides in a container of 95% ethanol (change alcohol solution daily).

b. Using a forceps, drain excess alcohol and flame slide prior to use.

c. Label the frosted ends with the information to identify the specimen or culture. Clinical specimens should be processed and handled in a biosafety cabinet, including the preparation of Gram stain slides. When working in a BSL 2 cabinet, personnel protective equipment should be used, including gowns and gloves, and laboratory staff should be trained to follow other BSL 2 recommendations (21).

2. For direct smears, prepare a monolayer of organisms sufficiently dense for easy visualization but sparse enough to reveal characteristic arrangements. As a guideline, newspaper print should be visible through the smear.

◩ **NOTE:** When working from the same pipette or swab, always prepare the Gram stain and then sequentially inoculate culture plates so that the smear is representative of the original cellular and microbial composition of the clinical specimen.

a. Body fluids, bronchoalveolar lavage fluid (BAL), and CSF

(1) Place 5 or 6 drops of sample plus 1 drop of 37% formalin into a cytospin specimen chamber. Follow procedure for operation of centrifuge from manufacturer.

◩ **NOTE:** Use of a cytospin slide centrifuge to concentrate body fluids increases the Gram stain sensitivity and decreases time of standard centrifugation and examination, for more rapid results (14).

(2) As an alternative when the specimen is viscous or cloudy or the quantity is not sufficient for concentration, use a Pasteur pipette to transfer 1 or 2 drops of the specimen directly to the slide, after marking the location with a wax pencil. Allow the drop(s) to form one large drop. Do not spread the fluid. Optionally, add a second drop of fluid to the same area to increase the concentration of fluid for examination (10).

b. Urine specimens

(1) Place 10 μl of well-mixed, uncentrifuged urine onto a glass slide marked with a wax pencil to indicate the location of the sample drop. A ring slide may also be used for ease in locating the specimen.

(2) Air dry without spreading.

c. Specimens received on swabs

(1) Request a separate swab for adequate smear preparation.

◩ **NOTE:** Many culturette systems provide two swabs in the container, eliminating problems with sufficient swabs for each test requested.

(2) Roll the swab gently across the slide to avoid destruction of cellular elements and disruption of bacterial arrangements.

V. PROCEDURE *(continued)*

(3) Alternatively, when only one swab is received, place the swab in a small amount of saline or culture broth, cap tube, and vortex. Squeeze the swab against the side of the tube, and use swab to prepare smear. Use the remaining suspension to inoculate culture media.

◩ **NOTE:** Never mix open tube vigorously. Avoid creation of aerosols.

d. Specimens not received on swabs: aspirates, exudates, sputa, etc.

(1) Transfer specimen to the cleaned slide.

(a) Specimens received by the laboratory in a syringe with the needle still attached should be rejected because of the risk of a needless sharps exposure by laboratory staff. The physician should be immediately phoned to recollect the sample and send it in the proper container.

◩ **NOTE:** Establish a policy for the proper collection and transport of clinical specimens not collected on swabs. Educate the physicians that needles must be removed from the syringe and the syringe cap secured prior to transport to avoid leakage.

(b) Select purulent or blood-tinged portions of pus or sputum with a sterile applicator stick, pipette, or wire loop.

(c) Spread the sample over a large area of the slide to form a thin film.

(2) For extremely thick or purulent specimens

(a) Dilute in a drop of saline on the slide for easier smear preparation.

(b) Alternately, place the specimen on one slide, cover it with second slide, press the slides together, and pull them apart (Fig. 3.2.1–1). Remove excess material on the side of slides with a disinfectant-soaked paper towel.

e. Dried material or very small amounts of clinical specimen

(1) Emulsify specimen in 0.5 ml of sterile broth. Vortex, if necessary.

(2) Use a sterile Pasteur pipette to transfer 1 drop to a slide.

(3) Use the pipette tip to spread the drop into an even thin film.

f. Biopsy specimens and tissue sections

(1) Touch preparation (Fig. 3.2.1–2)

(a) Place tissue in sterile petri dish, and mince with sterile scissors or surgical scalpel.

(b) With sterile forceps to hold pieces, touch the sides of one or more of the minced fragments to a sterile glass slide, grouping the touches together for easier examination.

(2) Thin-smear preparation (Fig. 3.2.1–1)

(a) When specimen sample is soft tissue or thick exudate, prepare smear by taking a small portion of tissue, and place it on sterile slide with a swab or sterile forceps.

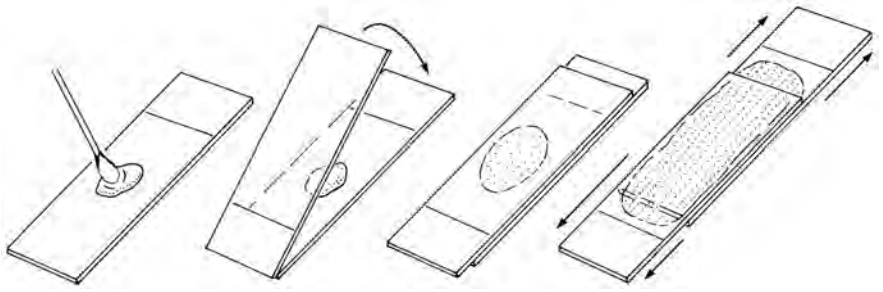


Figure 3.2.1–1 Thin-smear preparation.

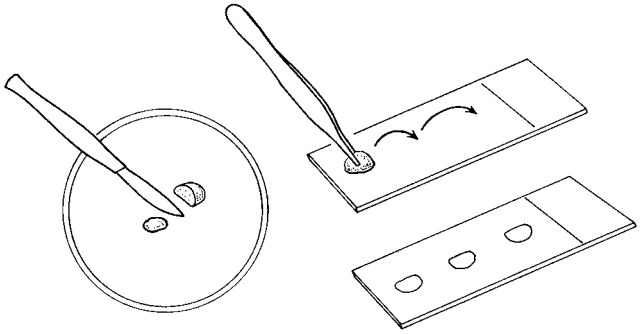


Figure 3.2.1–2 Touch preparation of smear.

V. PROCEDURE *(continued)*

(b) Place second sterile slide over sample, and press slides together.
(c) Separate slide by sliding the two away from each other.

(3) If there is no other option, use a ground specimen preparation (*see* procedure 3.13.1). Spread 1 drop to the size of a dime.

◩ **NOTE:** Homogenizing or grinding tissue specimens before preparing the smear will often destroy characteristic cellular entities and bacterial arrangements.

3. Broth cultures
- **a.** Prepare one smear per slide to avoid washing off of "dried" liquid from one area to another during staining.
- **b.** Use a sterile Pasteur pipette (or a venting needle or syringe adapter for containers with septa, such as blood culture bottles, to avoid manipulating a needle and syringe) to transfer 1 or 2 drops to the slide.
- **c.** Spread drop into an even thin film.
- **d.** For charcoal-containing medium, prepare blood culture smear as for hematology blood count, using a second slide at a 45° angle to spread a thin layer of cells on first slide.

4. Colonies from solid media
- **a.** Place a drop of sterile saline or water on slide. Distilled water may distort cellular morphology of fragile organisms.
- **b.** Transfer a small portion of colony with a sterile applicator stick, wire needle, or loop.
- **c.** Gently mix to emulsify. The resulting smear should be slightly cloudy and homogeneous (adjust saline drop size, area of smear, and/or inoculum to achieve optimum results). If swirl lines are evident as the smear dries, the inoculum was too heavy, the drop of saline was too small, and/or the smear was spread over too small an area.

B. Smear fixation

1. Air dry slides in a biosafety cabinet or covered on a slide warmer at 60°C until dry.

2. Heat fixation
- **a.** Pass air-dried slides two or three times through a flame, or hold the slide against the front of a microincinerator for 5 to 10 s. To avoid distortions, do not overheat.
- **b.** Allow slide to cool before staining.

3. Alternatively, fix with methanol to prevent the lysis of RBCs, produce a cleaner background, and prevent washing off of liquid specimens (12).
- **a.** Place a few drops of methanol on air-dried slide for 1 min, drain off remaining methanol without rinsing, and allow slide to air dry again.
- **b.** Do not use heat before staining.

V. PROCEDURE *(continued)*

C. Staining procedures (*see* Table 3.2.1–1 for comparison of methods)

◩ **NOTE:** Do not apply stains, water, or decolorizer directly to specimen area. Apply drops near the frosted end of the slide, allowing reagent to flow over the remaining surface.

1. Hucker's modification

◩ **NOTE:** Hucker's modification is widely used for routine work. The decolorizer chosen will alter decolorization times. Acetone-alcohol gives consistent results, but 95% ethanol is preferred for students and less experienced personnel. Acetone is a more rapid decolorizer with a shorter range of reproducibility and is recommended for experienced personnel only (1). It is most useful for specimens containing an appreciable number of host cells.

a. Flood the fixed smear with the crystal violet solution. Allow the stain to remain for 30 s.

b. Decant crystal violet, and rinse slide gently with running tap water.

◩ **NOTE:** Excessive rinsing in this step could cause crystal violet to be washed from gram-positive cells. Apply the flow of water to the underside of the angled slide to ensure a gentle flow across the smeared side.

c. Rinse off excess water with iodine solution, and then flood the slide with fresh iodine solution. Allow iodine to remain for 30 s.

d. Rinse off iodine gently with flowing tap water.

e. Decolorize by letting the reagent flow over the smear while the slide is held at an angle. Stop when the runoff becomes clear. Adjust decolorization time to thickness of smear and type of decolorizer used.

f. Remove excess decolorizer with gentle flow of tap water.

g. Flood the slide with counterstain and allow to remain for at least 30 s and for ≥1 min with fuchsin stains. Use one of the following counterstains.

(1) Safranin

(2) 0.1 to 0.2% basic fuchsin (5, 6)

(3) Carbol fuchsin or 0.1 to 0.8% basic fuchsin counterstain for detecting faintly staining gram-negative organisms (23). See Table 3.2.1–1 for decolorizer options.

Table 3.2.1–1 Gram stain modifications, recommended reagents, timing, and uses

Stain and use	Hucker's		Carbol fuchsin		Kopeloff's	
	Reagent	Time	Reagent	Time	Reagent	Time
Initial stain	Crystal violet	30 s	Crystal violet	30 s	Alkaline crystal violet: flood with solution A; add 5 drops of solution B	2–3 min
Iodine	Gram's iodine	30 s	Gram's iodine	30 s	Kopeloff's iodine	≥2 min
Decolorizer	Acetone-alcohol	~1–5 s	95% ethanol	~30 s	3:7 acetone-alcohol: rinse immediately after applying	
Counterstain	Safranin[a]	30 s	Carbol fuchsin or 0.8% basic fuchsin	≥1 min	Kopeloff's safranin	10–30 s
Recommended use	General bacteriology		*Bacteroides* spp. *Fusobacterium* spp. *Legionella* spp. *Campylobacter* spp. *Brucella* spp. and other faintly staining gram-negative organisms		Anaerobes Diagnosis of bacterial vaginosis (Appendix 3.2.1–3)	

[a] Or, preferably, use 0.1 to 0.2% basic fuchsin as a counterstain (5).

V. PROCEDURE *(continued)*

h. Remove excess counterstain with a gentle flow of tap water.

i. Drain slide, and air dry it in an upright position, or use a commercial slide drier.

2. Kopeloff's modification

◩ **NOTE:** Kopeloff's modification is recommended for better visualization and differentiation of anaerobes, which may easily overdecolorize and stain faintly with Hucker's modification (7–9). It is recommended for vaginal smears to diagnose bacterial vaginosis (*see* Appendix 3.2.1–3).

a. Flood fixed smear with solution A (crystal violet). Add approximately 5 drops of solution B (5% sodium bicarbonate). Blow slide to mix. Then allow the stain to remain for 15 to 30 s or up to 2 min, but do not allow it to dry on the slide.

b. Rinse slide gently with Kopeloff's iodine.

c. Apply fresh Kopeloff's iodine for at least 2 min.

d. Hold the slide in a slanted position, and apply decolorizer. Rinse immediately.

e. Counterstain with Kopeloff's safranin for at least 30 s.

f. Gently rinse excess counterstain with running tap water. Drain slide, and air dry.

◩ **NOTE:** The Gram staining procedure differentiates gram-positive and gram-negative bacteria due to structural differences between the cell envelopes. The animation in reference 4 shows the molecular interactions that occur for gram-positive and gram-negative microbial envelopes during stepwise addition of the Gram stain reagents.

D. Examine the direct smear microscopically.

1. Evaluate the general nature of the smear under low power (defined as 10× objective with 10× eyepiece) (24).

a. Observe for stain crystals.

(1) If an excess of precipitated stain is observed, decolorize and restain slide.

(2) Alternatively, prepare another Gram-stained smear.

(3) If precipitate continues, use freshly filtered crystal violet or counterstain in a clean container.

b. Determine if smear has been properly decolorized.

(1) Depending on the source of the specimen, the background should be generally clear or gram negative.

(2) If WBCs are present, they should appear completely gram negative.

(3) If slide is overdecolorized, completely decolorize and restain slide.

c. Determine if thickness of smear is appropriate. For proper interpretation, areas must be no more than one cell thick, with no overlapping of cells. Prepare new slide if unreadable.

d. For smears prepared from clinical specimens, examine several fields (10 for urine and 20 to 40 for other specimens) under low power for evidence of inflammation.

(1) Observe distribution of organisms and cells.

(2) Determine areas representative of inflammation or purulence and areas of apparent contamination with squamous epithelial cells (SECs). If no purulence is seen, choose areas of apparent necrosis, inflammatory cell debris, and mucus.

2. If cells are present, determine the average count of WBCs and epithelial cells in 20 to 40 representative fields that contain cells.

Table 3.2.1–2 Gram-positive organisms found in direct smears from some clinical sources

Organism(s)	Gram stain morphology[a]	Frequent sources	Comments and additional tests or media that may be included
Actinomyces spp.	Thin, beaded, branched gram-positive filaments; may be within sulfur granules with peripheral clubs	Cervicofacial, thoracic, abdominal, and pelvic abscesses and drainages; pleural fluid; bronchial washings	Modified acid-fast stain; if sulfur granules present, wash and crush in THIO
Nocardia spp.	Long, thin, branching, beaded, gram-positive or irregularly staining bacilli; in culture smears, may be pleomorphic with branching and coccoid forms	Sputum, bronchial washings, biopsy material, purulent exudates, CSF, blood	Modified acid-fast stain; if mixed microbiota, mycobacterial decontamination procedures may be used; plate incubated at 45°C may enhance recovery; use CYE[b] or Thayer-Martin agar.
Mycobacterium spp.	Gram-positive beaded or gram-neutral bacilli; often found inside macrophages; bacilli may be short to long, banded or beaded, and/or slightly curved; some species appear pleomorphic and coccoid	Respiratory tract, urine, blood, biopsy material, CSF	Confirm with acid-fast stain. Add AFB culture.
Corynebacterium spp.	Gram-positive pleomorphic, club shaped, irregularly staining bacilli or coccobacilli with palisading and/or angular arrangements	Blood, tissue aspirates, skin lesions, wounds, indwelling catheters, prosthetic heart valves, upper and lower respiratory tracts	Add selective and differential media for *Corynebacterium diphtheriae,* if suspected.
C. jeikeium	Often small coccobacilli resembling streptococci		
Propionibacterium spp.	Gram-positive, very pleomorphic "diphtheroid" forms that may branch	Blood, CSF, other body fluids, skin lesions	Common skin contaminant during needle aspiration
Listeria monocytogenes	Gram-positive small to medium coccobacilli that may be pleomorphic; occur in short chains or palisades; may be confused with corynebacteria or enterococci	CSF, blood, amniotic fluid, placental or fetal tissue	Direct wet mount for tumbling motility; if mixed microbiota, cold enrichment may be used
Erysipelothrix rhusiopathiae	In tissue, long, slender, gram-positive bacilli; in blood, small "coryneforms"	Skin lesions, biopsy material, tissue aspirates, blood	Associated with occupational or animal contact
Lactobacillus spp.	Medium, straight, uniform gram-positive bacilli with rounded ends; may form chains or spirals; sometimes short and coccobacillary	Usually involved in mixed infections; rarely from blood, CSF	Recovery may be improved by anaerobic incubation; normal vaginal, mouth, and gastrointestinal tract microbiota
Bacillus spp.	Medium to large square-ended gram-positive bacilli with parallel sides with or without spores; some species have spores that swell sides; may stain gram variable or gram negative with age	May be clinically relevant from any source in compromised patient or intravenous-drug abuser; also intraocular	Frequent culture contaminants; may cause ocular infections

Table 3.2.1–2 *(continued)*

Organism(s)	Gram stain morphology[a]	Frequent sources	Comments and additional tests or media that may be included
Clostridium perfringens	Gram-positive large "boxcars" with no spores; may stain gram negative	Blood, wounds, intra-abdominal	Add egg yolk agar incubated anaerobically; absence of inflammatory cells may indicate clostridial myonecrosis; normal gastrointestinal tract microbiota
Clostridium spp.	Gram-positive, -variable, or -negative bacilli with or without spores; bacilli may be large, slender and short, or long; sometimes form coils; often smaller than *Bacillus* spp.	Blood, intra-abdominal, wounds, abscesses	Normal gastrointestinal and genital tract microbiota
S. pneumoniae	Gram-positive cocci in pairs, lancet shapes, short chains	Lower respiratory tract, blood, CSF, sterile fluids	Quellung test may be used on selected clinical specimens.
Enterococcus spp.	Gram-positive cocci in pairs, short chains; may resemble pneumococci	Urine, wounds, blood, intra-abdominal abscesses	Normal gastrointestinal tract microbiota; common cause of superinfections in patients treated with expanded-spectrum cephalosporins
Streptococcus spp.	Round to oval gram-positive cocci, occasionally elongated; in pairs and/or short to long chains; nutritionally variant streptococci often seen as highly pleomorphic, gram-variable to gram-negative coccobacilli with pointed ends and spindle shapes	Blood, CSF, respiratory tract, multiple other sources	May be difficult to distinguish from corynebacteria and lactobacilli
Aerococcus viridans	Gram-positive cocci in pairs, tetrads, clusters	Blood, CSF	
Staphylococcus spp.	Gram-positive cocci in pairs, tetrads, clusters	Abscesses, drainages, wounds, respiratory tract, blood, tissue, sterile fluids, indwelling catheters	Normal microbiota, especially skin, nares
S. aureus	May often be characterized by very uniform, geometric clusters of small cocci, whereas coagulase-negative species are often irregular and more pleomorphic, with greater size variation		
Rothia mucilaginosa	Large gram-positive cocci in pairs, tetrads	Blood in compromised patients, peritoneal dialysates	Normal oral microbiota

[a] See references 15 to 17 and 20.

[b] CYE, charcoal-yeast extract agar.

Table 3.2.1–3 Gram-negative organisms seen in direct smears from some clinical sources

Organism(s)	Gram stain morphology[a]	Frequent sources	Comments
Enterobacteriaceae	Straight thick bacilli; short to medium length with rounded ends; antimicrobial agent-affected organisms may be pleomorphic, filamentous, and/or irregularly staining	Urinary tract, multiple other sources	Includes organisms that cause gastroenteritis and bacterial dysentery; also normal gastrointestinal tract microbiota; nosocomial strains may be multiply resistant to antimicrobial agents
Pseudomonas spp.	Thin bacilli; medium length to long with rounded to pointy ends; often in pairs; antimicrobial agent-affected organisms may appear filamentous, coiled, and/or pleomorphic	Lower respiratory tract, wounds, eyes, multiple sites in compromised patients	Nosocomial strains may be multiply resistant to antimicrobial agents
Haemophilus spp., *Pasteurella* spp., fastidious gram-negative bacilli	Small coccoid to bacillary forms; pleomorphic; often with filamentous forms; may be faintly staining	Blood, sterile fluid (including CSF), lower respiratory tract, abscesses, wounds, eyes, genital tract	Inoculate CHOC.
Legionella spp.	Pleomorphic slender bacilli of variable lengths that may stain pale; may not take stain in clinical specimens	Lower respiratory tract	Add special growth media; direct fluorescent antibody stains and molecular probes available; acid wash method may be used to enhance recovery from specimens with mixed microbiota
Fusobacterium nucleatum, *Capnocytophaga* spp.	Long slender bacilli with tapered to pointed ends; "needlelike"; may be in pairs, end to end, or filamentous	Respiratory tract, wounds, blood, abscesses	Endogenous microbiota
Fusobacterium necrophorum, *Fusobacterium mortiferum*, or *Fusobacterium varium*	Highly pleomorphic bacilli with rounded to tapered ends; pale and irregularly staining, with bizarre forms and round bodies	Wounds, blood, abscesses	Endogenous microbiota
Bacteroides spp.	Pleomorphic straight bacilli with possible irregular to bipolar staining	Wounds, blood, abscesses	Direct fluorescent antibody stain available; endogenous microbiota
Vibrio spp.	Slightly curved to straight bacilli	Stool, wounds	If mixed microbiota, selective medium (TCBS) recommended
Campylobacter spp. (*Helicobacter* spp.)	Thin, curved bacilli, including S shapes, gull wings, and long spiral forms	Stool, blood, gastric biopsy samples	Microaerophilic or capnophilic atmosphere required; if mixed microbiota, 42°C incubation recommended for recovery of thermophilic species
Acinetobacter spp.	Medium to large cocci in pairs; occasionally coccoid, bacillary, and filamentous forms; often resistant to decolorization	Urine, lower respiratory tract, blood, sterile fluids, wounds, abscesses, tissues, stool	Multiple sites in compromised patients
Neisseria spp., *Moraxella catarrhalis*	Medium to large cocci in pairs and tetrads, coffee bean shaped; no bacilli seen	Genital tract, urine, lower respiratory tract, blood, sterile fluids, wounds, abscesses	If mixed microbiota, selective medium may be used to enhance recovery of *Neisseria gonorrhoeae*
Veillonella spp.	Tiny cocci in sheets or clumps	Wounds, blood	Endogenous microbiota

Table 3.2.1–3 *(continued)*

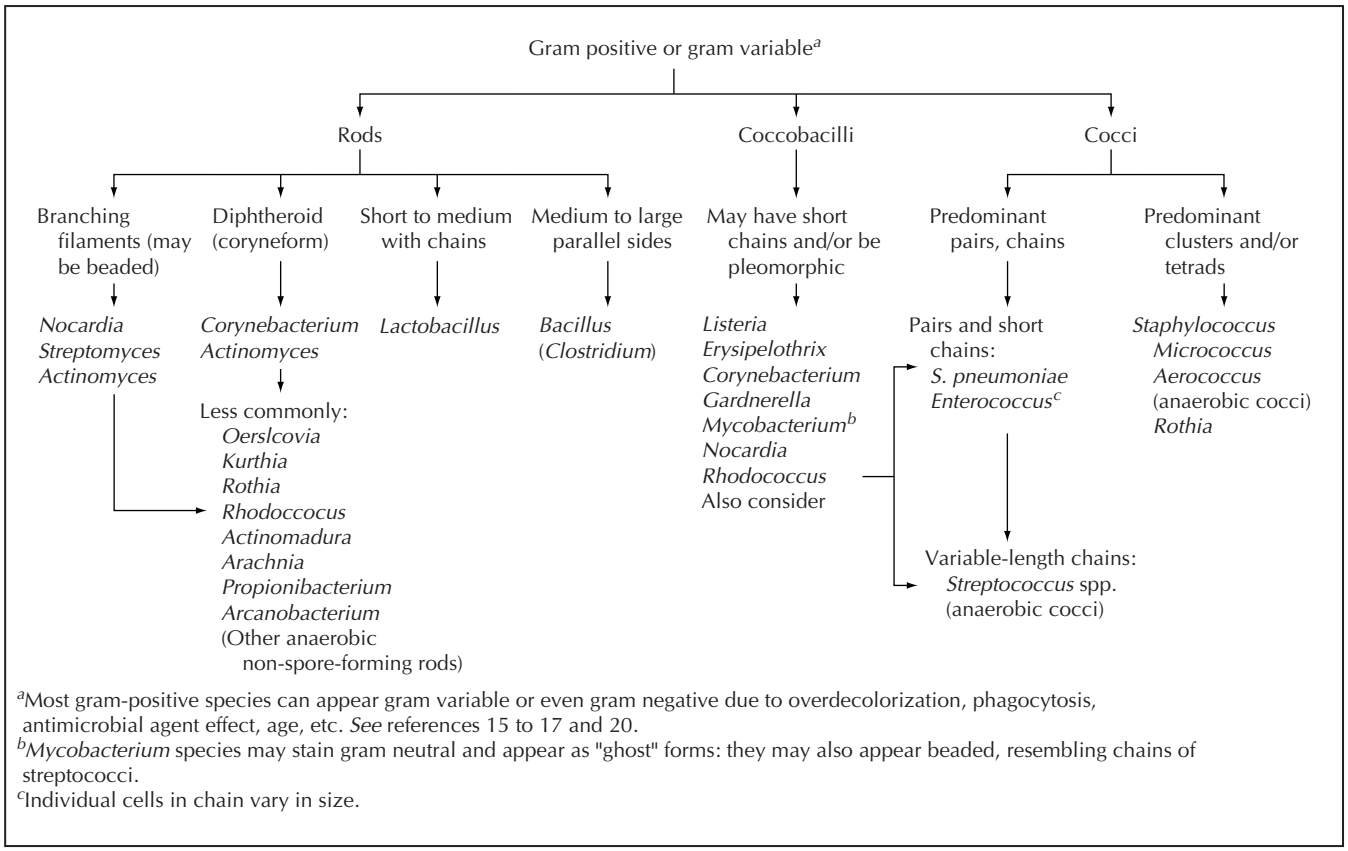
Organism(s)	Gram stain morphology[a]	Frequent sources	Comments
Pneumocystis carinii	Gram-negative spherical cysts (5–7 μm) often containing rosette of eight gram-negative intracystic bodies, or cluster of gram-negative cysts surrounded by halos in background of amorphous gram-negative material	Open lung and transtracheal biopsy materials, bronchial washings and lavage specimens, sputum	Confirm with Grocott methenamine-silver, toluidine blue O, Gram-Weigert, or direct or indirect fluorescent antibody stain.
Blastomyces dermatitidis	Gram-variable, broad-based, thick-walled yeast cell with figure-eight appearance	Bronchial washings, sputum, purulent exudates, skin lesions	
Microsporidia	Gram-variable spherical cells (1–4 μm), thicker at one end	Stool, respiratory, cornea, urine	Confirm with chromotrope stain
Cryptococcus neoformans	Partially or completely gram-positive round yeast cell with clear or red-orange halo; yeast cells may appear stippled or gram neutral	CSF, blood, biopsy material, sputum, bronchial washings, cutaneous lesions	Confirm capsule with India ink; direct antigen detection procedures may be used on CSF; inoculate urea slant.
Candida spp.	Gram-positive budding yeast cell with or without pseudohyphae; may also appear stippled or gram neutral	Sputum, urine, blood, biopsy material, vaginal discharge, upper respiratory tract	Endogenous microbiota
Malassezia furfur	Bottle-shaped yeast cells in compact clusters, usually with short hyphal elements	Skin scrapings, blood drawn through catheter lines, hyperalimentation fluids	Inoculate lipid-enriched medium.

[a] See references 18 to 20.

V. PROCEDURE *(continued)*

3. In a representative area with a preponderance of inflammation or purulence using the oil immersion lens (defined as 100× objective with 10× eyepiece), examine 20 to 40 fields to observe cell morphology and Gram reaction.
   a. If rare or no organisms are seen from a normally sterile-site specimen, but the specimen appears purulent, or the specimen looks suspicious, perform a more extensive review of the slide.
   b. Refer to Tables 3.2.1–2 and 3.2.1–3 for characteristic morphologies that may be seen in smears from clinical specimens.

4. Preservation of direct smear slides
   a. Drain or gently blot excess oil from slide and save slides for further evaluation for a minimum of 1 week to allow a confirmatory review, especially if culture or other laboratory test results are inconsistent.
   b. Should a slide need to be restained to repeat Gram stain or prepare a special stain to confirm findings suggested by a Gram stain, remove the immersion oil with xylene or a xylene substitute and decolorize smear. Then restain slide.
   c. For slide libraries and teaching collections that will be stored for longer periods, remove immersion oil with xylene and overlay smear with a sealer to prevent fading.



[a]Most gram-positive species can appear gram variable or even gram negative due to overdecolorization, phagocytosis, antimicrobial agent effect, age, etc. *See* references 15 to 17 and 20.
[b]*Mycobacterium* species may stain gram neutral and appear as "ghost" forms: they may also appear beaded, resembling chains of streptococci.
[c]Individual cells in chain vary in size.

Figure 3.2.1–3 Typical Gram stain morphologies of gram-positive and gram-variable genera.

V. PROCEDURE *(continued)*

E. Examination of broth and plate smears
   1. Specify Gram stain morphology (e.g., gram-positive cocci in clusters).
   2. Specify probable genus or organism group based on Gram stain morphology, colonial morphology, atmospheric requirements (aerobic or anaerobic), and results of any rapid biochemical tests (e.g., probable *Haemophilus* spp. for small pleomorphic aerobic gram-negative bacilli from transparent colonies with a "mousy" odor growing only on CHOC).
   3. Refer to Fig. 3.2.1–3 and 3.2.1–4 for typical morphological descriptions of bacterial genera.

F. When discarding stained smears, handle as biological waste. Treat slides as sharps, since they may puncture biohazard bags. If cardboard boxes are used, seal with tape prior to discarding.

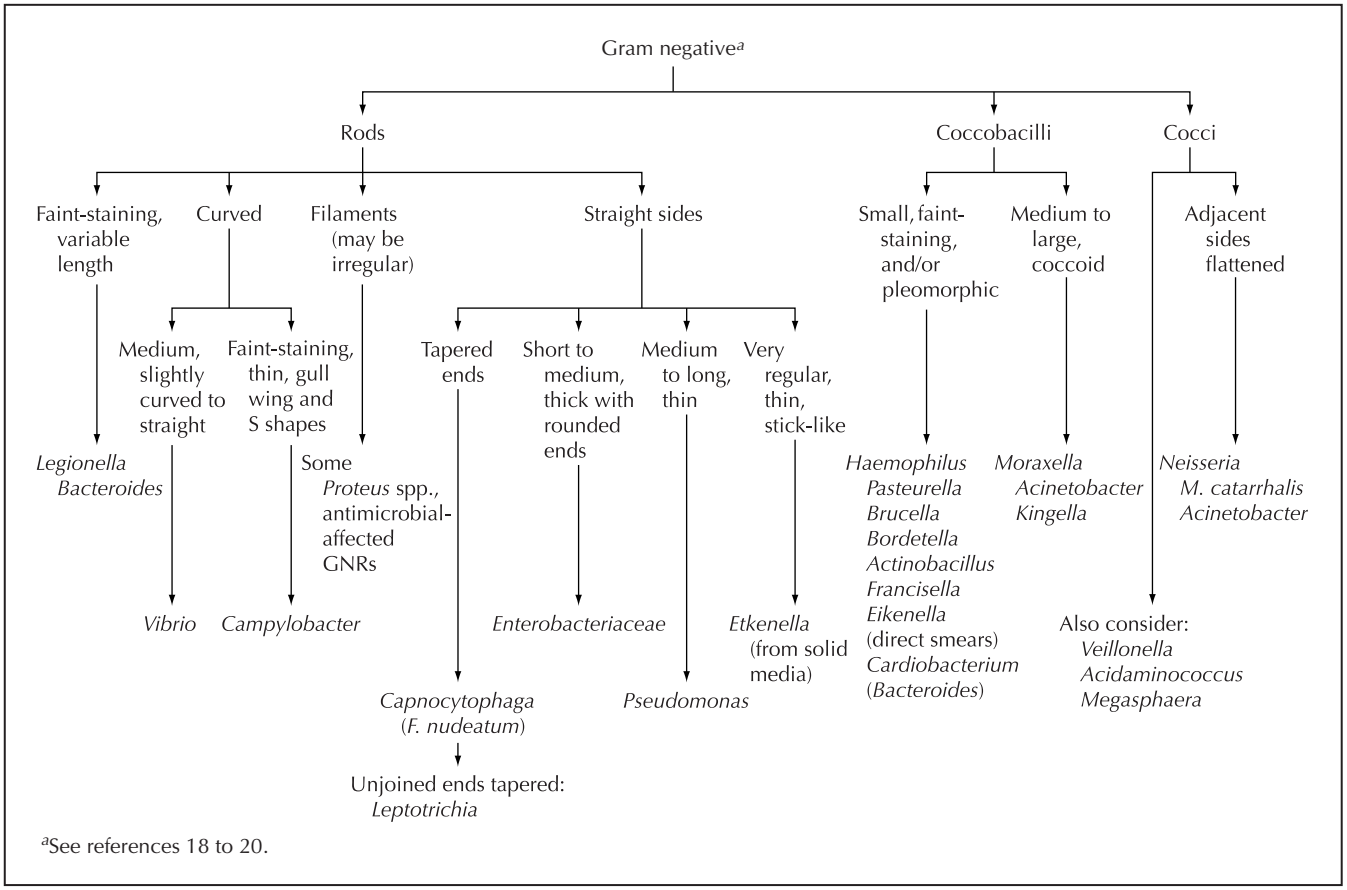


Figure 3.2.1–4 Typical Gram stain morphologies of gram-negative genera.

POSTANALYTICAL CONSIDERATIONS

VI. INTERPRETATION

A. Low-power observations

1. WBCs and RBCs suggest infectious process.

◩ **NOTE:** Neutropenic patients may have few WBCs, but they may have necrotic debris and protein in the background.

2. SECs, food debris, etc., suggest an improperly collected specimen (*see* Appendix 3.2.1–2)

3. Certain microorganisms (parasitic forms, branching hyphae, etc.) indicate infectious process.

B. Oil immersion observations

1. Observe microorganisms for characteristic morphology and presentation. See Fig. 3.2.1–3 and 3.2.1–4 for species consistent with each morphology.

2. Gram reaction

a. Gram positive: deep violet

b. Gram negative: pink or red (carbol fuchsin counterstains have a more intense color)

c. Gram variable: both gram-positive and gram-negative cells with the same morphology

◩ **NOTE:** This may result from a smear of uneven thickness, incomplete decolorization, overdecolorization, presence of older cells, damage of cell walls, or gram-variable nature of the particular organism.

VI. INTERPRETATION
(continued)

d. Gram neutral: taking up neither the crystal violet nor the counterstain
◩ **NOTE:** These cells appear colorless against a generally gram-negative background and may be intracellular. This reaction has been seen in smears of clinical specimens when fungal elements or *Mycobacterium* spp. are present (3).

3. Staining characteristics, predominant shapes of microorganisms, relative sizes, and characteristic arrangements: see Table 3.2.1–4

C. The presence of microorganisms from a normally sterile site is likely to indicate infection with an organism.

D. Physician offices may perform Gram stains on unspun urine. The presence of microorganisms is likely to indicate a bacterial count of $\geq 10^5$ CFU/ml in unspun urine (procedure 3.12).

E. The presence of large numbers of a single type of microorganism in a noninvasively collected specimen, especially if associated with WBCs, is likely to indicate infection.

F. Be wary of interpretations made from observing very few organisms (especially in the absence of inflammation or if the organisms are unevenly distributed), because collection tubes, slides, and media may harbor nonviable bacteria. *For critical specimens, where the results will define an infectious process (e.g., CSF smears), prepare a second smear to confirm rare findings of microorganisms.*

Table 3.2.1–4 Common descriptions of bacterial Gram staining characteristics

Bacterial Gram staining characteristics	Common descriptions
Staining characteristics	Even, bipolar, beaded, stippled, barred, irregular
Predominant shapes	Round, coccoid, coccobacillary, rod, filament, yeastlike
Ends of cells[a]	Rounded, pointed, tapered, flattened, clubbed (swollen), concave
Sides	Parallel, ovoid (bulging), concave, irregular
Axis	Straight, curved, spiral
Pleomorphism[b]	Variation in shape
Relative size[c]	
Overall	Minute or tiny (<0.3 μm), small (~0.3–0.5 μm), medium, or large
Length	Short (~0.5–1 μm), medium, long, or filamentous (10–30 μm)
Width	Thin, medium, or thick
Pleomorphic	Variation in size
Characteristic arrangements	Singles, pairs, chains, tetrads, clusters, palisades, Chinese letters, packets, angular forms (V and L shapes), etc.

[a] Swelling of sides can suggest the presence of spores but may also be due to the presence of vacuoles, inclusions, marked pleomorphism, or irregular staining. Phase-contrast microscopy or a spore stain may be helpful in observing bacterial endospores.

[b] The descriptive term "diphtheroid" or "coryneform" is used to describe gram-positive bacteria that are pleomorphic, club shaped, or irregularly staining or that have palisading and/or angular arrangements (V and L shapes).

[c] Sizes listed are only guidelines. The average size of an RBC is 7 μm, and cytoplasmic granules in neutrophils average 0.2 to 0.3 μm.

VII. REPORTING RESULTS

A. If no organisms or cells are detected in a smear of a clinical specimen, report "No organisms seen" or "No cells seen," respectively.

◩ **NOTE:** While there is no scientific basis for a policy on enumeration of cells and bacteria in stained smears, the following standard is the most applicable to all specimens and will provide consistency in the laboratories. The enumeration of cells is based on the publications documenting the relationship between cells and contaminated respiratory specimens (Appendix 3.2.1–2), and the enumeration of bacteria is based on the publications for counts for female genital specimens (Appendix 3.2.1–3). *Counts should be performed only in areas representative of inflammation or necrosis, if present.*

B. Determine number of cells and bacteria (Table 3.2.1–5).

Table 3.2.1–5 Reporting Gram stain results[a]

Enumeration of cells under low-power objective[b]	Description of the types of cells to report	Enumeration of bacteria under oil immersion objective[c]	Description of the morphology of bacteria[d]
Count each type of cell and report: 1+ (rare or occasional): <1/LPF 2+ (few): 1–9/LPF 3+ (moderate): 10–25/LPF 4+ (heavy): >25/LPF	ECs PMNs RBCs Host cellular material	Count bacteria and yeasts from areas associated with cells and report: 1+ (rare or occasional): <1/OIF 2+ (few): 1–5/OIF 3+ (moderate): 6–30/OIF 4+ (heavy): >30/OIF	Gram positive Cocci in pairs (and chains) Cocci in clusters Large bacilli Small bacilli Branching bacilli Coryneform bacilli Gram negative Diplococci Bacilli Bacilli, filamentous (or pleomorphic) Gram variable: coccobacilli Budding yeast cells Pseudohyphae

[a] Determine the number of cells and bacteria in 20 to 40 fields of the smear using an 18- to 22-mm lens field of view. Skip fields where there are no cells or bacteria, and do not average these fields in the counts if there are fields where cells and/or bacteria are present.

[b] The Gram stain is not a stain that demonstrates host cellular morphology, and the main cells routinely enumerated are WBCs (i.e., polymorphonuclear cells), which determine if the sample is purulent as an indication of infection. For other types of cells, there is no clinically useful reason to report cells present in amounts of <10/40 fields from a Gram stain. LPF, low-power field.

[c] Ignore one or two microorganisms on the entire slide, unless the results can be reproduced on a second smear and only then if it is from an invasively collected specimen. OIF, oil immersion field.

[d] Microbiologists should be encouraged to specify Gram stain morphology and resemblance to organism group consistent with the source if the presentation is classic; however, use caution when in doubt, as other species may mimic the typical presentations, including (i) gram-positive cocci in pairs, consistent with *Streptococcus pneumoniae*; (ii) small gram-negative coccobacilli, consistent with *Haemophilus*; (iii) tiny gram-positive bacilli, consistent with *Listeria*; and (iv) gram-negative diplococci, consistent with *Neisseria*.

VII. REPORTING RESULTS *(continued)*

C. Refer to Appendixes 3.2.1–2 and 3.2.1–3 for special reporting of respiratory specimens and female genital specimens, respectively.

D. *Notify the caregiver or physician of record, depending on local policy, of any clinically significant findings (any bacteria from a normally sterile site). Document notification, including date and time of notification.*

VIII. LIMITATIONS

A. The sensitivity of the Gram stain is 10^5 cells/ml or 10^4/ml if the specimen has been prepared with the cytocentrifuge (14). This is particularly applicable to the smear of a drop of urine, where an average of one bacterial cell per field from an examination of 20 fields corresponds to a count of $\geq 10^5$ CFU/ml.

B. Gram stain of cytocentrifuged BAL with one or more organisms per OIF correlates with active bacterial pneumonia (20).

C. Use results of Gram stains in conjunction with other clinical and laboratory findings. Use additional procedures (e.g., special stains, inclusion of selective media, etc.) to confirm findings suggested by Gram-stained smears (3).

D. Careful adherence to procedure and interpretive criteria is required for accurate results. Accuracy is highly dependent on the training and skill of microscopists (22).

E. Additional staining procedures, such as acridine orange (procedure 3.2.2), are recommended for purulent clinical specimens in which no organisms are observed by the Gram stain method.

F. Gram stain-positive, culture-negative specimens may be the result of contamination of reagents and other supplies, presence of antimicrobial agents, or failure of organisms to grow under usual culture conditions (medium, atmosphere, etc.).

G. False Gram stain results may be related to inadequately collected specimens or delays in transit.

REFERENCES

1. **Bartholomew, J. W.** 1962. Variables influencing results, and the precise definition of steps in Gram staining as a means of standardizing the results obtained. *Stain Technol.* **37:**139–155.
2. **Bottone, E. J.** 1988. The Gram stain: the century-old quintessential rapid diagnostic test. *Lab. Med.* **19:**288–291.
3. **Brown, M. S., and T. C. Wu.** 1986. The Gram stain morphology of fungi, mycobacteria, and *Pneumocystis carinii*. *J. Med. Technol.* **3:**495–499.
4. **Cavanaugh, D., and M. Keen.** 2005. *The Gram Stain: an Animated Approach.* http://www.microbelibrary.org.
5. **Chapin, K.** 2007. Principles of stains and media, p. 182–191. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
6. **Chapin, K. C., and T.-L. Lauderdale.** 2007. Reagents, stains, and media: bacteriology, p. 334–364, *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
7. **Clarridge, J. E., and J. M. Mullins.** 1987. Microscopy and staining, p. 87–103. *In* B. J. Howard (ed.), *Clinical and Pathogenic Microbiology.* The C. V. Mosby Co., St. Louis, MO.
8. **Conn, H. J., M. A. Darrow, and V. M. Emmel (ed.).** 1960. Stains for microorganisms in smears, p. 226–229. *In Staining Procedures Used by the Biological Stain Commission,* 2nd ed. The Williams & Wilkins Co., Baltimore, MD.
9. **Dowell, V. R., and T. M. Hawkins.** 1979. *Laboratory Methods in Anaerobic Bacteriology: CDC Laboratory Manual.* Center for Disease Control, Atlanta, GA.
10. **Forbes, B. A., D. F. Sahm, and A. S. Weissfeld.** 2007. Meningitis and other infections of the central nervous system, p. 828–829. *In Bailey's & Scott's Diagnostic Microbiology,* 12th ed. Mosby Elsevier, St. Louis, MO.
11. **Forbes, B. A., D. F. Sahm, and A. S. Weissfeld.** 2007. Role of microscopy, p. 78–92. *In Bailey's & Scott's Diagnostic Microbiology,* 12th ed. Mosby Elsevier, St. Louis, MO.
12. **Mangels, J. I., M. E. Cox, and L. H. Lindberg.** 1984. Methanol fixation: an alternative to heat fixation of smears before staining. *Diagn. Microbiol. Infect. Dis.* **2:**129–137.
13. **Murray, P. R., and J. A. Washington.** 1975. Microscopic and bacteriologic analysis of sputum. *Mayo Clin. Proc.* **50:**339–344.

REFERENCES *(continued)*

14. **Shanholtzer, C. J., P. Schaper, and L. R. Peterson.** 1982. Concentrated Gram stain smears prepared with a cytospin centrifuge. *J. Clin. Microbiol.* **16:**1052–1056.
15. **Smith, A. C., and M. A. Hussey.** 2005. *Gram Stain: Gram-Positive Rods.* http://www.microbelibrary.org.
16. **Smith, A. C., and M. A. Hussey.** 2005. *Gram Stain: Gram-Variable Rods and Cocci.* http://www.microbelibrary.org.
17. **Smith, A. C., and M. A. Hussey.** 2005. *Gram Stain: Gram-Positive Cocci.* http://www.microbelibrary.org.
18. **Smith, A. C., and M. A. Hussey.** 2005. *Gram Stain: Gram-Negative Cocci.* http://www.microbelibrary.org.
19. **Smith, A. C., and M. A. Hussey.** 2005. *Gram Stain: Gram-Negative Rods.* http://www.microbelibrary.org.
20. **Tenover, F., and J. V. Hirschmann (edited and revised by R. Buxton).** 2007. *Interpretation of Gram Stains and Other Common Microbiologic Slide Preparations.* http://www.microbelibrary.org.
21. **U.S. Department of Health and Human Services.** 1999. *Biosafety in Microbiology and Biomedical Laboratories*, 4th ed. U.S. Department of Health and Human Services stock no. 017-040-00547-4. U.S. Government Printing Office, Washington, DC.
22. **Washington, J. A.** 1986. Rapid diagnosis by microscopy. *Clin. Microbiol. Newsl.* **8:**135–137.
23. **Weaver, R. E., and J. C. Feeley.** 1979. Cultural and biochemical characterization of the Legionnaires' disease bacterium, p. 20–25. *In* G. L. Jones and G. A. Hebert (ed.), *"Legionnaires": the Disease, the Bacterium and Methodology*. Center for Disease Control, Atlanta, GA.
24. **Wiedbrauk, D. L.** 2007. Microscopy, p. 173–181. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.

SUPPLEMENTAL READING

Chapin-Robertson, K., S. E. Dahlberg, and S. C. Edberg. 1992. Clinical and laboratory analyses of cytospin-prepared Gram stains for recovery and diagnosis of bacteria from sterile body fluids. *J. Clin. Microbiol.* **30:**377–380.

Church, D., E. Melnyk, and B. Unger. 2000. Quantitative Gram stain interpretation criteria used by microbiology laboratories in Alberta, Canada. *J. Clin. Microbiol.* **38:**4266–4268.

APPENDIX 3.2.1–1

Include QC information on reagent container and in QC records.

Preparation of Gram Stain Reagents

A. Hucker's crystal violet

1. Crystal violet stock solution

   crystal violet (90 to 95% dye content) …40 g
   ethanol, 95% ……400 ml

   Dissolve (may take overnight) and mix in a glass bottle, label with a 1-year expiration date, and store at room temperature.
   Caution: *Ethanol is flammable.*
2. Ammonium oxalate solution (1%)

   ammonium oxalate (reagent grade) …….16 g
   distilled water ……1,600 ml

   Dissolve and mix in a brown glass bottle, label with a 1-year expiration date, and store at room temperature.
3. Crystal violet working solution

   crystal violet stock solution ……40 ml
   ammonium oxalate solution (1%) …….160 ml

   Filter crystal violet stock solution into a glass bottle. Allow to filter completely, and then filter ammonium oxalate solution. Label with earliest expiration date of stock solutions.

B. Gram's iodine

1. Stock Lugol's iodine solution

   iodine crystals (reagent grade) ……25 g
   potassium iodide (reagent grade) …….50 g
   distilled water ……500 ml

   Mix or let stand until dissolved in a brown glass bottle, label with a 6-month expiration date, and store at room temperature.

APPENDIX 3.2.1–1 *(continued)*

2. Sodium bicarbonate, 5% (wt/vol)

sodium bicarbonate ($NaHCO_3$), reagent grade ……50 g
distilled water ……1,000 ml

Dissolve in a glass bottle, label with a 1-year expiration date, and store at room temperature.

3. Gram's iodine

stock Lugol's iodine solution ……60 ml
distilled water ……220 ml
sodium bicarbonate, 5% ……60 ml

Mix in a brown glass bottle, label with a 6-month expiration date, and store at room temperature.
Caution: *Iodine and potassium iodide are corrosive. Avoid inhalation, ingestion, and skin contact. Do not store near acids.*

C. Counterstains

1. Safranin
   a. Safranin stock solution

safranin O (certified) ……5.0 g
ethanol, 95% ……200 ml

Dissolve in glass bottle, label with a 1-year expiration date, and store at room temperature.
Caution: *Ethanol is flammable.*
   b. Safranin working solution

safranin stock solution ……20 ml
distilled water ……180 ml

Combine in a glass bottle, label with a 1-year expiration date, and store at room temperature.

2. Basic fuchsin, 0.1, 0.2, or 0.8% (wt/vol)

basic fuchsin (reagent grade) ….. 1, 2, or 8 g
ethyl alcohol ……100 ml
distilled water ……900 ml

Add basic fuchsin to a brown glass bottle. Slowly add ethyl alcohol and let sit overnight. Filter through Whatman no. 1 filter paper. Add distilled water. Label with a 1-year expiration date, and store at room temperature.

3. Carbol fuchsin counterstain
   a. Solution A

basic fuchsin (reagent grade) ……3 g
ethanol, 95% ……100 ml

   b. Solution B

melted phenol crystals ……50 ml
distilled water ……950 ml

Dissolve basic fuchsin in ethanol in a brown glass bottle (solution A). Add phenol to distilled water in a separate flask (solution B). Add solution B to solution A, label with a 1-year expiration date, and store at room temperature.
Caution: *Ethanol is flammable, and phenol is corrosive. Avoid inhalation, ingestion, and skin contact.*

D. Kopeloff's modification (for anaerobes)

1. Alkaline crystal violet
   a. Solution A

crystal violet (90 to 95% dye content) …10 g
distilled water ……1,000 ml

Dissolve in a glass bottle, label with a 1-year expiration date, and store in glass-stoppered bottle at room temperature.

APPENDIX 3.2.1–1 *(continued)*

b. Solution B: sodium bicarbonate, 5% (wt/vol)

sodium bicarbonate ($NaHCO_3$), reagent grade	50 g
distilled water	1,000 ml

Dissolve in a glass bottle, label with a 1-year expiration date, and store at room temperature.

2. Iodine (Kopeloff's modification)

sodium hydroxide (NaOH), reagent grade	4 g
distilled water	25 ml
iodine crystals (reagent grade)	20 g
potassium iodide (reagent grade)	1 g
distilled water	975 ml

Dissolve NaOH in 25 ml of distilled water in a brown glass bottle. Add iodine and potassium iodide, and dissolve them well. Gradually add 975 ml of distilled water, mixing well after each addition.
Caution: *Iodine and potassium iodide are corrosive. Avoid inhalation, ingestion, and skin contact.*

3. Decolorizer: 3:7 acetone-alcohol

ethanol, 95%	700 ml
acetone (reagent grade)	300 ml

Combine and mix in a brown glass bottle, label with a 1-year expiration date, and store at room temperature.
Caution: *Ethanol and acetone are flammable.*

4. Safranin counterstain (Kopeloff's)

safranin O, certified	20 g
ethanol, 95%	100 ml
distilled water	1,000 ml

In a 1,000-ml glass bottle, add only enough ethanol to the safranin to dissolve it (approximately 50 ml). Add distilled water to safranin solution, label with a 1-year expiration date, and store at room temperature. (Basic fuchsin, 0.8% [wt/vol], may also be used for counterstain.)
Caution: *Ethanol is flammable.*

APPENDIX 3.2.1–2

Rejection Criteria for Sputum and Endotracheal Aspirates for Culture

I. RATIONALE

Despite the frequency of lower respiratory tract infection, diagnostic studies to detect and identify the etiologic agent are insensitive (8). Whether to perform a Gram stain or a culture has been the topic of repeated studies with conflicting conclusions from professional societies, particularly for evaluating cases of community-acquired pneumonia (2, 3, 7). Part of the problem with the Gram stain is the variability of sampling for smears and cultures (1, 6, 9). Everyone does agree that the culture of poorly collected respiratory specimens is a wasteful use of laboratory resources and can lead to erroneous reporting and treatment of patients (3, 5, 10). For laboratories that receive respiratory specimens for smear and culture, the following generally accepted policy should be followed.

II. REJECTION CRITERIA

A. Do not reject sputum and endotracheal aspirates for culture for *Legionella* or AFB, or specimens from cystic fibrosis patients.

B. Examine 20 to 40 fields from sputum smears under low power and endotracheal smears under both low power and oil immersion using an 18- to 22-mm lens field of view. Average the number of cells in representative fields that contain cells. Reject the following for culture, as poorly collected or not consistent with a bacterial infectious process.

1. Sputum: ≥10 SECs/LPF (3, 5, 10)

◩ **NOTE:** If the number of WBCs is 10 times the number of SECs and there is 3 to 4+ of a *single* morphotype of bacteria, accept the specimen for culture. Some

APPENDIX 3.2.1–2 *(continued)*

authors suggest using >25 SECs/LPF as a criterion, but generally too few sputa are rejected with this policy (10).

2. Tracheal aspriates from adults: ≥10 SECs/LPF or no organisms seen (4)
3. Tracheal aspriates from pediatric patients: no organisms seen (11)

◩ **NOTE:** if no organisms are seen in a specimen with numerous (4+) WBCs and cellular debris, it might be useful to flood the smear with acridine orange (procedure 3.2.2) and observe using fluorescent microscopy to confirm the absence of organisms in the debris. Both pseudomonads and *Haemophilus* can be missed in such smears, because they cannot be distinguished among the cellular debris (Mary K. York, personal observation). In addition, *Legionella* can be visualized with the acridine orange stain, although WBCs are often lacking in this infectious process.

III. REPORTING

When rejecting the specimen for culture, report one of the following as appropriate.

A. "Smear contains ≥10 squamous epithelial cells per low power field, suggestive of poor quality; culture not performed. Please recollect if clinically indicated."
B. "Smear is negative for bacteria after examination of 40 fields; culture not performed. Contact laboratory if further studies are clinically indicated."

IV. FOLLOW-UP

A. Notify the caregiver that the specimen will not be cultured.
B. Charge for the smear but not for the culture.

◩ **NOTE:** Particularly for outpatient specimens and those that are grossly purulent, the culture should be inoculated immediately upon receipt without waiting for the results of the smear. Plates from rejected specimens can be discarded or separated from the other cultures and examined later to validate the laboratory policy and staff competency.

References

1. **Cooper, G. M., J. J. Jones, J. C. Arbique, G. J. Flowerdew, and K. R. Forward.** 2000. Intra- and inter-technologist variability in the quality assessment of respiratory tract specimens. *Diagn. Microbiol. Infect. Dis.* **37:**231–235.
2. **Mandell, L. A., T. J. Marrie, R. F. Grossman, A. W. Chow, R. H. Hyland, and The Canadian Community-Acquired Pneumonia Working Group.** 2000. Canadian guidelines for the initial management of community-acquired pneumonia: an evidence-based update by the Canadian Infectious Diseases Society and the Canadian Thoracic Society. *Can. Respir. J.* **7:**371–382.
3. **Mandell, L. A., R. G. Wunderink, A. Anzueto, J. G. Bartlett, G. D. Campbell, N. C. Dean, S. F. Dowell, T. M. File, Jr., D. M. Musher, M. S. Niederman, A. Torres, and C. G. Whitney.** 2007. Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. *Clin. Infect. Dis.* **44:**S27–S72.
4. **Morris, A. J., D. C. Tanner, and L. B. Reller.** 1993. Rejection criteria for endotracheal aspirates from adults. *J. Clin. Microbiol.* **31:**1027–1029.
5. **Murray, P. R., and J. A. Washington.** 1975. Microscopic and bacteriologic analysis of sputum. *Mayo Clin. Proc.* **50:**339–344.
6. **Nagendra, S., P. Bourbeau, S. Brecher, M. Dunne, M. LaRocco, and G. Doern.** 2001. Sampling variability in the microbiological evaluation of expectorated sputa and endotracheal aspirates. *J. Clin. Microbiol.* **39:**2344–2347.
7. **Niederman, M. S., L. A. Mandell, A. Anzueto, J. B. Bass, W. A. Broughton, G. D. Campbell, N. Dean, T. File, M. J. Fine, P. A. Gross, F. Martinez, T. J. Marrie, J. F. Plouffe, J. Ramirez, G. A. Sarosi, A. Torres, R. Wilson, and V. L. Yu.** 2001. Guidelines for the management of adults with community-acquired pneumonia. Diagnosis, assessment of severity, antimicrobial therapy, and prevention. *Am. J. Respir. Crit. Care Med.* **163:**1730–1754.
8. **Reimer, L. G., and K. C. Carroll.** 1998. Role of the microbiology laboratory in the diagnosis of lower respiratory tract infections. *Clin. Infect. Dis.* **26:**742–748.
9. **Roson, B., J. Carratala, R. Verdaguer, J. Dorca, F. Manresa, and F. Gudiol.** 2000. Prospective study of the usefulness of sputum Gram stain in the initial approach to community-acquired pneumonia requiring hospitalization. *Clin. Infect. Dis.* **31:**869–874.
10. **Wong, L. K., A. L. Barry, and S. M. Morgan.** 1982. Comparison of six different criteria for judging the acceptability of sputum specimens. *J. Clin. Microbiol.* **16:**627–631.
11. **Zaidi, A. K., and L. B. Reller.** 1996. Rejection criteria for endotracheal aspirates from pediatric patients. *J. Clin. Microbiol.* **34:**352–354.

APPENDIX 3.2.1–3

Reporting Gram-Stained Vaginal Smears To Diagnose Bacterial Vaginosis and Vaginitis

I. RATIONALE

Bacterial vaginosis (BV) is a clinical syndrome characterized by an abnormal vaginal discharge in women in childbearing years accompanied by a rise in pH from 4.5 and an amine (fishy) smell, especially after addition of KOH to the discharge (6). The microbiota of the vagina shifts from predominantly lactobacilli to a mixture of *Gardnerella vaginalis*, *Prevotella* spp., *Mobiluncus* spp., and often other anaerobes and *Mycoplasma hominis* (1, 5). Greater than 10^7 CFU of these microorganisms per g of vaginal fluid are present with a decrease in the number of lactobacilli, which are no longer the predominant microorganisms (5). In addition, clue cells, vaginal SECs coated with bacteria such that the cell borders are obliterated, can also be present. BV is a risk factor for obstetric sequelae such as low birth weight and premature delivery. The syndrome has been associated with preterm birth, miscarriage, amniotic infections, and postpartum endometritis (2, 3). Treatment with topical metronidazole or clindamycin is effective, but the syndrome can recur (1). Preliminary diagnosis is usually made in the physician's office by checking the pH of the discharge with an indicator stick and adding KOH to detect the odor, although the usage of these inexpensive tests is reportedly low. The clinic-based microscopic evaluation of the vaginal fluid by wet mount is the most frequently used test, but laboratory-based testing is sometimes desired because no microscope is available or the evaluation of the wet mount is inconclusive.

The Gram stain with scoring of relative amounts of microbial morphotypes is the definitive laboratory method for diagnosis (4). *G. vaginalis* is a small gram-negative to gram-positive pleomorphic bacillus, varying from coccobacillus to longer forms up to 2 to 3 μm; cells can palisade or appear coryneform and irregularly shaped. *Mobilucus* organisms are curved gram-negative rods. The relative numbers of lactobacilli (medium to large gram-positive rods) compared to gram-negative curved rods and gram-variable coccobacilli aid in the diagnosis of BV.

The Gram stain can be useful in the diagnosis of candidiasis in women, when the timing does not allow evaluation of the discharge in the physician's office by wet mount, pH, and odor. Vaginal culture for yeasts is more sensitive than Gram stain and may be useful for the detection of yeasts in women with symptoms consistent with yeast vaginitis whose wet mount examinations are negative. Candidiasis is characterized by a white discharge and a normal pH below 4.2. The Gram stain is less helpful in the diagnosis of purulent vaginitis, characterized by a macroscopically yellowish-green, foul-smelling discharge containing ≥30 WBCs per high-power field. The most common etiologic agent of this infection is *Trichomonas vaginalis*, which is seen on wet mount but not Gram stain.

II. SPECIMEN

Collect vaginal secretions from posterior fornix using sterile cotton or Dacron swab.

III. METHOD

Perform the Gram stain with Kopeloff's modification and 0.1% basic fuchsin counterstain. After flooding the slide with crystal violet, add 5 drops of sodium bicarbonate and blow slide to mix. Let stand for 15 s. Continue staining as usual for Gram stain. Examine for host cells and bacteria in the same manner as for a routine Gram stain, and, additionally, score as indicated in Table 3.2.1–A1, *only for women in childbearing years and postmenopausal women on estrogen replacement therapy.*

IV. QUALITY CONTROL

To ensure that smears are being read correctly, prepare a collection of smears showing a variety of scores between 0 and 10. Use these smears for competency testing and training. To determine the accuracy of the interpretation in the teaching collection, perform a culture of each specimen that shows either a preponderance of lactobacilli (scores of 0 to 3) or a preponderance of *Gardnerella* (scores of 7 to 10).

A. Inoculate CHOC and incubate for 48 h.
B. Determine the relative amount of lactobacilli (catalase negative, greening of the agar) compared to *Gardnerella* (nonhemolytic, catalase-negative, tiny Gram-variable bacilli) in the third and fourth quadrants of the plate.
C. Do *not* use selective or differential media to perform the correlation.

APPENDIX 3.2.1–3 *(continued)*

Table 3.2.1–A1 Standardized scoring method for evaluation of Gram stains for BV

Quantitation of bacterial morphotype[a]	Points scored per morphotype				
	None	1+	2+	3+	4+
Medium to large gram-positive rods	4	3	2	1	0
Small gram-negative or -variable rods	0	1	2	3	4
Curved gram-negative or -variable rods	0	1	1	2	2

[a] Modified from Nugent et al. (4) using quantitation from the Gram stain procedure. Circle in each row the number that corresponds to the quantitation visualized in the smear. Add circled numbers to arrive at total score. Interpret as follows: 0 to 3, normal; 4 to 6, intermediate; and 7 to 10, BV.

D. Cultures with 3 to 4+ lactobacilli should correlate with scores of 0 to 3. Cultures with 3 to 4+ *Gardnerella* should correlate with scores of 7 to 10.

V. REPORTING

Follow routine Gram stain reporting method to enumerate and report the following.

A. WBCs and RBCs
B. Clue cells
C. Yeasts
D. Generally pathogenic morphotypes, such as intracellular gram-negative diplococci consistent with *Neisseria* spp.

Then report one of the following based on the score in Table 3.2.1–A1: 0 to 3, "Morphotypes consistent with normal vaginal microbiota"; 4 to 6, "Mixed morphotypes consistent with transition from normal vaginal microbiota"; or 7 to 10, "Mixed morphotypes consistent with bacterial vaginosis."

References

1. **Hay, P.** 2000. Recurrent bacterial vaginosis. *Curr. Infect. Dis. Rep.* **2:**506–512.
2. **Holst, E., A. R. Goffeng, and B. Andersch.** 1994. Bacterial vaginosis and vaginal microorganisms in idiopathic premature labor and association with pregnancy outcome. *J. Clin. Microbiol.* **32:**176–186.
3. **Kimberlin, D. F., and W. W. Andrews.** 1998. Bacterial vaginosis: association with adverse pregnancy outcome. *Semin. Perinatol.* **22:**242–250.
4. **Nugent, R. P., M. A. Krohn, and S. L. Hillier.** 1991. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. *J. Clin. Microbiol.* **29:**297–301.
5. **Roy, S., M. Sharma, A. Ayyagari, and S. A. Malhotra.** 1994. A quantitative microbiological study of bacterial vaginosis. *Indian J. Med. Res.* **100:**172–176.
6. **Spiegel, C. A.** 1999. Bacterial vaginosis: changes in laboratory practice. *Clin. Microbiol. Newsl.* **21:**33–37.

3.2.2 Acridine Orange Stain

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Acridine orange is a fluorochromatic dye which binds to nucleic acids of bacteria and other cells. Under UV light, acridine orange stains RNA and single-stranded DNA orange; double-stranded DNA appears green, as first described by Strugger and Hilbrich in 1942 (4). When buffered at pH 3.5 to 4.0, acridine orange differentially stains microorganisms from cellular materials (2). Bacteria and fungi uniformly stain bright orange, whereas human epithelial and inflammatory cells and background debris stain pale green to yellow. Nuclei of activated leukocytes stain yellow, orange, or red due to increased RNA production resulting from activation. Erythrocytes either do not stain or appear pale green.

II. SPECIMEN HANDLING

Observe standard precautions.

◩ **NOTE:** The acridine orange stain is an optional stain that can be helpful in detecting organisms not visualized by Gram stain, often due to a large amount of host cellular debris.

A. For apparent plate growth that is not visualized by Gram stain (e.g., *Mycoplasma*), prepare smear as for Gram stain.

B. Broths

1. Gram stain-negative blood culture bottles that are detected as positive by the instrument

2. Broths that look turbid but are negative by Gram stain

C. For direct specimens (urine, CSF, body fluids), when WBCs are seen but no organisms are seen, or isolated, and physician requests additional studies for a difficult diagnosis, make a smear from the specimen as for Gram stain.

III. MATERIALS

A. Acridine orange stain (*see* Appendix 3.2.2–1; also available from most microbiology stain supply vendors)

1. Use the stain directly from the bottle.

2. Store at 15 to 30°C in the dark.

B. Methanol, absolute

C. Glass microscope slides

D. Heat block at 45 to 60°C (optional)

E. Immersion oil suitable for fluorescence

F. Fluorescent microscope with the following

1. Filter system for fluorescein isothiocyanate, i.e., maximum excitation wavelength of 490 nm and mean emission wavelength of 520 nm

2. ×1,000 magnification (×100 oil immersion objective)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Examine the acridine orange staining solution for color and clarity. The solution should be clear, orange, and without evidence of precipitate.

B. Each time of use, stain a prepared slide of known bacteria, such as *Escherichia coli* mixed with staphylococci, and examine for the desired results. Record results and refer out-of-control results to the supervisor.

1. Gram-negative rods and gram-positive cocci are fluorescent (orange) (1).

2. Background is nonfluorescent (green-yellow) (5).

V. PROCEDURE

◩ **NOTE:** Acridine orange is a carcinogen when absorbed through the skin. Wear gloves when working with this stain.

Observe standard precautions.

A. Prepare a smear of the specimen on a clean glass slide as for Gram stain (procedure 3.2.1). Spread specimen thinly with a sterile stick.

B. Allow to air dry.

C. Fix smear with methanol by flooding the slide, draining the excess; allow to air dry.

D. Flood slide with acridine orange stain for 2 min.

E. Drain the excess stain and rinse thoroughly with tap water.

F. Allow to air dry. The slide may be *gently* blotted on a clean sheet of filter paper or paper towel to decrease drying time.

G. Read smear on the fluorescent microscope at $\times 400$ to $\times 1{,}000$ (oil immersion). Look for the distinct morphology of bacteria or fungi. No coverslip is needed.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Record results of stain as negative or positive with the morphology of the organisms seen.

B. Review Gram stain to see if organisms can be recognized.

C. If organisms are not seen on direct Gram stain, report the following: "Culture (or specimen) positive for bacteria by acridine orange stain; bacteria not seen in Gram-stained preparation."

D. If the acridine orange stain is positive from a blood culture, subculture for the most likely organism based on the morphology.

E. If the smear is negative from a direct specimen, report "No bacteria seen by acridine orange-stained smear."

VII. INTERPRETATION

A. Bacteria and fungi fluoresce bright orange. The background appears black to yellow green. Human epithelial and inflammatory cells and tissue debris fluoresce pale green to yellow. Activated leukocytes will fluoresce yellow, orange, or red, depending on the level of activation and amount of RNA produced, whereas erythrocytes either do not fluoresce or fluoresce pale green.

B. The presence of one or more organisms per oil immersion field correlates with a colony count of approximately $\geq 10^5$ CFU/ml if unconcentrated specimen has been used to prepare the smear.

C. *Ureaplasma* and *Mycoplasma* can be visualized by this method.

VIII. LIMITATIONS

A. Nuclei or granules from disintegrated activated leukocytes, and certain types of debris, may fluoresce in acridine orange-stained smears. These may be differentiated from microorganisms on the basis of morphology.

B. Acridine orange staining does not distinguish between gram-negative and gram-positive organisms. The Gram reaction may be determined by Gram staining directly over the acridine orange after removal of the immersion oil. Acridine orange staining may also be done over Gram stain (after removal of oil) if necessary.

C. Intracellular organisms may be more difficult to see by the acridine orange stain, due to the staining of cellular nuclei.

D. The acridine orange stain will detect some organisms, especially those that are gram negative, when they are poorly visualized by Gram stain.

E. The sensitivity of the acridine orange smear is approximately 10^4 bacteria/ml (3).

REFERENCES

1. **Graham, D.** 2002. Escherichia coli *O157:H7*, Figure 3. http://www.microbelibrary.org.
2. **Kronvall, G., and E. Myhre.** 1977. Differential staining of bacteria in clinical specimens using acridine orange buffered at low pH. *Acta Pathol. Microbiol. Scand. Sect. B* **85:**249–254.
3. **Lauer, B. A., L. B. Reller, and S. Mirrett.** 1981. Comparison of acridine orange and Gram stains for detection of microorganisms in cerebrospinal fluid and other clinical specimens. *J. Clin. Microbiol.* **14:**201–205.
4. **Strugger, S., and P. Hilbrich.** 1942. Die fluoreszenzmikroskopische Unterscheidung lebender und toten Bakterienzeillen mit Hilfe des Akridinorangefärbung. *Dtsch. Tieraerztl. Wochenschr.* **50:**121–130.
5. **Wong, A. L.** 2002. *Food-borne Pathogens on Surfaces Found in the Food Processing Industry*, Figure 2. http://www.microbelibrary.org.

SUPPLEMENTAL READING

De Brauwer, E., J. Jacobs, F. Nieman, C. Bruggeman, and M. Drent. 1999. Test characteristics of acridine orange, Gram, and May-Grünwald-Giemsa stains for enumeration of intracellular organisms in bronchoalveolar lavage fluid. *J. Clin. Microbiol.* **37:**427–429.

Hoff, R. G., D. E. Newman, and J. L. Stanek. 1985. Bacteriuria screening by use of acridine orange-stained smears. *J Clin. Microbiol.* **21:**513–516.

Hunter, J. S. 1993. Acridine orange staining as a replacement for subculturing of false-positive blood cultures with the BACTEC NR 660. *J. Clin. Microbiol.* **31:**465–466.

Kasten, F. H. 1967. Cytochemical studies with acridine orange and the influence of dye contaminants in the staining of nucleic acids. *Int. Rev. Cytol.* **21:**141–202.

Larson, A. M., M. J. Dougherty, D. J. Nowowiejski, D. F. Welch, G. M. Matar, B. Swaminathan, and M. B. Coyle. 1994. Detection of *Bartonella (Rochalimaea) quintana* by routine acridine orange staining of broth blood cultures. *J. Clin. Microbiol.* **32:**1492–1496.

APPENDIX 3.2.2–1

Include QC information on reagent container and in QC records.

Preparation of Acridine Orange Stain

I. MATERIALS

A. Acridine orange powder, 20 mg

B. Sodium acetate buffer, 190 ml

1. Solution A: 100 ml of 1 M $CH_3COONa\text{-}3H_2O$
2. Solution B: 90 ml of 1 M HCl

Caution: *Acridine orange is a carcinogen; avoid all contact with skin. Avoid exposure to powder aerosol by performing reagent preparation in a fume hood and by wearing gloves.*

II. PROCEDURE

A. Acetate buffer: combine solutions A and B.

B. Add additional solution B as necessary to yield pH between 3.5 and 4.

C. Dissolve powder in buffer until solution is clear of precipitate.

D. Store stain away from light (e.g., brown bottle) at 15 to 30°C.

◩ **NOTE:** The final acridine orange concentration will be about 100 mg/liter.

Supplemental Reading

Lauer, B. A., L. B. Reller, and S. Mirrett. 1981. Comparison of acridine orange and Gram stains for detection of microorganisms in cerebrospinal fluid and other clinical specimens. *J. Clin. Microbiol.* **14:**201–205.

3.2.3 Wet Mount for Detection of Leukocytes and Microorganisms

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The presence of WBCs is one factor suggestive of an invasive infectious process. Wet mount examination of noninvasively collected specimens can be useful for rapid, inexpensive evaluation for WBCs and the detection of microorganisms such as yeasts, campylobacters, and *Trichomonas vaginalis.* The method can be done without expensive supplies and equipment, allowing rapid intervention into the disease process in the outpatient setting. The sensitivity of the technique varies with the experience of the microscopist and is generally about 60%.

Fecal leukocytes are produced in response to bacteria that invade the colonic mucosa, e.g., infections caused by *Shigella*, *Campylobacter*, some *Salmonella* organisms, enteroinvasive *Escherichia coli*, and *Yersinia.* Fecal leukocytes are also found in cases of ulcerative colitis, Crohn's disease, amebic dysentery, and antimicrobial agent-associated colitis, associated with *Clostridium difficile* toxin. Leukocytes are not associated with *E. coli* Shiga toxin infections, which is a useful characteristic of the presentation of the disease, since treatment with antimicrobial agents is contraindicated (6). Because of the variability in results for the presence of WBCs in stool and sensitivities of 50 to 60% in gastroenteritis (9) and as low as 14% for *C. difficile* colitis (10), the test cannot be used as a screening test but rather, as one of several tests to evaluate a patient's condition. It is most useful in the outpatient setting for timely evaluation of the patient, since culture confirmation of gastroenteritis is usually delayed several days. In the test, fecal samples from patients are examined microscopically at $\times 400$ magnification for the presence of fecal leukocytes. As an alternative, especially if there is a delay in transport of the specimen, the *LEUKO*-TEST, a rapid latex agglutination test for the presence of elevated levels of lactoferrin, can be done from stool specimens from adult patients, children, and infants not being breastfed (3–5). The latex particles are coated with antibody to lactoferrin (a breakdown product of fecal leukocytes).

Urinary leukocytes are present in infectious processes such as cystitis, glomerulonephritis, and catheter-associated urinary tract infection, and the report of their presence (pyuria) is helpful in the determination of disease. When WBCs are counted in a hemacytometer, the test has a high sensitivity for disease in infants (8). Urinary wet mounts can also demonstrate the presence of motile trichomonads, although the sensitivity is lower than that of vaginal wet prep or culture (7). Additionally, the detection of WBCs in urine can be determined by the dipstick for leukocyte esterase (*see* Appendix 3.12–1 in procedure 3.12 for detailed method).

The presence of WBCs is one factor suggestive of an invasive reproductive tract infectious process, including pelvic inflammatory disease and cervical infection due to *Chlamydia trachomatis* or *Neisseria gonorrhoeae.* A wet mount of vaginal fluid can demonstrate the presence of leukocytes, specialized epithelial cells coated with bacteria known as "clue cells," *T. vaginalis*, and yeasts. This rapid test is useful in the timely detection of bacterial vaginosis and vaginitis. Large numbers of WBCs are associated with *T. vaginalis* infection. Bacterial vaginosis is a clinical syndrome characterized by a shift in the vaginal microbiota from the dominant microbiota of *Lactobacillus* spp. to a mixed microbiota of *Gardnerella vaginalis*, *Prevotella* spp., *Mobiluncus* spp., and *Mycoplasma hominis.* Budding yeasts and/or pseudohyphae of yeasts can also be identified in the vaginal fluid and are an indicator of yeast vaginitis.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

◩ **NOTE:** Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for caregivers. It is the responsibility of the laboratory director or designee to educate physicians and other caregivers on proper specimen collection.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

A. Specimen collection

1. Fresh stool specimen

a. Collect stool specimen for wet mount into a sterile, leakproof widemouthed container (unpreserved).

b. If fresh stool cannot be transported and processed for wet mount within 2 h after collection, place in 10% formalin and/or polyvinyl alcohol (preserved). Preserved stool may be indefinitely transported and stored prior to wet mount exam.

c. Store specimen for lactoferrin assay at 4°C for up to 48 h either in no preservative or in Cary-Blair or similar culture transport system. Dilute 1:50 for testing.

◩ **NOTE:** Stool wet mounts should be discouraged due to their low sensitivity and specificity. A Gram stain or methylene blue stain may be used to detect leukocytes.

2. Urine

a. Collect urine specimen into a sterile, leakproof widemouthed container and store at 4°C.

b. If fresh urine cannot be transported and processed for wet mount within 2 h after collection, place in a sterile transport tube containing preservative (boric acid). Preserved urine may be indefinitely transported and stored prior to wet mount exam.

3. Vaginal fluid

a. Collect vaginal fluid/discharge from the posterior fornix using sterile cotton or Dacron swab.

b. Slides for wet mount must be immediately prepared and examined (within 15 to 20 min after collection) for WBC and trichomonad evaluation.

c. Vaginal swabs for wet mount that cannot be immediately examined should be placed in Amies' gel transport system (Copan Diagnostics, Inc., Corona, CA) for submission to the laboratory (1).

d. Vaginal swabs for trichomonad evaluation that cannot be immediately examined by wet mount of smear should be placed in InPouch TV culture medium for submission to the laboratory (1).

4. For information about InPouch TV culture medium, see reference 2.

B. Rejection criteria

1. Reject fresh stool delayed in transport and received greater than 2 h after collection.

2. Reject vaginal swabs that are not received in transport media if they are delayed in transport and received greater than 2 h after collection.

a. Vaginal smears prepared at the time of specimen collection are acceptable for WBC evaluation for up to 24 h after collection.

b. Vaginal swabs must be submitted in Diamond's culture medium or they should be rejected for trichomonad evaluation (1).

III. MATERIALS

A. Reagents

1. Saline (0.9% NaCl), preferably at 35°C

2. Loeffler's methylene blue (optional)

a. Dissolve 0.3 g of methylene blue in 30 ml of ethyl alcohol.

b. Add 100 ml of distilled water.

3. Microscope slides and coverslips

4. *LEUKO*-TEST (TechLab, Blacksburg, VA)—alternative for fecal specimens

5. Chemstrip LN (Bio-Dynamics, Division of Boehringer Mannheim Diagnostics, Indianapolis, IN)—alternative for urine (*see* Appendix 3.12–1 in procedure 3.12)

B. Equipment

1. Phase-contrast (preferred) or bright-field microscope

2. Hemacytometer for infant urine

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Controls for methylene blue stain and saline

1. Negative control: each day of use, add 1 drop of saline to 1 drop of methylene blue and examine at ×400. No bacteria or cells should be visible.

2. Positive control: each day of use, examine 1 drop of buffy coat from an EDTA-collected blood sample for cellular components.

B. Positive controls are not needed for wet mounts if no reagents are added, but evaluate competency with an ongoing program using positive and negative smears showing WBCs, clue cells, and trichomonads. Unfortunately, this is generally accomplished by viewing a collection of photographs.

C. Follow package insert for controls for the *LEUKO*-TEST.

V. PROCEDURE

Observe standard precautions.

A. Specimen preparation

Wear gloves at all times during preparation and reading of smears.

1. Fecal specimens

a. Place 2 drops of saline separated by a wax pencil line on a microscope slide.

b. Add a small drop of methylene blue to one side and a saline drop to the other.

c. Mix each drop with a small portion of stool (preferably liquid stool or sample with blood or mucus).

2. Vaginal and urine specimens

a. Place 1 or 2 drops of specimen onto slide.

b. If vaginal swab is transported in Amies' gel, suspend into 2 or 3 drops of saline before placing onto the slide.

3. For urine specimens from pediatric patients, place the drop of urine in a hemacytometer (8).

B. Apply coverslip and examine by using the high dry objective, preferably under phase-contrast optics with a 10× eyepiece for a magnification of ×400.

C. Observe for and count leukocytes, RBCs, monocytes, and epithelial cells (clue cells in vaginal specimens) in 10 to 20 fields to get an accurate determination of the specimen content.

◩ **NOTE:** Clue cells are squamous epithelial cells with bacteria attached to the top and sides. Clue cells appear as granular cells with an undefined edge.

D. Observe for parasites that are motile, such as trichomonads.

E. In fecal specimens, observe the saline wet prep for bacteria with typical darting "campylobacter" motility.

F. Observe for budding yeast cells and pseudohyphae in vaginal exudate and urine but not in fecal specimens, where they are part of the normal microbiota.

G. Dispose of slides in sharps container, wipe microscope with 70% ethanol, and remove gloves.

H. Follow the package instructions for the latex agglutination procedure for the *LEUKO*-TEST.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report wet mount results (Table 3.2.3–1).

VI. REPORTING RESULTS *(continued)*

Table 3.2.3–1 Outline for reporting wet mount results from different specimens

Specimen	Cells[a]	Microorganisms
Stool	Report WBCs/HPF.	Report the presence of *Campylobacter*—"Motile organisms suggestive of *Campylobacter* spp. present."
Urine	Report WBCs/microliter. Report RBCs/microliter.	Report the presence of bacteria. Report the presence of budding yeasts and pseudohyphae.
Vaginal[b]	Report WBCs/HPF. ◩ **NOTE:** Vaginal infections with *T. vaginalis* are usually accompanied by >30 WBCs/HPF.	Report the presence of *T. vaginalis* (see procedures 9.6.6 and 9.9.4 for details). Report the presence of budding yeasts and pseudohyphae. Report the presence of clue cells.

[a] Report the number of WBCs and RBCs per high-power field (HPF). Average several fields and report as follows: (i) heavy = ≥5/HPF, (ii) moderate = 1 to 4/HPF, (iii) few = <1/HPF, and (iv) none = 0/HPF.

[b] If the vaginal specimen is negative for parasites and was not preserved, or was not processed within 15 min, report "Unable to determine the presence of *Trichomonas vaginalis* in unpreserved specimen or because of delay in transport."

VII. INTERPRETATION

A. Urine
- **1.** Greater than 5/HPF is considered positive for pyuria. Such a result has a specificity of 90% for predicting catheter-associated infection with greater than 10^5 CFU/ml but a sensitivity of only 37% (procedure 3.12).
- **2.** Greater than 10/μl with the hemacytometer method is reported to have a sensitivity of 84% and a specificity of 90% for predicting pyuria in infants (8).

B. Fecal WBCs
- **1.** The presence of >5/HPF had a sensitivity of 63.2% and a specificity of 84.3% in one study (9), but in another study a 52% sensitivity was seen only if >1 WBC/HPF was used as the cutoff (10).
- **2.** The results of fecal WBCs were not statistically or clinically significantly different from those of lactoferrin testing (9).
- **3.** *If WBCs are not present and RBCs are present in a stool submitted for culture, always perform a culture for* E. coli *O157 or a Shiga toxin test (6).*

C. For vaginal specimens, the sensitivity of testing by wet mount for WBCs is not as important as the examination for clue cells, yeasts, and *T. vaginalis,* although *T. vaginalis* is more common in specimens with WBCs.
- **1.** The presence of budding yeast is associated with candidiasis.
- **2.** The presence of clue cells is indicative of bacterial vaginosis.

VIII. LIMITATIONS

A. Activated leukocytes disintegrate easily, decreasing the sensitivity of the assay with delays in transit.

B. The wet mount is dependent on the expertise of the microscopist, whose work should be reviewed at frequent intervals.

C. WBCs are often confused with trichomonads that are no longer moving, which can be avoided by careful examination for the characteristics of the parasite.

D. If there is a question in reading the test, the cells, but not yeasts, will dissolve with the addition of KOH (*see* procedure 8.3).

E. Delays in transit decrease the ability to detect trichomonads.

REFERENCES

1. **Beverly, A. L., M. Venglarik, B. Cotton, and J. R. Schwebke.** 1999. Viability of *Trichomonas vaginalis* in transport medium. *J. Clin. Microbiol.* **37:**3749–3750.
2. **Borchardt, K. A., M. Z. Zhang, H. Shing, and K. Flink.** 1997. A comparison of the sensitivity of the InPouch™ TV, Diamond's, and Tichosel media for detection of *Trichomonas vaginalis. Genitourin. Med.* **73:**297–298.
3. **Fine, K. D., F. Ogunji, J. George, M. D. Niehaus, and R. L. Guerrant.** 1998. Utility of a rapid fecal latex agglutination test detecting the neutrophil protein, lactoferrin, for diagnosing inflammatory causes of chronic diarrhea. *Am. J. Gastroenterol.* **93:**1300–1305.
4. **Guerrant, R. L., V. Araujo, E. Soares, K. Kotoff, A. A. M. Lima, W. H. Cooper, and A. G. Lee.** 1992. Measurement of fecal lactoferrin as a marker for fecal leukocytes. *J. Clin. Microbiol.* **30:**1238–1242.
5. **Huicho, L., V. Garaycochea, N. Uchima, R. Zerpa, and R. L. Guerrant.** 1997. Fecal lactoferrin, fecal leukocytes and occult blood in the diagnostic approach to childhood invasive diarrhea. *Pediatr. Infect. Dis. J.* **16:**644–647.
6. **Iida, T., A. Naka, O. Suthienkul, Y. Sakaue, R. L. Guerrant, and T. Honda.** 1997. Measurement of fecal lactoferrin for rapid diagnosis of enterohemorrhagic *Escherichia coli* infection. *Clin. Infect. Dis.* **25:**167.
7. **Lawing, L. F., S. R. Hedges, and J. R. Schwebke.** 2000. Detection of trichomonosis in vaginal and urine specimens from women by culture and PCR. *J. Clin. Microbiol.* **38:**3585–3588.
8. **Lin, D. S., F. Y. Huang, N. C. Chiu, H. A. Koa, H. Y. Hung, C. H. Hsu, W. S. Hsieh, and D. I. Yang.** 2000. Comparison of hemocytometer leukocyte counts and standard urinalyses for predicting urinary tract infections in febrile infants. *Pediatr. Infect. Dis. J.* **19:**223–227.
9. **Ruiz-Pelaez, J. G., and S. Mattar.** 1999. Accuracy of fecal lactoferrin and other stool tests for diagnosis of invasive diarrhea at a Colombian pediatric hospital. *Pediatr. Infect. Dis. J.* **18:**342–346.
10. **Savola, K. L., E. J. Baron, L. S. Tompkins, and D. J. Passaro.** 2001. Fecal leukocyte stain has diagnostic value for outpatients but not inpatients. *J. Clin. Microbiol.* **39:**266–269.

APPENDIX 3.2.3–1

Demonstration of *Treponema pallidum* in Specimens Using Dark-Field Microscopy

I. PRINCIPLE

Dark-field microscopy is used to demonstrate the presence of motile *Treponema pallidum* in lesions or aspirates in early-stage syphilis prior to healing of lesions (1–4).

II. MATERIALS

A. Dark-field microscope with parfocal 10×, 40× to 45×, and 100× oil immersion objectives, 10× oculars, dark-field immersion condenser (single or double deflecting), and a 6.0- to 6.5-V high-intensity lamp with variable transformer for regulating light intensity.
B. Microscope slides, 1 by 3 in.
C. Coverslip, 22 by 22 mm
D. Immersion oil, nondrying

Observe standard precautions.

III. SPECIMEN

Collect serous fluid from lesion for examination prior to antimicrobial therapy.

A. Clean surface of lesion with saline, and blot dry.
B. Gently remove any crusts, and discard.
C. Abrade superficially until slight bleeding occurs, using a needle, scalpel blade, or broken glass slide. Irrigate with sterile saline and wipe away the first few drops of blood, etc.
D. Apply gentle pressure at lesion base, touching *clear* exudate in ulcer base with a glass slide.
E. If no exudate is present, add a drop of saline to the lesion or insert a needle and syringe at lesion base, aspirate, and then draw a drop of saline into the needle. Express the material onto a slide.
F. Place coverslip immediately, and examine the slide by dark-field microscopy.

Observe standard precautions.

IV. MICROSCOPE EXAMINATION PROCEDURE

A. Search entire specimen with high dry objective for spiral organisms.
B. Center suspicious objects, and examine them under oil immersion objective.
C. Upon completion of examination, discard slide into container of appropriate disinfectant.

V. INTERPRETATION

A. *T. pallidum* organisms appear as delicate, corkscrew-shaped, rigid, uniform, tightly wound, deep spirals; coil appearance is maintained even while organisms are actively motile.

APPENDIX 3.2.3–1 *(continued)*

B. Observe for rotational motility around longitudinal base; backward and forward movement; flexion, bending, or twisting from side to side; and snapping motion.
C. Spirochetes are 6 to 14 μm long, which is slightly longer than the diameter of an erythrocyte.

VI. REPORTING RESULTS

A. When organisms are seen that have characteristic morphology, shape, and motility of *T. pallidum*, report "Treponemas resembling *T. pallidum* observed."
B. When no treponemas are observed, report "No treponemas resembling *T. pallidum* observed."

VII. LIMITATIONS

The specimen must be examined immediately (within 20 min) to observe motile organisms. For optimal sensitivity, up to three separately collected slides should be examined by dark-field microscopy to rule out *T. pallidum*. If immediate dark-field examination is not possible, air dry the slide and submit to a laboratory, such as the CDC Syphilis Diagnostic Immunology Section (http://www.cdc.gov; STD hotline, 1-800-232-4636), that has reagents for specific direct fluorescent-antibody examination for treponemes, or purchase commercial reagents (Virostat, Portland, ME) (4).

References

1. **Baron, E. J., G. H. Cassell, D. A. Eschenbach, J. R. Greenwood, S. M. Harvey, N. E. Madinger, E. M. Paterson, and K. B. Waites.** 1993. *Cumitech 17A, Laboratory Diagnosis of Female Genital Tract Infections.* Coordinating ed., E. J. Baron. American Society for Microbiology, Washington, DC.
2. **Chapin, K.** 2007. Principles of stains and media, p. 182–191. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
3. **Holmes, K. K., P. A. Mardh, P. F. Sparling, P. J. Wiesner, W. Cates, Jr., S. M. Lemon, and W. E. Stamm.** 1990. *Sexually Transmitted Diseases,* 2nd ed. McGraw Hill Book Co., New York, NY.
4. **Pope, V., S. J. Norris, and R. E. Johnson.** 2007. Treponema and other human host-associated spirochetes, p. 987–1003. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.

3.3.1 Paratechnical Processing of Specimens for Aerobic Bacteriology

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The initial processing of clinical specimens for bacteriology is a multifaceted endeavor involving a number of decision-making steps, including the need for processing the specimen for anaerobic bacteriology, mycology, virology, and parasitology, depending on the nature of the specimen. The need for direct tests, such as Gram stains, must also be considered. These issues will determine whether the specimen requires any pretreatment before inoculation.

First, one must consider the specimen type and its anatomic origin. The second step is the selection of primary isolation media to be used for each specimen type. The final step is the selection of incubation temperature and atmosphere. A biological safety cabinet should be used during the processing of all specimens. This procedure covers only general processing instructions for routine cultures. Refer to the Specimen Collection, Transport, and Handling portions of procedures 3.4 through 3.15 for processing by specific anatomic site and the use of specialized media for processing for specific organisms.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Prepare specimen collection instruction manual and make available to physicians and other caregivers for proper collection, timing, and transport of specimens. This can be achieved by copying the Specimen Collection, Transport, and Handling portions from each of the procedures that follow. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

◩ **NOTE:** Proper specimen collection is critical to isolating the causative agent of infection.

B. Along with the specimen, design a system to provide the following.

1. Demographics of the patient: name, address, age, sex, location in the hospital or clinic, unique patient identifying number, name of physician of record, name of the physician who is ordering the test, and ICD9 code or diagnosis.

2. Details of the specimen: type of specimen, anatomic site of collection (if variable), whether the specimen was collected from an invasive procedure (e.g., during surgery), and gross description, if variable.

3. Specific culture, stain, and antigen test requests.

C. Provide the above information on each request form, specimen container, and transport carrier. The patient demographic information, specimen information, and specific test requests provided on the laboratory requisition should match those on the specimen container label and the transport carrier.

D. Determine acceptability of specimen labeling and collection. Regulations for specimen labeling are outlined on the Centers for Medicare and Medicaid Services website (http://hcfa.gov). See section 2 of this handbook for general rejection criteria and each procedure that follows for specific rejection criteria by specimen type.

III. MATERIALS

A. The media used for common laboratory/aerobic cultures in bacteriology are listed in Table 3.3.1–1.
B. Stain reagents and supplies (*see* procedures 3.2.1 to 3.2.3)
C. Other supplies
  1. Sterile petri dishes
  2. Pasteur pipettes
  3. Sterile scissors, forceps, and scalpels
  4. Sterile test tubes
  5. Sterile swabs and sticks
  6. Inoculating loops
  7. System for grinding tissue
D. Equipment
  1. Biological safety cabinet
  2. Incubators (35 to 37°C; both 5% CO_2 and ambient air)
  3. Bacticinerator or flame burner with automatic shutoff (optional)

Table 3.3.1–1 Common laboratory media used for aerobic cultures[a]

Medium	Abbreviation	Type[b]	Atmosphere	Inhibitor(s)	Purpose
Nonselective agars					
Chocolate agar	CHOC	N	5–10% CO_2	None	To grow most bacteria, including *N. gonorrhoeae* and *Haemophilus*
Trypic soy agar with 5% defibrinated sheep blood or Columbia agar with 5% defibrinated sheep blood	BAP	N	5–10% CO_2 preferred; O_2 for special circumstances	None	To grow most bacteria and determine the type of hemolysis: alpha (green), beta (clear), or gamma (none). Will not support *N. gonorrhoeae*, *Haemophilus*, *Legionella*, or *Bordetella pertussis.*
Gram-negative rod selective agars					
MacConkey agar	MAC	S, D	O_2	Bile salts, crystal violet, lactose, neutral red	Gram-negative enteric agar that inhibits growth of gram-positive organisms and yeasts and inhibits the spreading of *Proteus.* Lactose-positive colonies are pink, and lactose-negative colonies are colorless.
Eosin-methylene blue	EMB	S, D	O_2	Eosin, methylene blue, lactose, sucrose	Gram-negative enteric agar that inhibits (but does not prevent) the growth of gram-positive organisms and some yeasts. Enhances the growth of *Candida glabrata* and some molds. Enteric rods have various colors. Lactose- and sucrose-fermenting colonies are dark.
Gram-positive selective agars					
Phenylethyl alcohol with 5% defibrinated sheep blood	PEA	S	5–10% CO_2	Phenylethyl alcohol	Inhibits most gram-negative bacteria. Used for growth of *Staphylococcus* and *Streptococcus* in mixed cultures. Has short shelf life.
Colistin-nalidixic acid Columbia agar with 5% defibrinated sheep blood	CNA	S	5–10% CO_2	Colistin and nalidixic acid	Inhibits most gram-negative bacteria. Used for growth of *Staphylococcus* and *Streptococcus* in mixed cultures.
Selective agar for neisseriae					
Thayer-Martin	TM	S	5–10% CO_2	Vancomycin, colistin, nystatin	To select for *Neisseria* in mixed cultures. For other related agars, see procedure 3.9.3.

Table 3.3.1–1 *(continued)*

Medium	Abbreviation	Type[b]	Atmosphere	Inhibitor(s)	Purpose
Broths: support the growth of most aerobic bacteria					
Brain heart infusion	BHI	E	Ambient air		BHI and TSB can be supplemented with 0.1% agar to support some anaerobic growth and with X and V factor or sheep blood to support growth of *Haemophilus* and other fastidious facultative anaerobic organisms. THIO does not support the growth of fastidious facultative anaerobic organisms.
Tryptic soy broth	TSB				
Brucella broth	BRUB				
Thioglycolate	THIO				
Chopped-meat broth	CMB				
Fastidious anaerobic broth (4)	FAB				

[a] For enteric selective and differential media used for stool cultures (e.g., Hektoen, XLD, and chromogenic agars), refer to procedure 3.8.1.

[b] N, nutrient; D, differential; S, selective; E, enrichment.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. For agar media, inspect each shipment or batch for cracks in media or plastic petri dishes, thin or unequal fill, hemolysis, evidence of freezing, desiccation, bubbles, and visible contamination and report deficiencies to manufacturer.

B. Maintain records of date received or prepared, lot number, and expiration date of each medium.

C. QC media, whether prepared in-house or procured from commercial vendors, with one or more organisms expected to grow and one or more organisms expected to be inhibited, if applicable. See Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22-A3 (1) and procedure 14.2 for details, including preparation of stock cultures with controlled inocula of microorganisms.

D. *Exception:* For media listed in Table 2 of CLSI document M22-A3 (1) and procured from commercial sources, omit QC and verify that the vendor has controlled the media according to procedures outlined in document M22-A3.

E. Test each lot of CHOC and TM for QC, whether purchased commercially or prepared in-house. Refer to procedure 3.9.3 for QC of TM and related selective agars. For QC of CHOC, use the following positive controls; a negative control is not needed. Incubate plates for 24 to 48 h in 5 to 7% CO_2 at 35°C.

Test organism	Result
Neisseria gonorrhoeae ATCC 43069 or ATCC 43070	Growth
Haemophilus influenzae ATCC 10211	Growth

F. Check and record incubator temperatures daily; include in records readings of humidity and CO_2 concentrations, if applicable.

G. Set up a QA program to ensure that the specimens received are of the best possible quality (*see* section 14). Troubleshoot problems and follow with necessary revisions to existing procedures to improve quality.

H. Prepare an alternative testing policy to be used when the laboratory is unable to perform testing if reagents, personnel, and methods of communication are not available. The following are examples.

1. If media cannot be supplied by vendor, contact alternative vendor. . . .

2. If incubator is inoperable, use alternative incubator located at. . . .

3. If computer system is not operable, send manual reports according to details in the computer crash plan.

V. PROCEDURE

Observe standard precautions.

A. Timing

The most important specimens must be inoculated to media first. Look through the cultures and pull out all those that are invasively collected. The preferred order of setting up cultures is listed in Table 3.3.1–2. The delay of inoculation and processing of certain specimens can affect the quality of the culture and ability to isolate pathogens.

B. Specimen pretreatment

1. Work in a biological safety cabinet. Wear gloves, laboratory coat, and other protective equipment when working with specimens.

2. Verify that the patient demographic information, specimen information, and specific test requests provided on the laboratory requisition match those on the specimen container label and the transport carrier. Assign a unique identifying number to the specimen at the time of accession.

3. Generally, look for the most purulent part of the specimen and use that for smears and culture.

a. Fluids, except urine

(1) For normally sterile body fluids

(a) Use the cytocentrifuge to concentrate smears from clear fluids.

(b) In addition to plate culture, inoculate broth culture, diluting the specimen 1:10 in broth.

(c) For joint and peritoneal fluid specimens, culture at least 10 ml of specimen.

◩ **NOTE:** The culture of large volumes of specimen has been shown to have a higher yield than centrifugation of specimens for aerobic bacteria. Generally inoculation of up to 10 ml into the aerobic and 10 ml into the anaerobic blood culture bottle will increase the yield of the causative agent from joint and peritoneal body fluids.

Table 3.3.1–2 Order of specimen processing for bacteriology when multiple specimens are received at the same time

Order	Common tests or specimens	Maximum time to processing
1	STATS: specimens from surgery and normally sterile sites are processed before STATs from nonsterile body sites.	20 min after receipt
2	*N. gonorrhoeae* cultures submitted on plates or unpreserved swabs	20 min after receipt
3	CSF (treat all as STAT)	20 min after receipt
4	BAL[a]	20 min after receipt
5	Tissues	1 h
6	Other body fluids	1 h
7	Abscesses	1 h
8	Unpreserved stools for culture	30 min after collection, or place in transport medium immediately[b]
9	Sputum and other lower respiratory cultures	1 h at room temp, 2 h at 4°C
10	Blood	4 h at room temp after collection
11	Swabs in transport tubes	8 h at 4°C
12	Urine	Up to 24 h at 4°C
13	Group A and B streptococcal cultures	8 h at 4°C

[a] BALs, bronchoalveolar lavage samples.

[b] *Shigella* sp. viability is compromised unless the specimen is placed in transport medium.

V. PROCEDURE *(continued)*

(2) For other liquid specimens, inoculate plates and smears using a swab or pipette dipped into the liquid.

b. Tissues

Refer to procedure 3.13 for details on preparation of tissues for smear and culture.

c. Swabs

(1) Reject dry swabs not submitted in transport medium.

(2) Use the swab to streak the first quadrant of the plate. If a second swab is submitted, use for smear preparation.

(3) If only one swab is received or several plates and smears are to be prepared, place swab into a small amount of broth and vortex. Ream swab to expel all fluid and discard. Then use a pipette to inoculate agar plate media and to prepare smears.

◩ **NOTE:** Use of the CultureSwab EZ II system (BD Diagnostic Systems) omits the need to extract the organisms from the swab.

d. For prosthesis specimens and other surgical foreign-object specimens with no visible tissue or purulent fluid that can be cultured, add culture broth to the sterile specimen container and incubate at 35°C for 18 h. Then subculture broth and perform a Gram stain. Hold broth with rest of culture. If the object is from a genital source (e.g., intrauterine device), touch specimen to a CHOC plate and to anaerobic plates prior to adding broth.

e. For catheter tips, refer to procedure 3.6 for quantitative and semiquantitative methods of culture.

f. Refer to procedure 3.12 for options to inoculate quantitative urine cultures.

4. Saving specimens

a. Hold urine and stool not in preservative only long enough to resolve any labeling problems. These specimens should be recollected rather than recultured if there are indications to do so.

b. Hold samples from other nonsterile sites for 1 to 2 days, usually at 4°C, to resolve any problems.

c. Hold specimens from normally sterile sites for 7 days at 4°C, if possible. Rotate specimens such that older ones are discarded in favor of recently cultured specimens.

d. Freeze CSF at −20°C in case PCR testing is requested after evaluation of cultures.

C. Medium choices

1. Table 3.3.1–1 outlines the common laboratory media used for aerobic cultures, including routinely used incubation atmospheres and medium inhibitors.

2. Inoculate most specimens onto a BAP.

3. In addition, inoculate specimens from normally sterile sites, genital sites, and respiratory sites to CHOC (or, optionally, horse blood agar for respiratory specimens).

4. Add additional media based on nosocomially significant microorganisms.

5. For specimens from nonsterile sites, inoculate to either MAC or EMB to select for these organisms within normal microbiota.

6. Use Columbia colistin-nalidixic acid agar (CNA) or phenylethyl alcohol agar (PEA) to select for gram-positive organisms in specimens potentially contaminated with gram-negative microbiota.

7. Historically, tissues from surgery and fluids from selected normally sterile sites have been inoculated into broth, but this has been shown to not be clinically relevant or cost-effective in improving the recovery of pathogenic organisms from these types of specimens (2, 3).

V. PROCEDURE *(continued)*

8. Refer to Table 3.3.1–3 and the individual procedures that follow for details of inoculation of stool cultures and cultures for specific organisms to special media. Table 3.3.1–3 provides recommendations for the initial examination and plating of clinical specimens to common laboratory media used for aerobic culture.

D. Perform Gram stains on most respiratory and wound cultures, all cultures from normally sterile sites, and urine and genital cultures, on request (Table 3.3.1–1). Gram stains are not performed on throat, nasal, stool, or catheter tip cultures. For smear preparation, see procedure 3.2.1.

1. If sterile glass slides are not used for the Gram stain, the culture should be inoculated first to avoid contamination. Never touch the nonsterile glass slide with a pipette or swab that will subsequently be used for culture. Alternatively, the Gram stain may be prepared prior to inoculation of the culture if sterile glass slides are used. Preparation of the Gram stain prior to inoculation of the culture may provide a better smear that accurately reflects the clinical cellular and bacterial contents of the original clinical specimen.

2. If there is insufficient specimen, omit the smear rather than the culture.

3. *If the smear result indicates a mixed culture and selective medium was not inoculated, add appropriate selective medium to the culture inoculation.*

4. Use a cytocentrifuge to prepare smears from normally sterile body fluids, especially CSF.

E. Inoculation technique

1. Label plates with at least the identifying number and date of culture. If convenient, label at least one plate with the anatomic site and patient name.

2. Automated inoculator

Automated specimen processing instruments are available for clinical microbiology laboratories processing a large volume of specimens and include the following.

a. The Walk-Away Specimen Processor (WASP) (Copan Diagnostics Inc.) allows the automatic streaking and planting of swabs, urine samples, and fecal samples using a single-instrument platform. A total of 180 urine samples, throat swabs, or nasal swabs can be processed per hour, but it can also manage complex plate medium sets used for wound swabs, vaginal swabs, and fecal samples. The WASP instrument utilizes several preprogrammed streak patterns, or laboratories may design their own streaking method for use by the instrument.

b. The Innova (Dynacon, Toronto, Ontario, Canada) instrument allows the automatic streaking and planting of most types of clinical microbiology specimens, including liquid-based flocked swabs. The Innova has a capacity of 200 containers an hour and is preprogrammed with streak patterns, or laboratories may design their own streaking technique for use by the instrument. Several other automated processors are also available, including the innoLAB instruments (http://www.dynacon.ca).

3. Manual inoculation

Generally inoculate onto plate by touching specimen to one quadrant with a swab, pipette, or sterile forceps containing the specimen.

a. Sterilize the inoculating loop in the microincinerator for 5 to 10 s. Allow to cool. Alternatively, use separate, sterile disposable loops or sticks.

b. Streak with gentle pressure onto one-fourth to one-third of the culture plate using the sterile loop, or stick, with a back-and-forth motion several times and without entering the area that was previously streaked. Avoid touching the sides of the petri dish.

Table 3.3.1–3 Recommended aerobic culture media for inoculation of common clinical specimens[a,b]

Specimen type/source	Gram stain	Aerobic media	Incubation	Comments
Abscesses				See procedure 3.13 and section 4 (Anaerobic Bacteriology) for further information on abscess sample processing.
Superficial	Yes	BA, CNA, CHOC	35–37°C/CO_2	
Deep[c]	Yes	MAC	35–37°C/O_2	
Catheter tips	Yes—touch prep			See procedure 3.6 for further information on catheter tip processing.
Dialysate	Yes (if fluid)			Dialysates should be directly inoculated into blood culture bottles for the best yield.
Ear swab/external drainage	Yes	BA, CNA, CHOC	35–37°C/CO_2	
		MAC	35–37°C/O_2	
Eye swab—conjunctiva	Yes	BA, CHOC, TM/MTM	35–37°C/CO_2	
		MAC	35–37°C/O_2	
Genital				
Cervix	Yes	All urogenital samples plated to:	35–37°C/CO_2	
Intrauterine device[c]	Yes—touch prep	BA, CNA, CHOC, TM/MTM, MAC	35–37°C/O_2	
Rectal	No			
Urethra/penis	Yes			
Vaginal	Yes	LIM or carrot broth subculture to BA		
Vaginal-rectal (group B Streptococcus screen)	No			
Lower respiratory tract				BCYE with and without antimicrobial agents improves recovery of *Legionella* spp. from BAL or BW. Sputa with a low Q score (e.g., saliva) should not be cultured.
BAL	Yes	BA, CHOC, MAC, BCYE	35–37°C/CO_2	
BW	Yes	BA, CAN, CHOC, MAC used for all ET/sputum cultures	35–37°C/O_2	
ET	Yes		35–37°C/CO_2	
Sputum (routine)	Q score		35–37°C/O_2	
Sputum (CF)	Yes			
Sterile fluids				Sterile fluids may be inoculated into blood culture bottles after appropriate stains (e.g., Gram, India ink, calcofluor white, acid-fast) or other tests are done (e.g., cryptococcal antigen).
CSF (LP)	Gram stain of Cytospin-prepared slides on all sterile fluids should be done	BA, CHOC for most fluids	35–37°C/CO_2	
CSF—shunt[c]		Add CNA, MAC for peritoneal, tympanocentesis, or vitreous/aqueous samples.	35–37°C/O_2	
Pericardial				
Pleural[c]				
Peritoneal[c]				
Synovial				
Vitreous/aqueous				
Other				

(continued)

Table 3.3.1–3 Recommended aerobic culture media for inoculation of common clinical specimens[a,b] *(continued)*

Specimen type/source	Gram stain	Aerobic media	Incubation	Comments
Tissues[c]				Bone marrow should be inoculated into blood culture bottles after appropriate stains (e.g., Gram, India ink, calcofluor white, acid-fast) are prepared.
Bone	All tissues should be examined by Gram stain.	BA, CHOC	35–37°C/CO_2	
Bone marrow		MAC	35–37°C/O_2	
Corneal scraping				
Liver				
Lung				
Muscle				
Other				
Upper respiratory tract				Gram stain only is done on mouth/gingival swabs. Specialized media are needed to recover *N. gonorrhoeae* (e.g., TM/MTM) Small children with CF are unable to produce sputa.
Gingiva	Yes	Culture not done	35–37°C/O_2	
Nose/nasopharynx	No	BA, MRSA CHROMagar	35–37°C/AnO_2	
Throat (routine)	No	BA + Taxos A	35–37°C/CO_2	
Throat (rule out GC)	No	Add CHOC, TM/MTM	35–37°C/AnO_2	
Throat (CF) (pediatrics)	No	BA + Taxos A, CHOC, MAC, MSA, BCSA	35–37°C/O_2	
Mouth	Yes	Culture not done		
Urine	No	BA, MAC, or chromogenic agar	35–37°C/O_2	All urine types are plated to the same routine media. Inoculation may be done manually or using an automated streaking instrument.
Wound				See procedure 3.13 and section 4 (Anaerobic Bacteriology) for further information on wound sample processing.
Superficial	Yes	BA, CNA, CHOC	35–37°C/CO_2	
Deep[b]	Yes	MAC	35–37°C/O_2	

[a] See reference 5 for detailed recommendations for Gram stain and aerobic and anaerobic plating media for bacteriology specimens or organisms.

[b] Abbreviations: BAL, bronchoalveolar lavage; BW, bronchial wash; ET, endotracheal; CF, cystic fibrosis; GC, gonococci; TM, Thayer Martin; MTM, modified Thayer Martin.

[c] Clinical specimens that should have anaerobic culture also performed provided the samples have been properly collected and transported to ensure anaerobiasis.

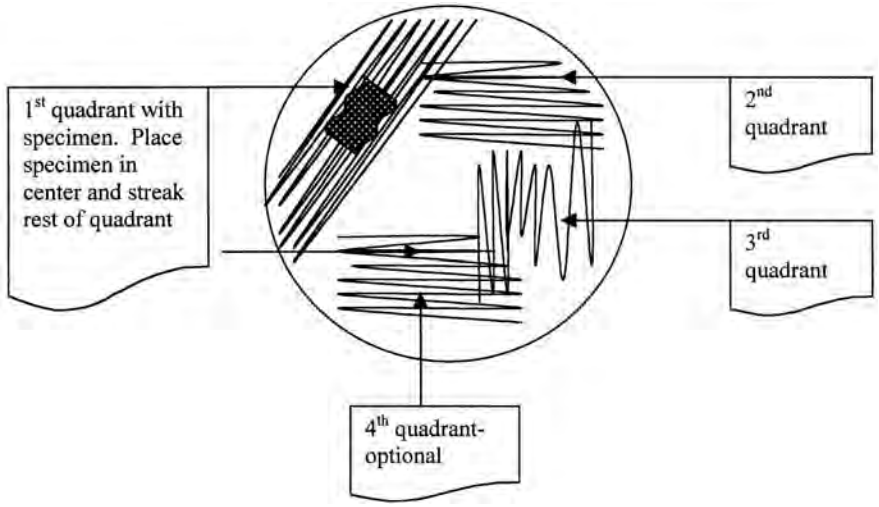

1st quadrant with specimen. Place specimen in center and streak rest of quadrant
2nd quadrant
3rd quadrant
4th quadrant-optional

Figure 3.3.1–1 Appropriate method to streak plate for isolation of bacteria. Inoculate first quadrant with a few drops or pieces of specimen or by rolling a swab on a small area.

V. PROCEDURE *(continued)*

c. Turn the plate a quarter turn. Pass the loop through the edge of the first quadrant approximately four times, while streaking into the second quadrant. Continue streaking in the second quadrant without going back to the first quadrant.

d. Rotate the plate another quarter turn and repeat the above procedure until one or two additional quadrants are streaked, as shown in Fig. 3.3.1–1.

4. For normally sterile specimens, use separate disposable loops, sticks, or needles for each plate. Alternatively, flame the loop or needle between each plate inoculation. Use biplates to save handling time and space (Fig. 3.3.1–2).

5. For inoculation of plates to detect beta-hemolysis, carefully stab the agar with the same loop both inside and outside the inoculated area to expose the organism to anaerobic conditions (Fig. 3.3.1–3) (usually done for throat cultures).

6. For urine, catheter tips, and quantitative cultures, do not streak in quadrants. Refer to the relevant procedures in this section.

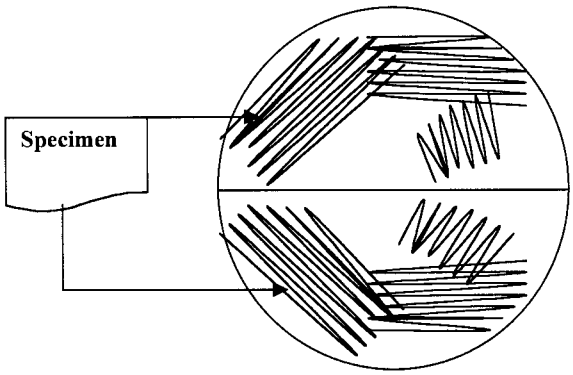


Figure 3.3.1–2 Inoculation of biplate, often used for specimens from sterile sites when blood agar and CHOC are needed. To avoid cross contamination of media with plate contaminants, always use a separate loop or flame loop between inoculation of each side of plate.

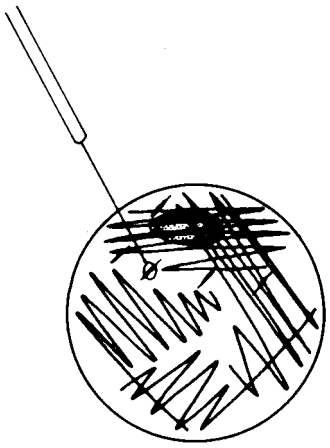


Figure 3.3.1–3 Optional method of streaking plate for throat cultures.

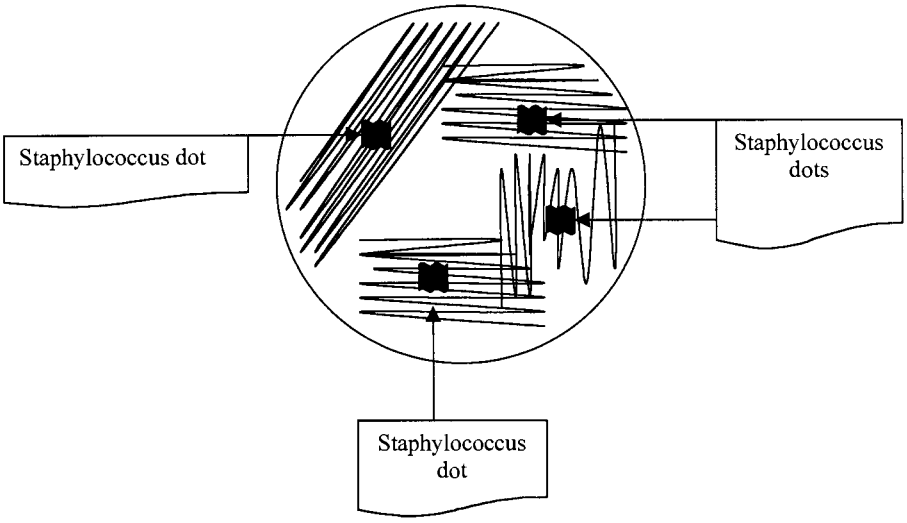


Figure 3.3.1–4 Alternative for detection of *H. influenzae* on BAP (e.g., respiratory specimens).

V. PROCEDURE *(continued)*

7. Optional: for respiratory specimens or aerobic wound cultures, cross streak or dot the BAP with coagulase-negative staphylococci after the specimen has been inoculated to the plate, to allow for recognition of *Haemophilus* (Fig. 3.3.1–4). This technique may be done instead of or in addition to the CHOC plate inoculation. Refer to procedure 3.17.44 for further details.

F. Incubation

1. Separate all inoculated direct plates and broths according to the sorting system used in the laboratory (e.g., alphabetical by patient name, by specimen type, by ward, etc.).

2. Generally place in order by workstation in canisters and incubate in a humidified incubator at 35°C.

a. Supply humidity by an automatic humidifier or by placing a large pan of water at the bottom of the incubator.

b. Check humidity control or water level daily to maintain constant humidity.

3. Provide 5 to 7% CO_2 for appropriate cultures, particularly those for possible isolation of *Streptococcus pneumoniae, Haemophilus influenzae,* and *Neisseria gonorrhoeae,* especially routine respiratory cultures, genital cultures, and normally sterile body fluid cultures. CO_2 can be supplied as follows.

a. By incubators with special controls and a CO_2 gas cylinder

b. By using an Anoxomat Mark II jar connected to a CO_2 supply (MART Microbiology; http://www.anoxomat.com). This method improves the recovery of fastidious microaerophiles or oxygen-sensitive organisms due to its unique rapid system for establishing optimal environmental growth conditions.

c. By using a gas-generating envelope in an airtight jar (Fig. 3.3.1–5) or plastic container *or*

d. By placing the medium in a bag with a CO_2-generating ampoule or tablet.

4. Do *not* incubate the following in increased CO_2.

a. Stool culture selective agar

b. *Legionella* cultures

c. *Bordetella pertussis* cultures

d. Throat cultures when using BAP without selective media with antimicrobial agents

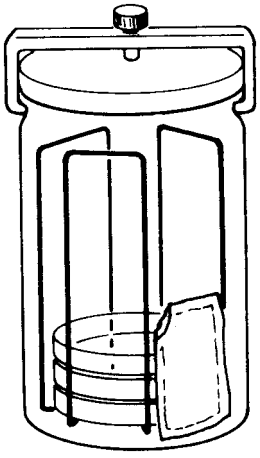


Figure 3.3.1–5 GasPak envelopes in airtight jar, used to produce 5 to 7% CO_2 atmosphere.

V. PROCEDURE *(continued)*

5. Increased CO_2 is optional for the following.
   - **a.** MAC or EMB
   - **b.** Broths and blood cultures
   - **c.** Group B streptococcal cultures
   - **d.** Diphtheria cultures
   - **e.** Urine cultures
6. Cultures for special organisms have special requirements. See the relevant procedures (e.g., for *Campylobacter*, *Bartonella*, and *Haemophilus ducreyi*).
7. Incubate anaerobic plates in an anaerobic environment, as discussed in section 4 of this handbook.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING SPECIMEN INFORMATION

A. Document association of specimen identifying number with patient demographics (name, patient identifier, patient location) and information on the specimen (anatomic site, date and time of collection, and name of person ordering the test).

B. Document type, container, and macroscopic description of specimen (e.g., color, consistency, etc.).

C. Document all problems with specimens, culturing, and test requests in laboratory report and notify the collecting location of such problems.

D. If ICD9 code is available, provide access to information to person examining culture.

VII. LIMITATIONS

A. False-positive cultures result from specimen mix-up and from contamination of media used for culture.

B. False-negative results are due to improper collection, delays in culture inoculation, inappropriate medium usage, and inappropriate incubation conditions.

REFERENCES

1. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
2. **Meredith, F. T., H. K. Phillips, and L. B. Reller.** 1997. Clinical utility of broth cultures of cerebrospinal fluid from patients at risk for shunt infections. *J. Clin. Microbiol.* **35:**3109–3111.
3. **Morris, A. J., S. J. Wilson, C. E. Marx, M. L. Wilson, S. Mirrett, and L. B. Reller.** 1995. Clinical impact of bacteria and fungi recovered only from broth cultures. *J. Clin. Microbiol.* **33:**161–165.
4. **Scythes, K. D., M. Louis, and A. E. Simor.** 1996. Evaluation of nutritive capacities of 10 broth media. *J. Clin. Microbiol.* **34:**1804–1807.
5. **Thomsen, R. B., Jr.** 2007. Specimen collection, transport, and processing: bacteriology, p. 291–333. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.

SUPPLEMENTAL READING

Baron, E. J., G. H. Cassell, D. A. Eschenbach, J. R. Greenwood, S. M. Harvey, N. E. Madinger, E. M. Paterson, and K. B. Waites. 1993. *Cumitech 17A, Laboratory Diagnosis of Female Genital Tract Infections.* Coordinating ed., E. J. Baron. American Society for Microbiology, Washington, DC.

Difco Laboratories. 1984. *Difco Manual,* 10th ed., p. 546–551, 1025–1026. Difco Laboratories, Detroit, MI.

MacFaddin, J. F. 1985. *Media for Isolation-Cultivation-Identification-Maintenance of Medical Bacteria,* vol. 1. The Williams & Wilkins Co., Baltimore, MD.

Miller, J. M. 1999. *A Guide to Specimen Management in Clinical Microbiology,* 2nd ed. American Society for Microbiology, Washington, DC.

Morris, A. J., S. J. Wilson, C. E. Marx, M. L. Wilson, S. Mirrett, and L. B. Reller. 1995. Clinical impact of bacteria and fungi recovered only from broth cultures. *J. Clin. Microbiol.* **33:**161–165.

Ray, C. G., J. A. Smith, B. L. Wasilauskas, and R. J. Zabransky. 1993. *Cumitech 14A, Laboratory Diagnosis of Central Nervous System Infections.* Coordinating ed., A. J. Smith. American Society for Microbiology, Washington, DC.

Runyon, B. A., M. R. Antillon, E. A. Akriviadis, and J. G. McHutchison. 1990. Bedside inoculation of blood culture bottles with ascitic fluid is superior to delayed inoculation in the detection of spontaneous bacterial peritonitis. *J. Clin. Microbiol.* **28:**2811–2812.

Sharp, S. E., A. Robinson, M. Saubolle, M. Santa Cruz, K. Carroll, and B. Baselski. 2003. *Cumitech 7B, Lower Respiratory Tract Infections.* Coordinating ed., S. E. Sharp. American Society for Microbiology, Washington, DC.

Silletti, R. P., E. Ailey, S. Sun, and D. Tang. 1997. Microbiologic and clinical value of primary broth cultures of wound specimens collected with swabs. *J. Clin. Microbiol.* **35:**2003–2006.

Simor, A. E., F. J. Roberts, and J. A. Smith. 1988. *Cumitech 23, Infection of the Skin and Subcutaneous Tissues.* Coordinating ed., J. A. Smith. American Society for Microbiology, Washington, DC.

Waites, K. B., M. A. Saubolle, D. F. Talkington, S. A. Moser, and V. Baselski. 2005. *Cumitech 10A, Laboratory Diagnosis of Upper Respiratory Tract Infections.* Coordinating ed., S. E. Sharp. American Society for Microbiology, Washington, DC.

Wilhelmus, K. R., T. J. Liesegang, M. S. Osato, and D. B. Jones. 1994. *Cumitech 13A, Laboratory Diagnosis of Ocular Infections.* Coordinating ed., S. C. Specter. American Society for Microbiology, Washington, DC.

3.3.2 Interpretation and Rapid Identification of Bacterial Growth on Primary Culture Media

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The initial interpretation of bacterial growth on primary culture media, which usually follows the first 24 to 48 h of incubation, is an opportunity for the skilled microbiologist to make a preliminary identification and to decide what additional tests and procedures must be performed to arrive at a definitive identification. Many of the tables in this procedure are useful for a staff training manual, but they need not be a part of the bench manuals for daily use.

II. SPECIMEN

Cultures of specimens incubated for routine bacterial culture at 35°C for 24, 48, or ≥72 h

III. MATERIALS

A. Reagents and biochemical tests

1. Aminolevulinic acid (ALA) test (procedure 3.17.3)
2. Bile solubility (procedure 3.17.6)
3. Bile-esculin (procedure 3.17.5)
4. Butyrate disk test (procedure 3.17.7)
5. Catalase reagent (procedure 3.17.10)
6. Coagulase and agglutination tests for detection of coagulase (procedures 3.17.13 and 3.17.14).
7. Gram stain reagents (procedure 3.2.1)
8. Hippurate test with ninhydrin (procedure 3.17.21)
9. Indole reagent (procedure 3.17.23)
10. Leucine amino peptidase (LAP) (procedure 3.17.26)
11. 4-Methylumbelliferyl-β-D-glucuronide (MUG) (procedure 3.17.34)
12. Mueller-Hinton (MH) agar for growth studies and disks (e.g., novobiocin) (procedure 3.17.4)
13. Optochin (procedure 3.17.38)
14. Ornithine decarboxylase (procedure 3.17.15)
15. Oxidase test reagent (procedure 3.17.39)
16. Pyrrolidonyl-β-naphthylamide (PYR) (procedure 3.17.41)
17. Urea disks (procedure 3.17.48)
18. Other tests for sugar fermentation, pigment enhancement, serology, DNA probes, gelatin, germ tube, H_2S, motility, and various kit identification systems

B. Supplies

1. Microscope
2. Microscope slides
3. UV (Wood's) light
4. Petri dishes and sterile sticks and inoculating loops

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that media meet expiration date and QC parameters per the current Clinical and Laboratory Standards Institute (formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

B. For QC requirements for biochemical tests, refer to the individual tests in procedure 3.17.

V. PROCEDURE

Observe standard precautions.

It is imperative that these cultures be handled in a biological safety cabinet.

For specimens from lymph nodes and tiny colonies from normally sterile sites, examine cultures in a biological safety cabinet (7).

A. Initial examination of primary plates

1. Describe colonial morphology for each agar medium.

a. Note the different types of colonies on each agar plate.

(1) When making these initial assessments, look at the culture plates from different angles and use direct illumination of the plate.

(2) Use a light with a magnifying lens to observe slow-growing or very small colonies, which are often hidden between the much larger rapidly growing colonies.

b. Describe the gross colonial morphology of each colony type. Refer to Table 3.3.2–1 for a list of terms.

c. Visual images of typical bacterial colony morphology are provided online at http://microbelibrary.org (1).

2. Enumerate each colony type per the criteria in Table 3.3.2–2.

a. Do not enumerate as CFU in cultures (other than urine, quantitative cultures, and catheter tips) since the specimen has not been quantitatively sampled.

b. On the work record, note the number of colonies on each of several media to distinguish suspected plate contamination from true presence of an organism in a culture.

c. Be careful when one mold colony is larger than several smaller ones. The smaller ones could have arisen from the larger one; all could be contaminants.

3. Interpret colonies on various primary culture media to determine the most likely genus. Refer to Table 3.3.2–3 for a list of common microorganisms and their characteristics on primary media. Refer to procedure 3.8.1 for media specific to the detection of stool pathogens.

a. Note the appearance of organisms on general-purpose primary media.

b. Be aware of organisms that will grow or are inhibited on various selective primary media. Commonly used primary culture media and the likely pathogens isolated from each medium are described in Table 3.3.2–4.

c. Observe medium changes in the vicinity of the bacterial colonies that provide some indication of bacterial activity. Different dyes, sugars, and other ingredients are incorporated into the media to detect end products of bacterial enzymatic activity.

d. Note hemolysis, which is helpful in characterizing microorganisms.

(1) Alpha-hemolysis is the reduction of hemoglobin to methemoglobin, producing a greenish discoloration; RBC membrane is intact on microscopic inspection.

Table 3.3.2–1 Terms to describe gross colonial morphology[a]

SIZE (diameter in mm)
Large = greater than 1mm in diameter
Medium = 1mm in diameter
Small = less than 1mm in diameter

SHAPE

Circular

Filamentous

Irregular

Punctiform

Rhizoid

Spindle

ELEVATION

Flat

Raised

Convex

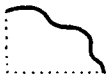
Dome shaped

Umbonate

Umbilicate

MARGIN (edge of colony)

Entire


Undulate

Lobate

Erose

Filamentous

Curled

COLOR White, Black, Cream, Orange etc.

SURFACE APPEARANCE
Glistening — Dull
Smooth — Rough
Granular — Creamy

DENSITY (ability to see through the colony)
Opaque = can not see through the colony
Transparent = can see through the colony
Translucent = only with light shining through

CONSISTENCY (best observed by picking up a colony with a loop or needle)
Butyrous (buttery) — Brittle
Viscid (sticky) — Membranous (pliable)
Friable (crumbles easily)

[a] See reference 1.

V. PROCEDURE *(continued)*

(2) Beta-hemolysis is the lysis of RBCs, resulting in a distinct, clear, colorless zone surrounding and under the colony. The RBC membrane is destroyed.
(3) Gamma-hemolysis indicates no hemolysis. No destruction of RBCs occurs, and there is no change in the medium.

Table 3.3.2–2 Enumeration guidelines

Report	If the observation is growth
Isolated from broth only	In broth only
Scant growth	One to five colonies. For one colony of skin microbiota, may wish to add note regarding possible contamination.
1+ or few	In first quadrant only, ignoring a few colonies in the second quadrant
2+ or moderate	Up to second quadrant, ignoring a few colonies in the next quadrants
3+ or numerous	Up to third quadrant, ignoring a few colonies in the fourth quadrant
4+ or numerous	Up to fourth quadrant
Number + CFU/ml	For urine and quantitative cultures
Number + CFU	For intravascular catheter tips

V. PROCEDURE *(continued)*

(4) Alpha-prime-hemolysis is a small zone of complete hemolysis that is surrounded by an area of partial lysis with green discoloration. Alpha-prime-hemolysis is best seen when magnification is used to observe the colonies.

e. Check for pigment production. If needed, take a swab and pick up some colonies to check for pigment against a white background.
(1) Water-soluble pigments produce a discoloration of the surrounding medium, best seen on colorless agars.
(2) Nondiffusible (carotenoid) pigments are confined to the colonies themselves.

f. Odor may be important in the evaluation of colonies. Many bacteria have distinct odors that are strong clues to their presence and/or identification.
(1) *Pseudomonas aeruginosa* is described as having a fruity, grape-like odor.
(2) *Eikenella corrodens* often smells like bleach or crackers.
(3) *Proteus* species have been described as smelling like devil's food cake or burnt chocolate.

◩ **NOTE:** Because of the danger of laboratory-acquired infections with microorganisms whose aerosols are infectious (7), laboratory personnel should not directly smell colonies. Plates should be opened at arm's length, and odors should be detected by what is obvious during this procedure. In addition, all cultures suspected of containing an organism that requires a higher level of containment should be tape sealed and worked on in a BSL 2 cabinet.

B. Preliminary testing

1. Day 1

a. Gram stain colonies that do not appear to be normal microbiota.

b. Perform initial rapid identification procedures (catalase, oxidase, indole, etc.) for clinically relevant colony types as correlated with Gram stain and most likely pathogen. See Table 3.3.2–5 for appropriate testing.

c. Some specimen types will yield mixed cultures of >3 morphotypes of bacteria. Depending on the source of the specimen and whether these colonies represent potential pathogens, report such cultures with morphologic descriptions and minimal workup.

Table 3.3.2–3 Colonial morphology on primary media[a]

Organism(s)	Morphology on:		
	BAP or CNA[b]	CHOC	MAC/EMB[c]
Escherichia coli	Gray, mucoid, flat or convex, not swarming, may be beta-hemolytic[d]	Same as BAP	Pink/dark center and may have green sheen
Proteus spp.	Flat, gray, spreading[d]	Same as BAP	Colorless
Pseudomonas aeruginosa	Flat, gray-green, rough, may have spreading margins, metallic sheen, may be extremely mucoid[d]	Same as BAP	Colorless
Neisseria gonorrhoeae	Inhibited	Small, gray, entire, sticky	Inhibited
Neisseria meningitidis	Medium to large, creamy and gray, alpha-hemolytic[d]	Same as BAP, no hemolysis	Inhibited
Haemophilus spp.	Inhibited	Gray, raised, smooth, may be mucoid	Inhibited
Moraxella catarrhalis	Whitish, medium to large, raised or dome shaped[d]	Same as BAP	Inhibited
Staphylococcus aureus	Large, convex, white-yellow, creamy, opaque, may be beta-hemolytic	Same as BAP, no hemolysis	Inhibited/may be pinpoint
Coagulase-negative staphylococci	White-gray, raised, creamy	Same as BAP	Inhibited
Beta-hemolytic streptococci	Pinpoint to medium, zone of beta-hemolysis (clear zone) translucent, dull, gray	Same as BAP, no hemolysis	Inhibited
Viridans group streptococci	Pinpoint to medium, white-gray, caramel odor, alpha-hemolysis	Same as BAP	Inhibited
Enterococcus spp.	Gray, medium, usually no hemolysis	Same as BAP	Inhibited/may be pinpoint
Streptococcus pneumoniae	Umbilicate, alpha-hemolysis, transparent, may be mucoid, flattened, or teardrop shaped	Same as BAP	Inhibited
Listeria monocytogenes	Whitish gray similar to group B streptococcus, flat, narrow zones of beta-hemolysis	Same as BAP, no hemolysis	Inhibited
Corynebacterium spp.	White, dry, may be sticky	Same as BAP	Inhibited
Yeast cells	White, creamy, bread odor, "feet" extending from colony	Same as BAP	Inhibited/pinpoint

[a] See reference 1.

[b] CNA, Columbia colistin-nalidixic acid agar.

[c] EMB reaction is listed after MAC reaction separated by a slash, if they differ.

[d] Inhibited on CNA.

V. PROCEDURE *(continued)*

d. Set up definitive biochemical tests or other identification protocols on clinically relevant isolates, if sufficient pure culture material is available and identification from rapid tests is not definitive per Table 3.3.2–5.

◩ **NOTE:** The recommendations in Table 3.3.2–5, except as noted in the table, include the minimum required to identify the organisms listed with

Table 3.3.2–4 Commonly used primary plating media[a]

Medium	Expected isolates	Comments
CHOC	Most microorganisms, including *Haemophilus influenzae*, *Neisseria gonorrhoeae*, and *N. meningitidis*	Low agar content provides increased moisture required by some organisms. Contains heme and often has enrichments, such as IsoVitaleX and cysteine for fastidious organisms. (CHOC without enrichment should not be used for routine cultures.)
BAP with 5% sheep blood	Gram-positive and gram-negative organisms	General-purpose medium. Hemolysis can be observed. Stabbing creates area of reduced O_2, which can demonstrate oxygen-labile hemolysin O.
PEA	Gram-positive organisms	Inhibits gram-negative bacilli.
CNA with 5% sheep blood	Gram-positive organisms	Colistin inhibits gram-negative organisms, and nalidixic acid inhibits *Proteus* spp. Hemolysis may be observed. Columbia agar base is enriched for gram-positive organisms.
EMB	Gram-negative enteric bacilli	Differentiates L + or Su + (black, purple, metallic sheen) from L − or Su − (colorless or transparent). *Pseudomonas aeruginosa* is violet with filamentous margin. L and/or Su fermenters are generally normal enteric bacteria. Gram-positive organisms are inhibited by EMB. The agar concentration can be increased to 5% to inhibit swarming of *Proteus* spp. Enterococci can appear pinpoint.
MAC	Gram-negative enteric bacilli	Differentiates L + (pink) from L − (colorless). Gram-positive organisms are inhibited by bile salts. The agar concentration can be increased to 5% to inhibit swarming of *Proteus* spp.
TM or MTM agar	*Neisseria gonorrhoeae*, *Neisseria meningitidis*	Vancomycin inhibits gram-positive organisms, colistin inhibits gram-negative organisms, and nystatin inhibits yeast cells. Trimethoprim lactate is added to inhibit *Proteus* spp. *Neisseria lactamica* may grow.
CHROMagar	Varies by type of medium	Selective and differential CHROMagars for the isolation of MRSA, VRE, group B streptococci, *E. coli* O157, *Salmonella* spp., uropathogens, and *Candida* spp. are commercially available.[b]

[a] Abbreviations: L, lactose; Su, sucrose; TM, Thayer-Martin; MTM, modified Thayer-Martin. For other abbreviations, refer to Table 3.3.1–1.

[b] Chromogenic substrates within the media produce unique colony changes that allow direct identification of the pathogen of interest after minimal additional testing from the primary plate (http://www.chromagar.com).

Table 3.3.2–5 Common pathogens and rapid and conventional methods to identify them when suspected from colony morphology listed in Table 3.3.2–3

Organism	Presumptive identification	Additional tests for definitive identification[a]	Special requirements
Gram-negative rods			
Escherichia coli	1. Oxidase negative 2. Indole positive 3. Hemolytic	If not hemolytic, then either lactose positive and PYR negative or MUG positive	If lactose negative or not known, PYR will not identify. Confirm non-lactose fermenting from sterile sites with kit.
Proteus mirabilis	1. Spreading 2. Indole negative	No other testing if ampicillin susceptible. If ampicillin resistant, could be *Proteus penneri.*	If ampicillin resistant, confirm with positive ornithine or negative maltose fermentation.
Proteus vulgaris	1. Spreading 2. Indole positive	No other testing for definitive identification	
Pseudomonas aeruginosa	1. Oxidase positive 2. Indole negative 3. Fruity odor of grapes	Odor is definitive. If not fruity odor, blue-green pigment is definitive.	If not fruity or blue-green pigment, has green, fluorescent pigment, and grows at 42°C.[b] If cystic fibrosis patient, check colistin or polymyxin B; must be susceptible.
Pseudomonas fluorescens/putida[b]	1. Oxidase positive 2. Indole negative	Fluorescent but no odor or growth at 42°C	Gelatin or lecithin will separate.
Vibrio/Aeromonas[b]	1. Oxidase positive 2. Indole positive 3. MAC positive	Do kit and growth on MH agar with and without salt to identify (procedure 3.8.1)	If no growth on MAC, could be *Pasteurella* (*see* Table 3.18.2–3)
Eikenella corrodens[b]	1. Gram-negative rod 2. Oxidase positive 3. Catalase negative 4. No growth on MAC 5. Nonhemolytic	Odor of bleach; ornithine positive	
Haemophilus influenzae	1. Gram-negative coccobacilli 2. Good growth on CHOC in *24 h* and *not* on BAP, *or* satellite growth on BAP around staphylococci	Negative ALA test for porphyrin	The nonpathogenic *Haemophilus haemolyticus* is beta-hemolytic on horse or rabbit blood agar and often on sheep BAP. It is ALA negative but often grows unaided on BAP since it hemolyzes the blood. *Francisella tularensis* takes 48 h to grow as a smaller colony on CHOC; it does not grow on BAP.
Gram-negative cocci			
Moraxella catarrhalis	1. Gram-negative diplococci 2. Oxidase positive 3. Colonies on BAP move when pushed	Positive butyrate test	Gram stain morphology of "diplococcus" separates from other *Moraxella* organisms, which are "coccobacilli"
Neisseria gonorrhoeae[b]	1. Gram-negative diplococci 2. Oxidase positive 3. No growth on nutrient or MH agar 4. 4+ positive catalase with 30% H_2O_2	Sugar fermentation positive for glucose only, *or* identification with *Neisseria* kit, DNA probe, *or* serology (*see* procedure 3.9.3)	More than one test needed to confirm if negative result or abuse case
Neisseria meningitidis[b]	1. Gram-negative diplococci 2. Oxidase positive 3. Growth on BAP	Sugar fermentation positive for glucose and maltose only, or identification with *Neisseria* kit	(*See* Table 3.18.2–1.) Use caution when handling culture.

(continued)

Table 3.3.2–5 Common pathogens and rapid and conventional methods to identify them when suspected from colony morphology listed in Table 3.3.2–3 *(continued)*

Organism	Presumptive identification	Additional tests for definitive identification[a]	Special requirements
Gram-positive cocci			
Staphylococcus aureus	1. Catalase positive 2. Gram-positive cocci in clusters 3. Tube or slide coagulase or latex agglutination test positive	No other testing for definitive identification, except nonhemolytic in urine, which need tube coagulase to confirm agglutination test	For isolates from sterile sites, do tube coagulase test for further accuracy.[b] See procedure 3.18.1.
Coagulase-negative staphylococci	1. Catalase positive 2. Gram-positive cocci in clusters 3. Tube or slide coagulase or latex agglutination test negative	Colony not sticky; check for *Staphylococcus saprophyticus* in urine (novobiocin resistant) and *Staphylococcus lugdunensis* in blood (PYR positive; ornithine positive[b])	For isolates from sterile sites, do tube coagulase test with 24 h of incubation for further accuracy.[b]
Streptococcus pyogenes (group A)	1. Gram-positive spherical cocci in pairs 2. Catalase negative 3. Hemolytic 4. Colony of >0.5 mm in diam with sharp edges	PYR positive	Confirm with serology or negative bile-esculin, if site is not respiratory.[b]
Streptococcus agalactiae (group B)	1. Gram-positive spherical cocci in pairs 2. Catalase negative 3. Small zone of hemolysis around translucent colony	CAMP positive or hippurate positive	If invasively collected specimen or not hemolytic, confirm hippurate with PYR (negative) and CAMP or serology.[b]
Viridans group streptococci[b]	1. Gram-positive cocci in pairs 2. Catalase negative 3. Alpha-hemolytic 4. Bile negative or colony whitish	PYR negative	From sterile sites, do LAP and susceptibility to vancomycin or kit identification. Other genera can mimic streptococci (e.g., *Aerococcus* and *Leuconostoc*).[b]
Enterococcus spp.	1. Gram-positive cocci in pairs 2. Catalase negative 3. Nonhemolytic	PYR positive Hemolytic colonies could be group A streptococci. If hemolytic, enterococci are bile-esculin positive.[b]	From sterile sites, do LAP (positive) to verify genus. *Lactococcus* cannot be ruled out.[b] Do motility to separate species and *Vagococcus.*
Streptococcus pneumoniae	1. Gram-positive, lancet-shaped cocci in pairs 2. Catalase negative 3. Alpha-hemolytic	Bile soluble or Quellung positive	If bile-resistant but typical colonies, confirm with optochin susceptibility or DNA probe.[b]
Gram-positive rods			
Bacillus spp. (and related spore-forming genera)[b]	1. Large gram-positive rods 2. Catalase positive 3. Spores present	Motile (nonmotile should be checked for *Bacillus anthracis*)	*Bacillus cereus* group (not *B. anthracis*) is beta-hemolytic and penicillin resistant, with cells >1 μm in diam.
Coryneform rods[b]	1. Gram-positive rods, not large 2. Catalase positive 3. Nonhemolytic 4. Not pigmented 5. Nonmotile	Not branching, or partially acid-fast	Kits are useful to identify to the species level. See procedure 3.11.7 for *Corynebacterium diphtheriae* culture.
Gardnerella vaginalis[b]	1. Tiny gram-variable rod 2. Catalase negative 3. Tiny nonhemolytic colonies grow better on CNA[c] and CHOC than BAP	Beta-hemolytic on human blood or SPS[c] sensitive or hippurate positive	Confirmatory tests not needed for vaginal samples if direct Gram stain is consistent (Appendix 3.2.1–3).
Lactobacillus spp.[b]	1. Gram-positive rods 2. Catalase negative 3. Alpha-hemolytic	H_2S negative, vancomycin resistant	*Erysipelothrix* is H_2S positive and vancomycin resistant. Vancomycin susceptible could be *Actinomyces.*

Table 3.3.2–5 *(continued)*

Organism	Presumptive identification	Additional tests for definitive identification[a]	Special requirements
Listeria monocytogenes[b]	1. Tiny gram-positive rods without chaining 2. Catalase positive 3. Narrow zone of beta-hemolysis 4. Motile (tumbling) in wet mount	Bile-esculin positive and CAMP positive	If wet mount motility is questionable, check motility at 25°C but not at 35°C in semisolid agar.
Yeast cells	Budding yeast in smear	1. Yeasts can be presumptively identified as *Candida albicans* when there is the appearance of "feet" on BAP in <48 h and/or the germ tube is positive. 2. Tiny yeasts that are rapid trehalose positive or grow better on EMB are *Candida glabrata.*	Growth at 45°C separates *C. albicans* from *Candida dubliniensis.* See section 8 of this handbook for test methods and other identifications.

[a] For procedures on biochemical testing, refer to procedure 3.17; for information on multitest biochemical kit tests, refer to procedure 3.18.

[b] All testing requirements for identifications are found in NCCLS document M35 (6), *except* those noted by this footnote, which are taken from the references listed in Supplemental Reading and in procedures 3.18.1 and 3.18.2. The identifications from the NCCLS document are greater than 95% accurate without further testing.

[c] CNA, Columbia colistin-nalidixic acid agar; SPS, sodium polyanethol sulfonate.

V. PROCEDURE *(continued)*

a greater than 95% accuracy as presented in the guidelines of CLSI (formerly NCCLS) (6). Further testing even from normally sterile sites is not needed if there is a definite identification from performance of the tests listed.

e. Set up susceptibility tests as appropriate (references 2 and 3 and section 5).

f. Make subcultures of nonisolated colonies to appropriate media for later evaluation.

g. Reincubate all primary and subculture media for an additional 8 to 12 h.

h. Examine broth cultures by following the procedure below.

i. Prepare preliminary report.

j. When colonies are too small to determine their characteristics and incubation has been less than 18 h, report as "too young to evaluate." Reread plates later in the day.

2. Day 2

a. Read and record reactions and test results from previous day.

b. Reexamine plates and broth for changes and for colony morphologies not present the previous day.

c. Read anaerobic plates.

(1) Distinguish between organisms that are true anaerobes (i.e., those which grow markedly better or only under anaerobic conditions) and those that are facultative anaerobes (i.e., those which grow equally well aerobically and anaerobically) by performing Gram stains and comparing colonies on anaerobic plates to those present on aerobic plates.

(2) Note each colony type of suspected anaerobe, and subculture it to a section of CHOC and a section of an anaerobic medium; incubate the CHOC aerobically and the other anaerobically.

V. PROCEDURE *(continued)*

(3) Refer to section 4 of this handbook for further identification protocols for anaerobic bacteria.

d. Set up additional tests as needed, using references for unusual and difficult identifications (*see* procedures 3.18.1 and 3.18.2 and references listed in Supplemental Reading).

e. Prepare updated or final report, notifying appropriate persons of clinically or epidemiologically important results.

f. For positive cultures or isolates, whether complete identification is performed or not, hold a representative plate at room temperature for 5 days in case the clinician notifies the laboratory of the need for further studies.

g. Get immediate help from a supervisor or laboratory director when the identification or extent of identification is not clear. Delays in identification can affect patient care.

h. Perform susceptibility testing (section 5) for those organisms deemed significant, provided that standard methods are available for testing (2, 3).

i. Send subculture of significant isolates to reference laboratory when unable to identify or when confirmation is required or desired. See procedure 15.5 for details for packaging and shipping.

3. For additional days, follow up identification and susceptibility testing procedures until all relevant isolates have been identified; then send a final updated report.

◩ **NOTE:** When a culture of a slow-growing organism, e.g., *Nocardia,* is requested, media should be held and examined for 7 days. (*See* section 6 of this handbook.)

4. Isolates from positive cultures of sterile fluids (i.e., cerebrospinal, pleural, synovial, peritoneal, and pericardial), blood cultures, and tissues (i.e., bone, liver, brain, lung, etc.) should be saved for a minimum of 1 year so that further studies can be performed on the organism(s) if required for clinical or epidemiological purposes.

a. Save all primary pathogens for each patient.

b. Contaminants recovered from sterile-site cultures such as skin microbiota do not need to be saved unless their clinical relevance in a particular infection has not been definitively established.

c. Use one of the following methods for temporary isolate storage.

(1) Inoculate onto semisolid medium, such as sulfide-indole-motility agar (procedure 3.17.22) or cysteine Trypticase agar.

(2) Hold strict anaerobes in chopped meat.

(3) Hold fastidious organisms on CHOC slants or deeps overlaid with mineral oil.

d. For permanent storage, aerobic bacterial isolates should be frozen in TSB in 15% glycerol or in sterile skim milk at −70°C. Anaerobes should be frozen in rabbit blood or sterile skim milk.

5. Handling of broth cultures

Historically, tissues from surgery and fluids from selected normally sterile sites have been inoculated into broth, but this practice has been shown to be clinically neither relevant nor cost-effective in improving the recovery of pathogenic organisms from these types of specimens (4, 5). Prolonged incubation of broth cultures rarely enhances isolation of a primary pathogen and may result in overgrowth of commensal skin microbiota. The following procedure is included for laboratories continuing to perform broth cultures.

a. Incubate at 35°C and examine daily for 4 days.

V. PROCEDURE *(continued)*

b. For broths with apparent growth indicated by turbidity, fronds of growth, puff balls, pellicles, or sediment in the bottom of an otherwise clear tube, follow procedure below.

(1) If the direct plates have growth of fewer than three morphologic types and the broth is turbid, smear the broth for comparison with direct plates. Subculture if there is any suggestion that a different morphotype is present. Use appropriate plates to select for aerobes and anaerobes depending on the smear results.

(2) If direct plates are negative, smear and subculture turbid broths to BAP and CHOC, using care not to contaminate the broth. If the morphology is suggestive of anaerobes, inoculate anaerobic BAP.

(3) If an organism is seen in the broth but not on solid media on subculture, try more nutritious media or special incubation conditions to isolate the organism. Also, evaluate uninoculated broth for evidence of "dead" organisms in medium.

(4) If the culture grew coagulase-negative staphylococci and gram-positive cocci are in the broth, subculture the broth to rule out the presence of *Staphylococcus aureus.*

(5) When in doubt, resmear a turbid culture, but a subculture need not be repeated. Use of acridine orange stain may be helpful. See procedure 3.2.2.

(6) When broth is entered, record on tube the date and indicate that broth was smeared and subcultured. This will allow quick evaluation of broths on subsequent days.

(7) Broth cultures should be incubated and examined for growth for a minimum of 4 days at 35°C. Broth cultures that are positive for a primary pathogen within this time may be discarded once the culture report is finalized.

c. Broth cultures that remain negative after 4 days of incubation at 35°C may be held at room temperature for up to 7 days after inoculation and examined for obvious growth only prior to discard.

◩ **NOTE:** In the case of a clinically important infectious disease for which a pathogen has not been detected, refer specimen to a reference laboratory for molecular studies.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report results of direct smear within 1 h of receipt for specimens from invasive procedures and 4 h for specimens from other sites.

B. Reporting the presence of pathogens

1. Enumerate each pathogen as indicated in Table 3.3.2–2.

2. Report preliminary results on all cultures, including presumptive identifications.

3. When reporting identifications that are suggestive but for which not all biochemical testing has been completed, preface the organism name with "probable" or "presumptive."

4. If the identification changes after further testing, make a note of the change in the report.

5. If a species identification is not indicated, inform the physician by adding "NOS" (not otherwise specified) following the genus name and "spp."

VI. REPORTING RESULTS *(continued)*

6. Use the following guidelines to report repeated isolation of the same organism.

a. Do not perform full identification and susceptibility testing on microorganisms, if the patient has had a positive culture from the same source within the last (*x*) days with what apparently is the same organisms(s) and full identification and susceptibility testing were done on the previous isolate(s).

◩ **NOTE:** For determination of (*x*) days, a good general rule is to repeat identifications every 7 days, if the morphology is the same. An exception would be for nonhemolytic staphylococci, all of which should be checked with a coagulase test. Policies on how often to repeat antimicrobial susceptibility testing (AST) vary and should be based on evaluation of local AST results and therapies used to treat disease. General guidelines include 7 days for oxacillin-susceptible staphylococci and most gram-negative rods, 4 days for *P. aeruginosa* and selected other gram-negative rods, and 30 days for vancomycin-resistant enterococci. If extended-spectrum beta-lactamases are present locally, additional susceptibility surveillance may be indicated.

b. Ensure that the current organism is morphologically consistent with the previous isolate(s) prior to reporting them as identical. Perform minimal procedures to confirm the identification (oxidase, indole, catalase, etc.), if possible.

c. Report the genus and species identification.

d. When referring identification to prior identification, indicate in the report that the identification is "presumptive" followed by the following comment after the organism name: "Refer to culture from [date] for complete identification [and susceptibility testing]." Use caution so that referred cultures are not referred to referred cultures.

e. If susceptibility testing was performed (e.g., not sure it is the same, previous positive overlooked, etc.), record these results but do not report them, unless they differ from the prior result. Such reporting can distort the data in the antibiogram produced by the laboratory for epidemiological surveys.

7. Possible plate and broth contaminants

a. Review plates for possible plate contaminants (especially if the broth has no growth) before reporting.

◩ **NOTE:** If a broth turns positive with a gram-positive organism after the broth has been sampled, the organism may have been introduced at the time of sampling.

b. Do not report clear contaminants (those not on the streak).

c. For questionable contaminants, add the following notation to the report: "[Organism name] present in culture, cannot distinguish true infection from plate contamination; consider confirmation of isolate with appropriate follow-up culture."

d. Investigate repeated isolation of the same mold or skin microbiota, and perform cleaning of laboratory equipment to prevent recurrence. Special attention should be paid to the potential pathogenicity of a "contaminant" isolated repeatedly from the same or different anatomic sites.

C. Reporting mixed cultures

1. When three or more microorganisms of questionable clinical significance are isolated, report as a mixed culture.

Example: "Culture yields abundant growth of >3 colony types of enteric gram-negative bacilli. Please consult microbiology laboratory if more definitive studies are clinically indicated."

VI. REPORTING RESULTS *(continued)*

2. If only normal microbiota is observed, report as the following.
- **a.** Gastrointestinal
- **b.** Skin
- **c.** Genital
- **d.** Oral-nasal
- **e.** Staphylococcal skin (for wounds with only coagulase-negative staphylococci in adults)

  ◩ **NOTE:** Reporting coagulase-negative staphylococci may be indicated for cluster epidemics in neonatal units or intensive care units)
- **f.** Mixed anaerobic microbiota
- **g.** Mixed gram-positive microbiota, NOS (for mixed respiratory microbiota [staphylococci, diphtheroids, and enterococci] without viridans group streptococci)
- **h.** Alternatively, report as "No significant microorganisms isolated."

D. If no growth is observed on all media, send out report as "No growth in *x* days," where "*x*" is the number of days the culture has been incubated.
1. When the culture has been incubated the number of days required based on the source, document that the culture report is final.
2. If incubation is continued beyond the final date but no more reports will be issued unless it turns positive, add the comment "Cultures will be held for _____ days."

E. Telephone clinically or epidemiologically critical results to the appropriate persons. Such results include the following.
1. Anything isolated from normally sterile source (blood, CSF, peritoneal fluid, tissue, etc.)
2. Pathogens of serious clinical or epidemiologic concern, for example, *Neisseria gonorrhoeae, Streptococcus pyogenes, Clostridium difficile* toxin, enteric pathogens, and *Mycobacterium tuberculosis*
3. Take the initiative to immediately call any unusual, impressive finding that is of critical importance to patient care.

F. Document all testing on a hard copy or computerized work record.

G. Document reference laboratory identification when performed by reporting "Identification confirmed by" followed by reference laboratory name and address.

H. Document all processing and reporting errors in the report, and telephone report of such errors to the person who ordered the test.

VII. INTERPRETATION

A. Report culture results with emphasis on the clinical importance and relevance to the diagnosis and treatment of the patient.

B. Reporting normal microbiota, mixed cultures, or questionable contamination with the same level of detail as done for clinically significant pathogens can lead to erroneous diagnoses and treatment of the patient.

C. Use the Gram stain of the specimen as a guide to interpretation of results.

D. Bacterial cultures from normally sterile sites typically contain a low number of organisms so that recovery of isolates from these types of specimens may be difficult to achieve. The primary specimen Gram stain should be compared to the morphotypes recovered from culture. Anaerobes or fastidious bacteria may be present if an organism is seen in the direct Gram stain but is not recovered on aerobic culture. Other methods may need to be used to isolate the agent, including incubation of cultures under different atmospheric conditions, the use of specialized media and stains, and the use of DNA probes and immunological tests.

VI. INTERPRETATION *(continued)*

E. Decisions regarding the performance of susceptibility testing must also consider the significance of the organism and the ability of the susceptibility testing to provide information that is not already available; e.g., *S. pyogenes* organisms in throat cultures are uniformly susceptible to penicillin, and susceptibility testing for this antimicrobial is not indicated.

VIII. LIMITATIONS

A. Accurate reporting of culture results is limited to the expertise of the microbiologist who is able to recognize, evaluate, and pursue significant bacteria in the culture.

B. False-negative results occur when one of the following occurs.
- **1.** Cultures are delayed in processing.
- **2.** Incorrect atmosphere or temperature of incubation is used.
- **3.** Medium does not support growth of the microorganism.
- **4.** Organisms are present in low numbers or the volume of specimen cultured is too low to detect them.
- **5.** WBCs and other body defense factors inhibit growth.
- **6.** Collection or transport of the specimen was not optimal.
- **7.** The microorganism cannot be cultured by any method available at the present time.

C. False-positive results are due to the following.
- **1.** Mix-up of cultures from different patients
- **2.** Reporting of contaminants from the laboratory or collection process as pathogens in a culture

D. The lack of isolation of a pathogen does not necessarily mean that the laboratory was unable to detect the agent, because other diseases can have the same presentations as infectious diseases.

REFERENCES

1. **Breakwell, D., R. Robison, C. Adams, K. Smith, and R. MacDonald.** 2007. *Colony Morphology.* http://microbelibrary.org.
2. **Clinical and Laboratory Standards Institute.** 2006. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 7th ed. Approved standard M7-A7. Clinical and Laboratory Standards Institute, Wayne, PA.
3. **Clinical and Laboratory Standards Institute.** 2006. *Performance Standards for Antimicrobial Disk Susceptibility Tests*, 9th ed. Approved standard M2-A9. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Meredith, F. T., H. K. Phillips, and L. B. Reller.** 1997. Clinical utility of broth cultures of cerebrospinal fluid from patients at risk for shunt infections. *J. Clin. Microbiol.* **35:**3109–3111.
5. **Morris, A. J., S. J. Wilson, C. E. Marx, M. L. Wilson, S. Mirrett, and L. B. Reller.** 1995. Clinical impact of bacteria and fungi recovered only from broth cultures. *J. Clin. Microbiol.* **33:**161–165.
6. **NCCLS.** 2002. *Abbreviated Identification of Bacteria and Yeast.* Approved guideline M35-A. NCCLS, Wayne, PA.
7. **U.S. Department of Health and Human Services (Public Health Service), Centers for Disease Control and Prevention, and National Institutes of Health.** 2007. *Biosafety in Microbiological and Biomedical Laboratories*, 5th ed. U.S. Government Printing Office, Washington, DC.

SUPPLEMENTAL READING

Barrow, G. I., and K. A. Feltham (ed.). 2007. *Cowan and Steel's Manual for the Identification of Medical Bacteria*, 3rd ed. Cambridge University Press, New York, NY.

Forbes, B. A., D. F. Sahm, and A. S. Weissfeld. 2007. *Bailey and Scott's Diagnostic Microbiology*, 12th ed. Mosby, St. Louis, MO.

Garrity, G. (ed.). 2001. *Bergey's Manual of Systematic Bacteriology*, vol. 1. Williams & Wilkins, Baltimore, MD.

Garrity, G. (ed.). 2005. *Bergey's Manual of Systematic Bacteriology*, vol. 2. Williams & Wilkins, Baltimore, MD.

Garrity, G. (ed.). 2009. *Bergey's Manual of Systematic Bacteriology*, vol. 4. Williams & Wilkins, Baltimore, MD.

Garrity, G. (ed.). 2009. *Bergey's Manual of Systematic Bacteriology*, vol. 5. Williams & Wilkins, Baltimore, MD.

Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams. 1994. *Bergey's Manual of Determinative Bacteriology*, 9th ed. Williams & Wilkins, Baltimore, MD.

Horvath, R. S., and M. E. Ropp. 1974. Mechanism of action of eosin-methylene blue agar in the differentiation of *Escherichia coli* and *Enterobacter aerogenes. Int. J. Syst. Bacteriol.* **24:**221–224.

MacFaddin, J. (ed.). 1985. *Media for Isolation-Cultivation-Identification-Maintenance of Medical Bacteria.* Williams & Wilkins, Baltimore, MD.

Murray, P. R., E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.). 2007. *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.

Parks, L. C. (ed.). 2004. *Handbook of Microbiological Media by Ronald Atlas*, 3rd ed. CRC Press, Boca Raton, FL.

Thomsen, R. B., Jr. 2007. Specimen collection, transport, and processing: bacteriology, p. 291–333. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M.A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.

Truant, A. L. (ed.). 2002. *Manual of Commercial Methods in Microbiology.* ASM Press, Washington, DC.

Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar. 1996. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed. Williams & Wilkins, Baltimore, MD.

Winn, W. C., Jr., S. D. Allen, W. Janda, E. Koneman, G. Procop, P. Schreckenberger, and G. Woods (ed.). 2005. *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*, 6th ed. J. B. Lippincott, Philadelphia, PA.

3.4.1 General Detection and Interpretation

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

When bacteria or fungi overcome the host's normal defense mechanisms and enter the bloodstream through the lymphatics or from extravascular sites, they can quickly disseminate throughout the body, causing severe illness. In addition, the by-products of their metabolism can lead to septic shock, among the most serious complications of infectious diseases. *Rapid recognition and immediate institution of appropriate treatment are essential.* Laboratory diagnosis of bacteremia and fungemia depends on blood cultures, which are probably the most important cultures performed by the microbiology laboratory. Because the culture methods are so sensitive, the procedure must be carefully controlled beginning at the preanalytical stage (collection), to avoid the misinterpretation of a procurement-associated skin commensal microorganism as an agent of infection.

One blood culture usually consists of blood from a single venipuncture inoculated into two separate bottles to accommodate the volume of blood removed (usually 20 ml for adults), since optimal blood-to-broth ratios are 1:5 to 1:10. The use of more than one formulation of medium (usually one aerobic and one anaerobic bottle) for each blood culture generally maximizes recovery of all possible pathogens. Adequate volume is the single most important factor in the laboratory detection of microorganisms in the bloodstream; the more blood cultured, the more likely a culture will be positive (15, 17–19, 28). In one study of adults, increasing the total volume cultured from 20 to 40 ml increased the yield by 19%; increasing the volume from 40 to 60 ml increased the yield by an additional 10% (19). The total volume of blood drawn should be divided equally between at least two separate venipunctures, to allow evaluation of the recovery of a skin organism in a single positive set that could have resulted from contamination of the culture during collection (17). Sodium polyanethol sulfonate (SPS) is added as an anticoagulant, an antiphagocytic agent that inactivates complement, and a neutralizing agent to inhibit effects of many antimicrobial agents and antibacterial factors in blood (22). Media should contain 0.025 to 0.05% SPS; however, even at that level it can inhibit the recovery of some bacteria, such as *Neisseria* spp., *Streptobacillus moniliformis*, *Peptostreptococcus*, and *Gardnerella vaginalis* (1).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

◩ **NOTE:** Refer to procedure 3.3.1 for additional details. This portion can be copied for preparation of a specimen collection instruction manual for physicians and caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

A. See Appendix 3.4.1–1 for collection and testing for specialized microorganisms.

B. Collect blood aseptically by venipuncture or from previously placed lines.

◩ **NOTE:** Bone marrow culture should be reserved for culture for specific pathogens such as *Brucella*, *Salmonella*, *Listeria*, and fungi and mycobacteria; it adds little to detection of most other bacteria in the blood. Because of the complex nature of the collection procedure, it is more likely to result in a contaminated bacterial culture than a diagnostic one (M. K. York, unpublished data). Thus, routine cultures of bone marrow without a specific indication should be discouraged.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

C. Recommended total volume and numbers of blood cultures

1. Neonates to 1 year (<4 kg): 0.5 to 1.5 ml/tube, although at least 1.0 ml is preferred (18)

◩ **NOTE:** Two separate venipunctures are generally not possible.

2. Children 1 to 6 years old: 1 ml per year of age, divided between two blood cultures. For example, for a 3-year-old, draw 1.5 ml from each of two sites, for a total of 3.0 ml of blood. Consult with the physician, who is responsible for ordering the amount to be collected, especially if the child is below normal weight or has had previous venipuncture for other reasons.

3. Children weighing 30 to 80 lb: 10 to 20 ml, divided between two blood cultures

4. Adults and children weighing >80 lb: 30 to 40 ml, divided between two blood cultures

◩ **NOTE:** At least 20 to 30 ml of blood in two draws is the minimal requirement (19).

D. Timing of blood cultures

◩ **NOTE:** Although drawing blood cultures before or during the fever spike is optimal for recovery, volume is more important than timing in the detection of agents of septicemia.

1. When acute sepsis or another condition (osteomyelitis, meningitis, pneumonia, or pyelonephritis) requires immediate institution of antimicrobial agent therapy, draw two blood cultures of maximum volume consecutively from different anatomic sites before starting therapy.

2. For fever of unknown origin, subacute bacterial endocarditis, or other continuous bacteremia or fungemia, draw a maximum of three blood cultures with maximum volume.

3. When it is appropriate to draw blood cultures from patients on antimicrobial therapy, they should be drawn when antimicrobial agents are at their lowest concentration.

◩ **NOTE:** Use of a resin- and/or dematiaceous earth-containing medium may enhance recovery of microorganisms, especially staphylococci. However, appropriate dilution and action of some anticoagulants and antifoaming agents may also diminish the effect of antimicrobial agents. *If multiple cultures were drawn prior to the start of therapy, additional blood cultures from patients on antimicrobial therapy during the same febrile episode should be discouraged, since they are rarely positive* (11).

E. Skin antisepsis and collection of blood from venipuncture

1. Select a different venipuncture site for each blood culture.

a. If poor access requires that blood for culture be drawn through a port in an indwelling catheter, the second culture must be from a peripheral site, because cultures drawn through catheters can indicate catheter colonization but may not be indicative of sepsis (2).

b. Do not draw blood from a vein into which an intravenous solution is running.

c. Except from neonates, draw the two blood cultures in succession. If the phlebotomy must be performed at the same site (usually because of bad veins), perform the second venipuncture at that site.

2. Prepare the site.

a. Vigorously cleanse with 70% isopropyl or ethyl alcohol to remove surface dirt and oils. Allow to dry.

b. Swab or wipe concentric circles of tincture of iodine, moving outward from the center of the site.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

c. Allow the iodine to dry (about a minute), and avoid touching the site.

◩ **NOTE:** If povidone-iodine is used, it must be allowed to dry completely (about 2 min); 2% chlorhexidine gluconate in isopropyl alcohol may be used in place of tincture of iodine.

d. *For pediatric patients, omit the iodine step and clean two additional times with separate preparation pads saturated with 70% isopropyl alcohol or ethyl alcohol (26).*

3. Prepare the septum of the blood culture bottle and the rubber stoppers on bottles or tubes. Label the bottles with the patient name and the date and time of draw. Site of draw may also be listed.

4. Vigorously wipe septa with 70% alcohol and allow to dry completely, usually for 30 to 60 s.

◩ **NOTE:** Wiping the septum with iodine is usually unnecessary but may be considered if there is a history of problems with *Bacillus* spores or mold contamination.

5. While wearing gloves, insert the needle into the vein and withdraw the blood. Use a new needle if the first attempt is not successful. *Do not repalpate the skin after it is disinfected.*

6. *Apply a safety device to protect the phlebotomist from needle exposure.*

◩ **NOTE:** Safety devices consist of domes with internal needles that attach either to a syringe or directly to the tubing used in collecting the blood. The external port of these devices will accommodate a syringe or the end of a butterfly needle depending on the product (e.g., blood transfer device [catalog no. 364880; Becton Dickinson and Co., Paramus, NJ], BacT/Alert blood transfer device [bioMérieux Inc., Hazelwood, MO], Angel Wing adapter [Sherwood Davis & Geck, St. Louis, MO]).

7. Inoculate first the aerobic bottle and then the anaerobic bottle with *no more* than the manufacturer's recommended amount of blood.

a. For direct inoculation into the bottles from the needle apparatus, mark the side of the bottle with the manufacturer's recommended draw.

b. If using a needle and syringe, use the volume markings on the syringe to note the volume. *Hold the syringe plunger during transfer to avoid transfer of excess blood into bottles having a significant vacuum.*

◩ **NOTE:** There is no need to change the safety device between bottle inoculations (22).

8. Thoroughly mix bottles to avoid clotting.

9. After phlebotomy, dispose of needles in sharps container and remove residual tincture of iodine from the patient's skin by cleansing with alcohol to avoid development of irritation.

F. Collection of blood from intravascular catheters

◩ **NOTE:** Using either quantitative cultures (Appendix 3.4.1–2) or time to positive signal of cultures processed on an automated instrument, the comparison of cultures that are drawn through an indwelling intravenous catheter and through a peripheral site may be useful for diagnosis of catheter-related sepsis (2).

1. Label bottles with patient name, site of draw, and date and time of draw.

2. Disinfect the septum of the blood culture bottle and the rubber stoppers on bottles or tubes with 70% alcohol as for peripheral draw. Allow to dry completely, usually for 30 to 60 s.

3. Using two separate alcohol preps, scrub catheter hub connection for 15 s with 70% alcohol. Air dry.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

4. While wearing gloves, disconnect tubing or cap of catheter and attach syringe to collect discard blood (suggested amounts are 3 ml for adults and 0.2 ml for pediatric patients), which is not used for culture.
   ◩ **NOTE:** Avoid drawing from lines within an hour of completion of antimicrobial agent administration.
5. Using a new syringe, collect blood for culture through the hub. Quickly reconnect tubing.
6. Connect filled syringe to safety system adapter.
7. Holding the syringe plunger for control, inoculate the bottles with *no more* than the amount recommended by the manufacturer.
8. Thoroughly mix bottles to avoid clotting.

G. In addition to labeling the blood culture bottles or tubes with patient demographic information and collection time, indicate whether the collection was from a peripheral draw or a catheter draw and the initials of the phlebotomist.

H. Specimen transport

1. Do not refrigerate blood cultures. Generally hold at room temperature until processed, for a maximum of 4 h.
2. Refer to manufacturer's instructions for the appropriate method to store cultures prior to incubation in automated culture systems.
3. Provide method of transport that will ensure that bottles are not broken in transit. Ensure that users follow exactly the instructions for sending blood culture bottles through pneumatic tube systems.

I. Rejection criteria

1. Reject blood cultures that are received unlabeled.
2. Do not process if the tube or bottle is cracked or broken.
3. Labeled blood cultures are not rejected even if medium is expired, volume or number of bottles is insufficient, or bottles were received >12 h after collection, but develop an education plan to ensure that cultures are collected appropriately and numbers are not excessive. Document deficiency in report, as well as the effect on the reliability of the culture results.

III. MATERIALS

(See procedure 3.1 for vendor contact information.)

A. Media (*see* references 22 and 29 for review of literature on comparison of blood culture media listed below)

1. Usually inoculate one aerobic and one anaerobic medium for each culture. In addition, or instead of the above, inoculate a similar medium containing resin or dematiaceous earth to absorb antimicrobial agents.
   ◩ **NOTE:** For specialized patients (e.g., pediatric patients that do not have an abdominal abnormality), anaerobic cultures may not be indicated (6, 7, 16, 33). However, always inoculate at least two bottles for adults. Always include anaerobic cultures for febrile neutropenic patients and patients with diabetes or wound infections.
2. Several companies supply media for automated systems, formulated to maximize detection based on the indicator system.
   a. Bactec (BD Division Instrument Systems)
   b. ESP (Trek Diagnostics)
   c. BacT/Alert (bioMérieux Inc.)
   ◩ **NOTE:** Follow manufacturer's instructions for inoculation, special handling, and incubation of blood culture media. Most systems accommodate 10 ml per bottle for adult bottles and less for bottles designed for pediatric use.

III. MATERIALS *(continued)*

3. Alternatives to automated systems
   - **a.** Broth media in bottles to be observed manually (TSB, supplemented peptone, THIO, anaerobic BHI)
   - **b.** Biphasic media (agar and broth in one bottle)
     - (1) BBL Septi-Chek (BD Microbiology Systems)
       - (a) Agar consists of CHOC, MAC, and malt agar.
       - (b) Broth options are BHI, BHI supplemented, TSB, TSB with sucrose, Columbia, THIO, and Schaedler broth.
     - (2) PML biphasic (PML Microbiologicals, Inc.)
       - (a) Agar consists of CHOC and BHI agar.
       - (b) Broth is TSB.
   - **c.** Lysis-centrifugation system of ISOLATOR (Wampole Laboratories)

B. Reagents and media for biochemical tests

1. Agar plate media listed in Table 3.3.1–1
2. Gram stain reagents
3. Antimicrobial susceptibility testing (AST) system and beta-lactamase test (refer to section 5)
4. δ-Aminolevulinic acid (ALA) reagent (procedure 3.17.3)
5. Bile-esculin slants (procedure 3.17.5)
6. 10% Bile (sodium desoxycholate [procedure 3.17.6])
7. Catalase test reagent (procedure 3.17.10)
8. Coagulase rabbit plasma and (optionally) staphylococcal agglutination tests (procedures 3.17.13 and 3.17.14)
9. Disks (procedure 3.17.4)
   - **a.** 10 U of penicillin
   - **b.** 30 μg of vancomycin
   - **c.** 1 μg of oxacillin
   - **d.** Optochin (procedure 3.17.38)
   - **e.** 300 U of polymyxin B or 10 μg of colistin
10. Spot indole reagent (procedure 3.17.23)
11. Broth for motility (procedure 3.17.31)
12. Ornithine decarboxylase (procedure 3.17.15)
13. Oxidase test reagent (procedure 3.17.39).
14. L-Pyrrolidonyl-β-naphthylamide (PYR) substrate and developer (procedure 3.17.41)
15. Multitest gram-negative and gram-positive commercial automated, semiautomated, and manual kit identification systems, referred to as "kits." For detailed information on these products, refer to Evangelista et al. (9).
16. Media for identification of yeast and mold (*see* section 8)
    - **a.** CHROMagar (optional)
    - **b.** Calf serum for germ tube
    - **c.** India ink
    - **d.** Phenol oxidase test (caffeic acid disk or birdseed agar)
    - **e.** Rapid trehalose test (section 8)
    - **f.** Urea agar (procedure 3.17.48)
17. Other media as needed for special identifications
    - **a.** Acridine orange stain (procedure 3.2.2)
    - **b.** Media for H_2S detection (procedure 3.17.22)
    - **c.** Quellung test (procedure 3.17.42)
    - **d.** Leucine aminopeptidase (LAP) (procedure 3.17.26)
    - **e.** Salt (6.5%) broth (procedure 3.17.43)
    - **f.** Optional: serologic reagents for grouping *Salmonella* and *Shigella* (Appendix 3.8.1–1) or grouping beta-hemolytic streptococci into the Lancefield groups (procedure 3.11.8)
    - **g.** Rapid urea disk (procedure 3.17.48)

C. Supplies

1. 3-ml syringes with safety apparatus or venting needle
2. Alcohol (70 to 95%) and gauze
3. Microscope slides

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Verify that plate media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22. See procedures 14.2 and 3.3.1 for further procedures.

B. See individual tests in procedure 3.17 for biochemical test QC.

C. Blood culture bottles

1. For in-house-prepared culture media, verify that each lot of media will support the growth of bacteria likely to be present in blood cultures, including, but not limited to, the following microorganisms (as listed in Table 2 of CLSI document M22-A3 [4]).

a. Aerobic bottles

(1) *Pseudomonas aeruginosa* (ATCC 27853)

(2) *Streptococcus pneumoniae* (ATCC 6305)

b. Anaerobic bottles

(1) *Bacteroides fragilis* (ATCC 25285)

(2) *S. pneumoniae* (ATCC 6305)

c. Method of testing

(1) Prepare a broth culture of each microorganism equivalent to a 0.5 McFarland standard.

(2) Inoculate each bottle with 0.01 ml (10 µl) of suspension.

(3) Incubate for up to 5 days and observe for visible growth.

2. Maintain records provided by manufacturers of commercial blood culture systems, documenting their QC testing.

◩ **NOTE:** Regulatory agencies do not require routine QC checks by the user of commercially purchased blood culture bottles.

D. Check CHOC on biphasic medium paddles for growth of *Haemophilus influenzae* and *Neisseria gonorrhoeae*.

E. Prior to institution of a blood culture system, determine that it will support the growth of a variety of microorganisms, including fastidious gram-negative rods and cocci. Use bottles supplemented with human blood from volunteers for testing fastidious microorganisms.

F. Contamination

1. Have a system in place to determine if a culture has been collected from an intravascular catheter and whether a peripheral collection accompanied the line collection for adults. Educate clinicians on the need to collect both, to properly evaluate the culture results (2, 26).

2. Monitor positive blood culture results regularly for skin contamination.

a. For purposes of determination of contamination rate, consider only skin contaminants from venipuncture as significant. These consist of the following.

(1) Coagulase-negative staphylococci (excluding pediatric and line collections)

(2) *Bacillus* species

(3) *Corynebacterium* species

(4) *Propionibacterium* species

b. Exclude positive cultures as skin contaminants if more than one blood culture from the same patient is positive for any of the microorganisms listed above, provided that for coagulase-negative staphylococci the antibiograms are consistent with the isolates being the same strain.

c. Include as skin contaminants all positive cultures, even if both bottles are positive or only one culture was collected (20).

d. Calculate the contamination rate by dividing the number of cultures containing skin contaminants by the total number of cultures collected by venipuncture.

IV. QUALITY CONTROL
(continued)

3. Initiate additional training of staff involved with blood collection and processing if the contamination rate exceeds the expected rate for the institution. The rate should generally not exceed 3%, although many institutions have a goal to decrease the rate below 1% (26, 27) for cultures collected by venipuncture. When rates are high, separate calculations can be done for each phlebotomist and for each collection location.

G. Validate true-positive blood culture rates.
1. Calculate the true-positive culture rate by dividing the number of cultures positive by the total number of blood cultures received.
2. The rate should be in the 6 to 12% range. If it is too low, too many cultures might have been drawn; if it is too high, not enough cultures may have been collected.

H. Perform statistical analysis periodically to determine if the length of incubation is sufficient or can be decreased without loss of clinically meaningful information (3, 6, 8, 12, 23, 32).
1. Tabulate the number of cultures positive on days 4 through 7.
2. Delete those positives that are considered skin contaminants, as defined above.
3. Delete those positives that are positive in another culture collected within 24 h before or after the delayed positive culture.
4. For the remaining positive cultures, determine from chart review if they were considered contributing to true bacteremia.
5. Review completed results with the infectious disease service to determine what is a reasonable incubation period for cost-effective detection of bacteremia.

I. Use statistics to determine if bottles in use are appropriate for detection of local pathogens (14).

J. Monitor compliance with volume and number of bottles submitted and number of cultures collected from catheter lines without an accompanying peripheral culture.

V. PROCEDURE

Observe standard precautions.

It is imperative that these cultures be handled in a biological safety cabinet.

A. Laboratory processing of blood cultures

1. Safety
   - **a.** When working with blood cultures, keep the culture bottles within a biosafety cabinet or behind a shield, or wear a face mask.
   - **b.** Always wear gloves, because blood cultures contain material from patients that may harbor blood-borne pathogens.
   - **c.** Use needleless transfer devices or safety needles, and never recap them.
   - **d.** Dispose of needles and syringes in puncture-proof container.
2. Incubate blood cultures for the predetermined period at 35°C (usually 5 days, unless quality monitors indicate less time). In special circumstances (e.g., *Brucella* cultures), incubate for longer than standard policy. See procedure 3.4.2.
3. Maintain incubation conditions to allow recovery of microorganisms (follow manufacturer's instructions) and maintain rotation or agitation of the media if at all possible.
4. Examine the cultures at least daily, whether detection of positives is by visual inspection or by an automated system. For visual inspection, observe for hemolysis, turbidity, gas production, pellicle formation, "puffballs," and clotting, which are indicative of microbial growth (Table 3.4.1–1).

Table 3.4.1–1 Visible signs of growth caused by organisms commonly encountered in blood cultures

Microscopic observation	Associated microorganisms
Hemolysis	Streptococci, staphylococci, *Listeria* spp., clostridia, *Bacillus* spp.
Turbidity	Aerobic gram-negative bacilli, staphylococci, *Bacteroides* spp.
Gas formation	Aerobic gram-negative bacilli, anaerobes
Pellicle formation	*Pseudomonas* spp., *Bacillus* spp., yeast cells
Clotting	*Staphylococcus aureus*
Visible colonies ("puffballs")	Staphylococci, streptococci

V. PROCEDURE *(continued)*

5. For manual broth systems, perform at least one blind subculture to solid agar from visually negative bottles. Generally subculture after 72 h of incubation to detect most pathogens missed by visual inspection.
   ◩ **NOTE:** Subculture of automated systems has little clinical utility (13).
6. Biphasic systems
   a. For systems with agar paddle, remove plastic cap from bottle and securely attach paddle in biological safety cabinet while wearing gloves.
   b. Invert bottle to inoculate agar, and incubate at 35°C.
   c. Invert twice daily for the first 2 days and then daily for the remaining days of incubation. Observe broth and paddle for growth prior to each inversion.
7. In special circumstances when cultures appear to be negative, perform a Gram stain (procedure 3.2.1), wet mount (procedure 3.2.3), or acridine orange stain (procedure 3.2.2) from the culture or its sediment to determine the presence of organisms.
8. Discard negative bottles safely in infectious-waste container.

B. Culture methods for positive blood cultures

◩ **NOTE:** Since positive results with biphasic systems and ISOLATOR will be detected by the presence of colonies, subculture protocols for broth systems detailed below are not applicable. However, inoculation of biochemical tests for identification does apply.

1. Gram stain a thin smear from the broth or agar immediately when suggestive of growth 24 h a day for optimal patient care.
2. Following Table 3.4.1–2, subculture to agar media and biochemical tests based on the Gram stain results.
   ◩ **NOTE:** The extent of identification of isolates considered to be procurement-associated skin microorganisms should be determined in consultation with the institution's infectious disease specialists.
3. Develop and validate a laboratory protocol to inoculate a kit identification system for gram-negative rods and direct AST. Follow protocol, as indicated in Table 3.4.1–2. Examples of methods are listed below.
   ◩ **NOTE:** RBCs may interfere with some tests.
   a. Dilute 2 drops of blood culture in 5 ml of water or saline and inoculate gram-negative rod manual kit test, such as API 20E, that will support a low inoculum (9).
      ◩ **NOTE:** Citrate result is not reliable.
   b. Centrifuge in serum separator tube at 1,400 $\times$ *g* for 10 min, and adjust pellet on silicone layer equivalent to a 0.5 McFarland standard. Use inoculum for gram-negative kit identification system and susceptibility test system (25).

Table 3.4.1–2 Initial processing and reporting results from positive blood culture bottles[a]

Gram stain result	BAP	BAP with disks[b]	CHOC	Anaerobe plate	Other tests	Report
Positive cocci in clusters	×	Oxacillin Penicillin Vancomycin			1 or 2 drops to coagulase plasma at 35°C[c]; add AST[d] if coagulase positive or multiple "sets" positive	"Probable *Staphylococcus* spp."; update to "Probable *Staphylococcus aureus*" and notify physician if coagulase positive!
Positive cocci in chains or pairs	×	Penicillin Vancomycin Optochin CAMP test	×	×	1 drop to bile-esculin slant at 35°C[c]; AST[d] if bile-esculin positive (Optional: Quellung or slide bile solubility if lancet-shaped pairs)	"Probable *Streptococcus* spp."; update to "Probable *Enterococcus*" if bile-esculin positive or "Probable *Streptococcus pneumoniae*" if Quellung positive or bile soluble, and notify physician!
Small positive rods	×	Penicillin Vancomycin CAMP test	×	×	1 drop to bile-esculin slant at 35°C[c]; wet mount	"Gram-positive rods"; update to "Probable *Listeria*" if tumbling motile or bile-esculin positive
Large positive rods	×	Penicillin Vancomycin	×	Only if anaerobic bottle	For aerobic bottle, wet mount to rule out *B. anthracis*	"Gram-positive rods"; if diphtheroid-like or large and only in anaerobic bottle, report "Probable anaerobic gram-positive rods"
Negative rods	×	Polymyxin B or colistin disk	×	Only if anaerobic bottle	MAC or EMB; CNA or PEA; kit[d]; AST[d]; add BAP microaerobically if morphology is campylobacter-like	"Gram-negative rods" or "Probable *Campylobacter*" if smear indicates gull wing shapes, or "Probable *Haemophilus*" if morphology is consistent
Negative cocci	×	Penicillin	×	×	*Warning:* Staphylococci can appear gram negative; *Acinetobacter* organisms can appear as cocci.	"Gram-negative cocci" or "diplococci"; if tiny in sheets in anaerobic bottle, "anaerobic gram-negative cocci"
Yeast	×		×		CHROMagar; germ tube read at 2 h; India ink if round; 2 drops to urea slant at 35°C[c]	"Yeast"; update to "Probable *Cryptococcus*" if urease positive or capsules present, and notify physician! Update to "*Candida albicans*" if germ tube positive.

[a] Incubate aerobic plates in 5% CO_2 and anaerobic plates under anaerobic conditions. For any subsequent positive companion bottles or other positive bottles "collected" within the same 48 h as the first positive bottle, subculture only to BAP unless the Gram stain is different or the original culture did not grow on BAP. In addition, for subsequent positive cultures, inoculate MAC or EMB and either CNA (Columbia colistin-nalidixic acid agar) or PEA (phenylethyl alcohol agar) if gram-negative rods are present and a direct coagulase if gram-positive cocci in clusters are present.

[b] Disk testing is a screen for identification of *Lactobacillus, Erysipelothrix, Bacillus, Leuconostoc, Pediococcus,* and vancomycin-resistant and -dependent *Enterococcus* or *Staphylococcus* organisms that are penicillin susceptible (see procedure 3.17.4 for details on potency and QC). Disks will also detect mixed cultures but are *not* a substitute for a standard susceptibility test. A streak of *S. aureus* ATCC 25923 perpendicular to but not touching the line of the initial inoculum will aid in identification of group B streptococci and *Listeria.*

[c] Read test up to 4 h and update report and notify physician if test result is positive. Remove coagulase from incubator at ≤4 h and incubate at room temperature for remainder of 24 h.

[d] For methods for preparation of inoculum for direct testing of automated or manual commercial multitest kit identification systems and for AST, see text.

V. PROCEDURE *(continued)*

c. For MIC microtiter tray, place 1 drop of culture in 0.5 ml of BHI and incubate at 35°C for 3 to 4 h, with shaking; assume that it is equivalent to a 0.5 McFarland standard when entire volume is added to 5 ml of inoculation reagent. Check final inoculum with a colony count plate. Repeat AST if count is below 3×10^5/ml. Refer to section 5 for details on AST methods.

d. For disk method, add 10 drops of the blood culture or the broth of specimen in the case of a sterile fluid to 5 ml of TSB and inoculate Mueller-Hinton agar. If growth is not confluent at 18 h, repeat test or confirm with MIC method.

e. Alternatively, subculture to solid medium and incubate for 5 h. Scrape sufficient growth from plate to make inoculum for identification and AST. *This method is preferred for AST for* S. pneumoniae *but not staphylococci.*

4. Follow-up workup of positive blood culture isolates

a. Gram-negative rods growing on MAC or EMB

◩ **NOTE:** The presence of these isolates in blood cultures is most commonly associated with septic shock. Process and report as quickly as possible.

(1) As soon as results are available, read kit identification test and report results if there is a good probability that the result is accurate.
(2) Perform spot indole and oxidase to confirm kit results.
(3) Immediately report AST when available.
(4) Use caution to be sure that the culture is not mixed prior to reporting AST results (e.g., colistin or polymyxin B resistant). If the culture is mixed, contact physician to provide some information.
(5) For isolates identified as *Salmonella* or *Shigella*, perform serologic grouping to confirm identification.

b. *Haemophilus* or other fastidious gram-negative rods that do not grow on MAC or EMB

It is imperative that these cultures be handled in a biological safety cabinet.

◩ **NOTE:** If colistin or polymyxin B resistant, the organism may be a poorly staining gram-positive rod.

(1) Set up ALA test if growth is only on CHOC—*H. influenzae* organisms are negative. See Table 3.18.2–2.
(2) Do beta-lactamase test.
(3) Do catalase, oxidase, and rapid urea if growing on both BAP and CHOC, and see procedure 3.18.2; for *Brucella* identification, see procedure 3.4.2.

c. Staphylococci

(1) Do catalase. Should be positive.
(2) Confirm that it is not sticky or bright yellow. If clearly yellow, call it a *Micrococcus* sp. See procedure 3.18.1 for other testing. If it is sticky, see Table 3.18.1–4b for possible *Rothia mucilaginosa (Stomatococcus mucilaginosus).*
(3) If there is a large zone of inhibition (>20 mm) around the *penicillin* disk, perform beta-lactamase test from zone around *oxacillin* disk. Omit testing if the AST is available and shows penicillin resistance.
◩ **NOTE:** Penicillin-susceptible coagulase-negative strains may be *Micrococcus* (*see* procedure 3.18.1).
(4) Do latex agglutination test for clumping factor and protein A as a confirmation of a *negative* tube coagulase test. See charts for gram-positive cocci in procedure 3.18.1 if results are discrepant or for other confirmatory tests.
(5) Do PYR if tube coagulase is negative, regardless of slide coagulase result. If positive, set up ornithine decarboxylase to rule out *Staphylococcus lugdunensis.*

V. PROCEDURE *(continued)*

(6) Molecular methods may be used to rapidly identify staphylococci from positive blood cultures when the Gram stain is suspicious. The fluorescence in situ hybridization (FISH) method with peptide nucleic acid (PNA) probes was shown to have 100% sensitivity and 96% specificity in rapidly identifying *S. aureus* isolates within 2.5 h from positive blood culture bottles that contained gram-positive cocci in clusters (21). The *S. aureus* PNA FISH assay (AdvanDx, Woburn, MA) is based on a fluorescein-labeled PNA probe that targets a species-specific sequence of the 16S RNA of *S. aureus.*

The Xpert MRSA/SA BC (Cepheid, Sunnyvale, CA) is the first commercial real-time PCR assay available for the rapid identification of methicillin-susceptible and methicillin-resistant *S. aureus* from blood cultures. Several other companies also have molecular real-time PCR assays in development.

◩ **NOTE:** Isolation of *S. lugdunensis* is more often associated with endocarditis and poor outcome than isolation of other coagulase-negative staphylococci; thus, it should be differentiated from other species (24). It is also problematic for susceptibility testing, since the breakpoints for oxacillin susceptibility of coagulase-negative staphylococci do not correlate well (5). *S. lugdunensis* is rarely, if ever, resistant to oxacillin, although the MIC is generally ≥0.5 μg/ml. See section 5 for options for testing.

(7) Read and report AST.

(8) Check vancomycin disk. If resistant, see procedure 3.18.1 for identification. If coagulase positive, see section 5 for further AST on possible vancomycin-intermediate staphylococci.

d. Streptococci and other catalase-negative cocci

(1) *S. pneumoniae* (should have zones around optochin and vancomycin disks)

(a) Set up AST.

(b) If the growth is confluent and the zone size is >20 mm to penicillin, report preliminary result as "Susceptible to penicillin by nonstandard method."

(2) If beta-hemolytic

(a) Do catalase—should be negative

(b) Check CAMP. Report "*Streptococcus agalactiae*" if positive.

(c) Do PYR. If PYR positive and bile-esculin negative, report "*Streptococcus pyogenes.*"

(d) Otherwise, Lancefield group and report.

(3) Viridans group streptococci (have no zone around the optochin disk and a zone around the vancomycin disk).

◩ **NOTE:** *Leuconostoc* and *Pediococcus* look like streptococci, but they are vancomycin resistant. See Table 3.18.1–4.

(a) Do catalase—should be negative

(b) A repeat Gram stain from original broth should show chains and pairs.

(c) Do PYR (result should be negative). If PYR positive, refer to Table 3.18.1–4.

V. PROCEDURE *(continued)*

(d) Do LAP. If positive, report "Viridans group streptococcus" and identify further according to laboratory policy. If present in only one culture, call physician to see if it is a significant isolate for identification and AST.

◩ **NOTE:** For accuracy, strains may require several subcultures anaerobically prior to biochemical testing, especially with kit systems that require preformed enzymes for reactions. They also tend to present with various morphologies on plate culture. If there is any question of identification, set up growth at 42°C and 6.5% salt.

(e) If there is no zone around the penicillin disk, notify caregiver that strain may be penicillin resistant. Set up MIC method for susceptibility to penicillin and an extended-spectrum cephalosporin (*see* section 5).

(f) If there is no growth on BAP but growth on CHOC, set up satellite test (procedure 3.17.44) to determine if it is nutritionally variant streptococcus.

◩ **NOTE:** Nutritionally variant streptococci satellite around staphylococci.

(4) Enterococci

(a) Do catalase—should be negative or weakly positive

(b) Do PYR. If positive and bile-esculin was positive, report "*Enterococcus* species; identification to follow."

(c) Set up species identification and AST per policy.

(d) If vancomycin resistant by disk

i. Inoculate broth for motility (procedure 3.17.31) and incubate at 30°C for 2 h to rule out motile species. If nonmotile, report "Vancomycin resistant by preliminary test."

ii. Check for yellow pigment by sweeping with a cotton swab from direct BAP; swab will appear bright yellow.

iii. Confirm vancomycin result with MIC method.

iv. Use kit or sugar fermentation to differentiate *Enterococcus faecium* from *Enterococcus faecalis.*

(e) Molecular methods may be used to rapidly identify *Enterococcus* from positive blood cultures when the Gram stain is suspicious. The FISH method with PNA probes was shown to have 98.6% sensitivity and 100% specificity in rapidly identifying *E. faecalis* and other enterococcal isolates within 2.5 h from positive blood culture bottles that contained gram-positive cocci (10). The *E. faecalis*/OE PNA FISH assay (AdvanDx) is based on a fluorescein-labeled PNA probe that targets a species-specific sequence of the 16S RNA of *E. faecalis.*

(5) Other cocci: if sticky or vancomycin resistant or PYR positive and catalase negative or weakly positive and do not look like *Enterococcus* on Gram stain, see procedure 3.18.1.

e. Gram-positive rods

(1) Do catalase test.

(a) Catalase positive

i. Small rods with small zone of hemolysis and bile-esculin positive, set up motility tests at 22 and 35°C using semisolid agar. *Listeria* organisms are motile at 22°C. Check CAMP test. *Listeria monocytogenes* is positive.

V. PROCEDURE *(continued)*

ii. Large rods. Check for spores and motility.

◩ **NOTE:** *Bacillus cereus* is hemolytic and motile; *Bacillus anthracis* is nonhemolytic and nonmotile.

iii. If present in only one culture of several collected, limit workup of diphtheroids (bile-esculin-negative, catalase-positive rods) unless physician indicates that the isolate may be significant. If isolate is significant and is penicillin resistant (no zone around the penicillin disk), notify caregiver, even though the test is not standard. Identify with commercial kit and/or biochemical tests (*see* procedure 3.18.1).

◩ **NOTE:** *Corynebacterium jeikeium* is associated with catheter-related sepsis.

(b) Catalase-negative and vancomycin resistant: set up H_2S to rule out *Erysipelothrix* organisms, which are H_2S positive.

◩ **NOTE:** *Erysipelothrix* and often lactobacilli are vancomycin resistant. Notify physician of vancomycin result.

(2) Refer to gram-positive rod flowcharts (procedure 3.18.1) for reporting and further testing.

f. Potential anaerobic microorganisms: refer to section 4.

g. Yeasts: refer to section 8.

(1) Do phenol oxidase test if urease positive; omit test if patient has recent positive culture or cryptococcal antigen test.

(2) If urea negative

(a) Repeat germ tube if it was negative or questionable.

i. Call "*Candida albicans*" if germ tube positive.

ii. If hyphae are seen, call "*Candida*" species.

iii. If hyphae are not seen and isolate grows better on BAP than EMB, call "Yeast, not *Cryptococcus.*"

iv. If growth is better on EMB and the yeast is tiny on smear, call "Probable *Candida glabrata*" and set up rapid trehalose test.

(b) Read CHROMagar at 48 h for species identification, or see section 8 for other methods.

h. If there is no growth on initial subculture

(1) Subculture bottle anaerobically if anaerobic plates were not inoculated.

(2) Subculture to buffered charcoal-yeast extract agar for growth of *Afipia* or other fastidious gram-negative rods.

(3) Subculture to BAP and incubate microaerobically for campylobacters.

i. Hold positive cultures for at least a few weeks for further studies. See procedure 3.3.2 for methods to freeze isolates.

j. Molecular methods may be used to rapidly identify *Candida* species from positive blood cultures when the Gram stain is suspicious. The FISH method with PNA probes was shown to have 99% sensitivity and 100% specificity in rapidly identifying *C. albicans* and non-*C. albicans* isolates within 2.5 h from positive blood culture bottles that contained gram-positive yeast forms (31). The Yeast—*C. albicans* PNA FISH assay (AdvanDx) is based on a fluorescein-labeled PNA probe that targets a species-specific sequence of the 16S RNA of *C. albicans* (31). Other PNA FISH assays are also available for rapidly distinguishing *C. albicans* and *C. glabrata*, and the Yeast Traffic Light PNA FISH assay will distinguish most of the common *Candida* spp. that cause invasive infections and candidemia.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. For "No growth cultures," indicate the length of incubation: "No growth after *x* days of incubation" for both preliminary and final reports.

B. Positive cultures

1. Immediately report Gram stain results of all positive cultures, or additional organisms found in previously positive cultures, to the physician of record, with as much interpretive information as possible (using Table 3.4.1–2 for guidelines).

2. Follow immediately with a written or computer-generated report including the following.

a. Number of positive cultures compared with total number of specimens collected for specific patient.

◩ **NOTE:** There is no justification for telling the physician that one or both bottles in the set are positive, because the number of positive bottles in a set does not reliably differentiate contamination from true infection (20).

b. Date and time of collection and receipt

c. Date and time positive result is reported and whether it was from a catheter draw or a peripheral draw.

◩ **NOTE:** Such information is useful in the diagnosis of catheter-related sepsis (2).

d. Name, phone number, and location of person taking report

Example: Positive culture reported to Dr. X on 07/01/09 at 1300 h.

3. For single positive cultures with microorganisms generally considered skin contaminants (coagulase-negative staphylococci, viridans group streptococci, corynebacteria, *Propionibacterium* [30]), perform only minimal identification and do not perform AST. For single positive cultures with these potential skin contaminants (30), report result with a comment similar to the following: "One set of two positive. Isolation does not necessarily mean infection. No susceptibility tests performed. Contact laboratory for further information."

4. Provide genus and species identification as soon as possible, using tests in Table 3.3.2–5 and charts in procedures 3.18.1 and 3.18.2.

5. For subsequent positive cultures, it is not necessary to repeat biochemical testing if the microorganism has the same Gram and colony morphology as the first isolate. Perform a few spot tests (catalase, coagulase, indole, PYR, etc.) to verify that it is the same strain. Report as "Probable [genus and species]; refer to prior positive for complete identification and susceptibility testing."

VII. INTERPRETATION

A. The report of a positive culture generally means that the patient is bacteremic. However, skin microbiota may contaminate the culture, causing a false-positive result or pseudobacteremia. Pseudobacteremias have many other causes.

1. If organisms are seen but not cultured, dead organisms can be found in the medium components and produce a positive smear.

2. *Bacillus* or other bacteria can be present on the nonsterile gloves of the phlebotomist (34).

3. Laboratory contamination of equipment or supplies used in culture may contaminate the patient specimens.

VII. INTERPRETATION *(continued)*

B. Mixed cultures can be present and account for a significant number of bacteremias. Be aware of this when examining smears and plates.

C. Performance and reporting of AST are critical for timely patient care and increase the chance of appropriate therapy and cure.

VIII. LIMITATIONS

A. Low levels of organisms may not be detected in the incubation interval of the culture.

B. The media used may not support the growth of some organisms. Use of multiple formulations increases the yield.

C. SPS may inhibit the growth and viability of the organism.

D. Other diseases can present similarly to bacteremia, since there are many causes of fever of unknown origin.

E. Bacterial metabolism may not produce sufficient CO_2 for detection in automated systems.

F. There are a number of fastidious microorganisms that infect the blood that cannot be grown in routine culture of blood. These are presented further in the appendixes and other procedures that follow.

REFERENCES

1. **Baron, E. J., M. P. Weinstein, W. M. Dunne, Jr., P. Yagupsky, D. F. Welch, and D. M. Wilson.** 2005. *Cumitech 1C, Blood Cultures IV.* Coordinating ed., E. J. Baron. ASM Press, Washington, DC.
2. **Blot, F., G. Nitenberg, E. Chachaty, B. Raynard, N. Germann, S. Antoun, A. Laplanche, C. Brun-Buisson, and C. Tancrede.** 1999. Diagnosis of catheter-related bacteremia: a prospective comparison of the time to positivity of hub-blood versus peripheral-blood cultures. *Lancet* **354:**1071–1077.
3. **Bourbeau, P. P., and J. K. Pohlman.** 2001. Three days of incubation may be sufficient for routine blood cultures with BacT/Alert FAN blood culture bottles. *J. Clin. Microbiol.* **39:**2079–2082.
4. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Clinical and Laboratory Standards Institute.** 2008. *Performance Standards for Antimicrobial Susceptibility Testing.* Eighteenth informational supplement. Approved standard M100-S18. Clinical and Laboratory Standards Institute, Wayne, PA.
6. **Cornish, N., B. A. Kirkley, K. A. Easley, and J. A. Washington.** 1998. Reassessment of the incubation time in a controlled clinical comparison of the BacT/Alert aerobic FAN bottle and standard anaerobic bottle used aerobically for the detection of bloodstream infections. *Diagn. Microbiol. Infect. Dis.* **32:**1–7.
7. **Cornish, N., B. A. Kirkley, K. A. Easley, and J. A. Washington.** 1999. Reassessment of the routine anaerobic culture and incubation time in the BacT/Alert FAN blood culture bottles. *Diagn. Microbiol. Infect. Dis.* **35:**93–99.
8. **Doern, G. V., A. G. Brueggemann, W. M. Dunne, S. G. Jenkins, D. C. Halstead, and J. McLaughlin.** 1997. Four-day incubation period for blood culture bottles processed with the Difco ESP blood culture system. *J. Clin. Microbiol.* **35:**1290–1292.
9. **Evangelista, A. T., A. L. Truant, and P. P. Bourbeau.** 2002. Rapid systems and instruments for the identification of bacteria, p. 22–49. *In* A. L. Truant (ed.), *Manual of Commercial Methods in Microbiology.* ASM Press, Washington, DC.
10. **Forrest, G. N., M. C. Roghmann, L. S. Toombs, J. K. Johnson, E. Weekes, D. P. Lincalis, and R. A. Venezia.** 2008. Peptide nucleic acid fluorescent in situ hybridization for hospital-acquired enterococcal bacteremia: delivering earlier antimicrobial therapy. *Antimicrob. Agents Chemother.* **52:**3558–3563.
11. **Grace, C. J., J. Lieberman, K. Pierce, and B. Littenberg.** 2001. Usefulness of blood culture for hospitalized patients who are receiving antibiotic therapy. *Clin. Infect. Dis.* **32:**1651–1655.
12. **Han, X. Y., and A. L. Truant.** 1999. The detection of positive blood cultures by the AccuMed ESP-384 system: the clinical significance of three-day testing. *Diagn. Microbiol. Infect. Dis.* **33:**1–6.
13. **Hardy, D. J., B. B. Hulbert, and P. C. Migneault.** 1992. Time to detection of positive BacT/Alert blood cultures and lack of need for routine subculture of 5- to 7-day negative cultures. *J. Clin. Microbiol.* **30:**2743–2745.
14. **Ilstrup, D. M.** 1978. Statistical methods employed in the study of blood culture media, p. 31–39. *In* J. A. Washington II (ed.), *The Detection of Septicemia.* CRC Press, West Palm Beach, FL.

REFERENCES *(continued)*

15. **Ilstrup, D. M., and J. A. Washington II.** 1983. The importance of volume of blood cultured in the detection of bacteremia and fungemia. *Diagn. Microbiol. Infect. Dis.* **1:**107–110.
16. **James, P. A., and K. M. Al-Shafi.** 2000. Clinical value of anaerobic blood culture: a retrospective analysis of positive patient episodes. *J. Clin. Pathol.* **53:**231–233.
17. **Kellogg, J. A., F. L. Ferrentino, J. Liss, S. L. Shapiro, and D. A. Bankert.** 1994. Justification and implementation of a policy requiring two blood cultures when one is ordered. *Lab. Med.* **25:**323–330.
18. **Kellogg, J. A., J. P. Manzella, and D. A. Bankert.** 2000. Frequency of low-level bacteremia in children from birth to fifteen years of age. *J. Clin. Microbiol.* **38:**2181–2185.
19. **Li, J., J. J. Plorde, and L. G. Carlson.** 1994. Effects of volume and periodicity on blood cultures. *J. Clin. Microbiol.* **32:**2829–2831.
20. **Mirrett, S., M. P. Weinstein, L. G. Reimer, M. L. Wilson, and L. B. Reller.** 2001. Relevance of the number of positive bottles in determining clinical significance of coagulase-negative staphylococci in blood cultures. *J. Clin. Microbiol.* **39:**3279–3281.
21. **Oliveira, K., G. W. Procop, D. Wilson, J. Coull, and H. Stender.** 2002. Rapid identification of *Staphylococcus aureus* from blood cultures by fluorescence in situ hybridization with peptide nucleic acid probes. *J. Clin. Microbiol.* **40:**247–251.
22. **Reimer, L. G., M. L. Wilson, and M. P. Weinstein.** 1997. Update on detection of bacteremia and fungemia. *Clin. Microbiol. Rev.* **10:**444–465.
23. **Reisner, B. S., and G. L. Wood.** 1999. Times to detection of bacteria and yeasts in BACTEC 9240 blood culture bottles. *J. Clin. Microbiol.* **37:**2024–2026.
24. **Teong, H. H., Y. S. Leo, S. Y. Wong, L. H. Sng, and Z. P. Ding.** 2000. Case report of *Staphylococcus lugdunensis* native valve endocarditis and review of the literature. *Ann. Acad. Med. Singapore* **29:**673–677.
25. **Waites, K. B., E. S. Brookings, S. A. Moser, and B. L. Zimmer.** 1998. Direct bacterial identification from positive BacT/Alert blood cultures using MicroScan overnight and rapid panels. *Diagn. Microbiol. Infect. Dis.* **32:**21–26.
26. **Waltzman, M. L., and M. Harper.** 2001. Financial and clinical impact of false-positive blood culture results. *Clin. Infect. Dis.* **33:**296–299.
27. **Weinbaum, F. I., S. Lavie, M. Danek, D. Sixsmith, G. F. Heinrich, and S. S. Mills.** 1997. Doing it right the first time: quality improvement and the contaminant blood culture. *J. Clin. Microbiol.* **35:**563–565.
28. **Weinstein, M. P., S. Mirrett, M. L. Wilson, L. G. Reimer, and L. B. Reller.** 1994. Controlled evaluation of 5 versus 10 milliliters of blood cultured in aerobic BacT/Alert blood culture bottles. *J. Clin. Microbiol.* **32:**2103–2106.
29. **Weinstein, M. P., and B. Reller.** 2002. Commercial blood culture systems and methods, p. 12–21. *In* A. L. Truant (ed.), *Manual of Commercial Methods in Microbiology*. ASM Press, Washington, DC.
30. **Weinstein, M. P., M. L. Towns, S. M. Quartey, S. Mirrett, L. G. Reimer, G. Parmigiani, and L. B. Reller.** 1997. The clinical significance of positive blood cultures in the 1990s: a prospective comprehensive evaluation of the microbiology, epidemiology, and outcome of bacteremia and fungemia in adults. *Clin. Infect. Dis.* **24:**584–602.
31. **Wilson, D. A., M. J. Joyce, L. S. Hall, L. B. Reller, G. D. Roberts, G. S. Hall, B. D. Alexander, and G. W. Procop.** 2005. Multicenter evaluation of a *Candida albicans* peptide nucleic acid fluorescent in situ hybridization probe for characterization of yeast isolates from blood cultures. *J. Clin. Microbiol.* **43:**2909–2912.
32. **Wilson, M. L., S. Mirrett, L. B. Reller, M. P. Weinstein, and L. G. Reimer.** 1993. Recovery of clinically important microorganisms from the BacT/Alert blood culture system does not require testing for seven days. *Diagn. Microbiol. Infect. Dis.* **16:**31–33.
33. **Wilson, M. L., M. P. Weinstein, S. Mirrett, L. G. Reimer, R. J. Feldman, C. R. Chuard, and L. B. Reller.** 1995. Controlled evaluation of BacT/Alert standard anaerobic and FAN anaerobic blood culture bottles for detection of bacteremia and fungemia. *J. Clin. Microbiol.* **33:**2265–2270.
34. **York, M. K.** 1990. *Bacillus* species pseudobacteremia in AIDS patients traced to contaminated gloves in blood collection. *J. Clin. Microbiol.* **28:**2114–2116.

SUPPLEMENTAL READING

Barenfanger, J., C. Drake, and G. Kacich. 1999. Clinical and financial benefits of rapid bacterial identification and antimicrobial susceptibility testing. *J. Clin. Microbiol.* **37:**1415–1418.

Barenfanger, J., M. Short, and A. Groesch. 2001. Improved antimicrobial interventions have benefits. *J. Clin. Microbiol.* **39:**2823–2828.

Doern, G., R. Vautour, M. Gaudet, and B. Levy. 1994. Clinical impact of rapid in vitro susceptibility testing and bacterial identification. *J. Clin. Microbiol.* **32:**1757–1762.

Dunne, W. M., F. S. Nolte, and M. L. Wilson. 1997. *Cumitech 1B, Blood Cultures III.* Coordinating ed., J. A. Hindler. American Society for Microbiology, Washington, DC.

O'Grady, N. P., P. S. Barie, J. G. Bartlett, T. Bleck, G. Garvey, J. Jacobi, P. Linden, D. G. Maki, M. Nam, W. Pasculle, M. Pasquale, D. L. Tribett, and H. Masur. 1998. Practice guidelines for evaluating new fever in critically ill patients. *Clin. Infect. Dis.* **26:**1042–1059.

APPENDIX 3.4.1–1

Specialized Processing of Blood for Detection of Unusual Microorganisms or Conditions

A. Transfusion reactions
   Collect blood cultures both from the patient and from the transfused product.
   1. See procedure 13.13 for processing transfused blood.
   2. Never discard the transfused product; return it to the transfusion service.
   3. Always keep good records of what was cultured.

B. If the laboratory is notified that the isolation of unusual pathogens in blood is sought, perform an acridine orange stain (procedure 3.2.2) from the blood cultures prior to discarding bottles that do not indicate growth.

C. Inoculate special media or perform special testing listed below for the following organisms (listed alphabetically).
   1. *Bartonella* spp. (formerly *Rochalimaea*)
      Collect ISOLATOR tube and see procedure 3.4.3.
   2. *Borrelia* spp. (relapsing fever)
      a. Visualize in a Wright or Giemsa smear of citrated blood. Prepare smears as for malaria (*see* procedure 9.8). *Borrelia* organisms are 10 to 20 μm long and 0.2 to 0.5 μm wide, with uneven coils. Scan at ×400 and verify identification at ×1,000 magnification. Read at least 60 fields on a thin smear and 20 fields on a thick smear.
      b. Look for twisting motion in a dark-field exam.
      c. Concentrate by centrifugation at 200 × *g*, remove plasma, and examine buffy coat as described above.
      d. Culture and mouse inoculation are done in research laboratories. Blood is generally shipped cold.

      ◩ **NOTE:** *Borrelia burgdorferi* is best detected by PCR analysis of urine, blood, CSF, or lymph node aspirate.
   3. *Brucella* spp.
      Most blood culture systems will grow *Brucella* if incubation is extended with blind subculture; see procedure 3.4.2 for optional techniques and details.
   4. *Ehrlichia*
      a. This parasite can occasionally be detected as an intracellular morula or intracytoplasmic inclusion in the WBCs visualized in a Giemsa or Wright stain of blood or buffy coat smear.
      b. PCR and serologic methods are better tests.
   5. *Histoplasma* (*see* section 8)

      ◩ **NOTE:** Although yeast cells are usually detected in aerobic broth systems, culture using biphasic media, an ISOLATOR tube concentrate, or the buffy coat from an SPS or heparin tube to specialized fungal media may allow faster and more extensive recovery of *Histoplasma* (5). For *Cryptococcus,* the cryptococcal antigen test is recommended for rapid diagnosis. The Myco/F lytic bottle (BD Diagnostic Systems) is designed for enhanced recovery of yeasts and mycobacteria. Bone marrow specimens are excellent for recovery of *Histoplasma.*
   6. *Legionella* spp.
      Refer to procedure 3.11.4.
   7. *Leptospira* spp.
      Refer to procedure 3.14 prior to collection of specimens.
   8. *Malassezia furfur* (usually seen in catheter-related infections in infants receiving lipid-rich total parenteral nutrition) (3)
      a. Automated systems may not give a positive signal for *M. furfur* (5).
      b. Preferred method: collect a few milliliters of blood from the potentially infected catheter port.
         (1) Prepare slide by spreading blood in a thin film and perform Gram stain.
         (2) Streak BAP or noninhibitory fungal medium for isolated colonies.
         (3) Add a drop of pure virgin olive oil (sterilization not necessary) the size of a dime onto the agar surface to provide essential nutrients.

APPENDIX 3.4.1–1 *(continued)*

9. Mycobacteria (*see* section 7)

   ◩ **NOTE:** Collect specimen in special culture media supplied by the manufacturer of automated instruments or culture buffy coat from either SPS- or heparin-collected blood. ISOLATOR can also be used to concentrate the organisms. Testing should generally be limited to those patients with CD4 counts of less than 200 cells/μl, since the yield of mycobacteria is low from immunocompetent individuals. However, bone marrow is an appropriate specimen for culture from all patients.
10. Mycoplasmas (*see* procedure 3.15)
11. *Streptobacillus moniliformis* (cause of "rat bite fever" and "Haverhill fever")
    a. The specimens of choice are blood and joint fluid. Because this organism is inhibited by SPS and requires more blood to grow, inoculate *twice* the amount of blood or joint fluid recommended by the manufacturer into the blood culture bottle. Pediatric bottles are good for this. Growth appears as crumbs in the liquid. Alternatively, collect citrated blood and inoculate it into several THIO tubes (2 ml/10 ml of THIO).

    ◩ **NOTE:** Lymph nodes and abscesses are also good specimens for isolation of *S. moniliformis.* Inoculate as indicated below for subcultures.

    b. Culture specimen or subculture positive blood culture broths as follows.
       (1) Prepare agar with 20% rabbit, calf, or horse serum.
           (a) Melt 20 ml of BHI, TSA, or heart infusion agar.
           (b) Cool to 45 to 50°C.
           (c) Add 4 to 5 ml of rabbit, calf, or horse serum.
           (d) Mix, pour into petri dish, and allow to solidify.
           (e) Inoculate with a few drops from the specimen or the positive culture.
       (2) To THIO with 20% rabbit, calf, or horse serum added (2 ml/10 ml of broth), inoculate with a few drops from the positive culture or up to 2 ml of the specimen.
    c. Incubate plates in high humidity with 5 to 10% CO_2.
    d. *S. moniliformis* is gram negative and catalase, indole, nitrate, and oxidase negative and does not grow on MAC (6). It may be pleomorphic and show long filaments, chains, and swollen cells in broth culture. It is arginine positive. Confirmatory identification is usually done in a reference laboratory.

D. Broad-range PCR

Blood culture results from patients with suspected endocarditis may be falsely negative when fastidious or noncultivable bacteria are involved or when the patient has been treated prior to the start of antimicrobial therapy. Broad-range PCR that detects the 16S rRNA gene (i.e., conserved bacterial sequence) followed by nucleic acid sequencing for identification of the pathogen to the genus or species level may be helpful in these cases. Several broad-range PCR assays, including real-time quantitative assays, have been reported (1, 2, 4, 7). Real-time quantitative assays for broad-range PCR may improve the lower limits of molecular detection to the threshold required by this approach (i.e., blood may contain only 1 to 2 CFU/ml). In order to perform a broad-range PCR assay if a blood culture proves culture negative, a minimum 1- to 2-ml aliquot should be aseptically collected from the earliest set of blood culture bottles and frozen at −86°C.

References

1. **Goldenberger, D., A. Kunzli, P. Vogt, R. Zbinden, and M. Altwegg.** 1997. Molecular diagnosis of bacterial endocarditis by broad-range PCR amplification and direct sequencing. *J. Clin. Microbiol.* **35:**4759–4764.
2. **Harris, K. A., and J. C. Hartley.** 2003. Development of broad-range 16S rRNA PCR for use in the routine diagnostic clinical microbiology service. *J. Med. Microbiol.* **52:**685–691.
3. **Marcon, M. J., and D. A. Powell.** 1992. Human infections due to *Malassezia* spp. *Clin. Microbiol. Rev.* **5:**101–119.
4. **Rantakokko-Jalava, K., S. Nikkari, J. Jalava, E. Eerola, M. Skurnik, O. Meurman, O. Ruuskanen, A. Alanen, E. Kotilainen, P. Toivanen, and P. Kotilainen.** 2000. Direct amplification of rRNA genes in diagnosis of bacterial infection. *J. Clin. Microbiol.* **38:**32–39.
5. **Reimer, L. G., M. L. Wilson, and M. P. Weinstein.** 1997. Update on detection of bacteremia and fungemia. *Clin. Microbiol. Rev.* **10:**444–465.
6. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed. Williams & Wilkins, Baltimore, MD.
7. **Zucol, F., R. A. Ammann, C. Berger, C. Aebi, M. Altwegg, F. K. Niggli, and D. Nadal.** 2006. Real-time quantitative broad-range PCR assay for detection of the 16S rRNA gene followed by sequencing for species identification. *J. Clin. Microbiol.* **44:**2750–2759.

Supplemental Reading

Beebe, J. L., and E. W. Koneman. 1995. Recovery of uncommon bacteria from blood: association with neoplastic disease. *Clin. Microbiol. Rev.* **8:**336–356.

Brouqui, P., and D. Raoult. 2001. Endocarditis due to rare and fastidious bacteria. *Clin. Microbiol. Rev.* **14:**177–207.

APPENDIX 3.4.1–2

Processing of the Lysis-Centrifugation System of ISOLATOR

I. PRINCIPLE

The ISOLATOR system (Wampole Laboratories, Cranbury, NJ) consists of Vacutainer tubes for blood collection that contain lysing, antifoaming, and anticoagulating agents. The lysis of cells followed by centrifugation results in concentration of the microorganisms from the blood specimen into liquid Fluorinert, a water-immiscible substance. The Fluorinert mixture is cultured on plate media, resulting in simultaneous detection, enumeration, and isolation of organisms (2, 3). Because the system utilizes plates, it is not a sealed system; airborne contamination can be a problem in the inoculation and evaluation of the cultures (1, 4).

II. SPECIMEN

Collect specimen as for other blood cultures, and fill tube after careful disinfection of the stopper. Process within 8 h of collection.

III. MATERIALS

A. Collection tube contains a lysis fluid of saponin, polypropylene, SPS, Fluorinert, and EDTA. Draw is either 1.5 ml for pediatric and bone marrow patients or 10 ml for adults.
B. ISOSTAT press and rack
C. ISOSTAT supernatant and concentrate pipettes and white cap
D. Fixed-angle centrifuge
E. Plate media for desired culture

IV. PROCEDURE

A. Invert the tube several times to ensure adequate mixing at the time of collection.
B. Centrifuge at 3,000 $\times$ *g* for 30 min in a fixed-angle rotor.
C. Place tube in ISOSTAT press.
D. Wipe spun ISOLATOR tube, especially the top, with alcohol. Be generous with the alcohol; leave a drop on the top of the cap. Allow to dry. Do not disturb the microbial concentrate during the transfer. If it is disturbed, respin the tubes for another 30 min and allow the centrifuge to stop manually.
E. Disinfect ISOSTAT press hammer with alcohol and allow to dry.
F. Aseptically position cap over tube, and press down with ISOSTAT press. Do not touch top of tube. Gently pull the handle down as far as possible. The spike will penetrate the stopper and firmly seat the cap.

APPENDIX 3.4.1–2 *(continued)*

It is imperative that these cultures be handled in a biological safety cabinet.

G. Don gloves and place tube and plates for inoculation in biologic safety cabinet.
H. Aseptically remove a supernatant pipette from the pouch. Squeeze bulb of pipette, collapsing it completely, and insert pipette into tube through the cap membrane. Do not touch pipette stem. Release bulb slowly, allowing the supernatant blood to be drawn into the pipette. When bubbles appear, remove the pipette *without* squeezing the bulb, and discard in a biohazardous-waste container. If bubbles were not present in the pipette, repeat the procedure with another pipette.
I. Vortex the contents for at least 20 s.
J. Aseptically remove a concentrate pipette from the package. Squeeze the pipette bulb, collapsing it completely, and insert into the tube through the cap membrane so that the stem reaches the bottom of the tube. Release bulb slowly, allowing the pellet to be drawn into the pipette.
K. Remove pipette from tube and distribute the entire concentrate equally on media that will allow recovery of the organism sought. Spread evenly over surface of each plate, using separate loops or spreaders. Avoid the edges of the plates.
L. If the lysis-centrifugation system is used alone, inoculate a portion of the sediment onto anaerobic plates or into anaerobic broth for recovery of anaerobes.
M. Incubate the media under the correct conditions and for sufficient time to recover all pathogens sought.
N. Incubate agar side down for the first 24 h of incubation to maintain contact of the inoculum with agar and to prevent concentrate from dripping onto the lid. After the initial incubation, incubate plates agar side up. Generally plates are incubated for 4 days unless unusual fastidious organisms are suspected.

V. INTERPRETATION

A. The ISOLATOR system can be used for quantitative counting of microorganisms in blood. Use the formula below.

CFU/ml = total number of CFU on all plates/total number of plates on which organism is expected to grow ×(times) number of plates inoculated per blood volume

Example: Number of colonies: BAP—2; CHOC—3; CHOC—2; CHOC—1; CHOC—2. Therefore: 10 CFU/5 plates × 5 plates/1.5 ml = 7 CFU/ml (pediatric blood) or BAP—5; CHOC—7; CHOC—7; fungal selective agar—0. Therefore: 19/3 × 4/10 = 2.5 CFU/ml adult (10-ml tube).

B. The number of plate contaminants can be problematic in the interpretation of the results with this system. Generally one colony of coagulase-negative staphylococci is ignored, but the laboratory should work to achieve no contamination and monitor the results to reduce false-positive reporting of larger numbers of skin microbiota.

References

1. **Arpi, M., B. Gahrn-Hansen, and V. T. Rosdahl.** 1988. Contaminating coagulase-negative staphylococci isolated in a lysis-centrifugation (Isolator) blood culture system. Application of different epidemiological markers for deduction of mode of contamination. *APMIS* **96:**611–617.
2. **Henry, N. K., C. A. McLimans, A. J. Wright, R. L. Thompson, W. R. Wilson, and J. A. Washington II.** 1983. Microbiological and clinical evaluation of the Isolator lysis-centrifugation blood culture tube. *J. Clin. Microbiol.* **17:**864–869.
3. **Isenberg, H. D.** 1983. Clinical laboratory comparison of the lysis-centrifugation blood culture technique with radiometric and broth approaches, p. 38–54. *In* A. Balows and A. Sonnenwirth (ed.), *Bacteremia—Laboratory and Clinical Aspects.* Charles C Thomas, Springfield, IL.
4. **Thomson, R. B., Jr., S. J. Vanzo, N. K. Henry, K. L. Guenther, and J. A. Washington II.** 1984. Contamination of cultures processed with the Isolator lysis-centrifugation blood culture tube. *J. Clin. Microbiol.* **19:**97–99.

3.4.2 *Brucella* Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Brucella is a fastidious, aerobic, small, gram-negative coccobacillus that is slow growing and difficult to isolate. It is zoonotic, with four species being recognized as causing infection in humans: *Brucella abortus* (cattle), *Brucella melitensis* (goats, sheep, and camels), *Brucella suis* (pigs), and *Brucella canis* (dogs). Although species identification is of interest, it is *not* clinically important and is difficult to perform. Key characteristics of the species can be found in reference 9.

Infections are seen in essentially two patient populations. The first group is individuals who work with animals and who have not been vaccinated against brucellosis. This patient population includes farmers, veterinarians, and slaughterhouse workers. *B. abortus* and *B. suis* are the agents most likely to cause infections in this group of individuals. They become infected either by direct contact or by aerosol from infected animal tissues.

Brucellosis is also seen in individuals who ingest unpasteurized dairy products contaminated with brucellae. This is most likely to occur in individuals who travel to or migrate from rural areas of Latin American and the Middle East where disease is endemic in dairy animals, particularly goats and camels. *B. melitensis* is the most common agent seen in this patient population. Brucellae are included in the microorganisms at risk for being used in a bioterrorist event; refer to procedure 16.6 for further information on this agent's role in bioterrorism.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

It is imperative that these cultures be handled in a biological safety cabinet.

A. Aseptically collect blood or bone marrow (from the iliac crest [8]) during a fever episode.

B. Refer to procedure 3.4.1 for collection. Place specimen immediately in blood culture bottles.

◩ **NOTE:** CSF, lymph nodes, joint fluid, and liver or spleen biopsy specimens may be positive for *Brucella*, which will be detected by routine culture of these specimens. Follow this procedure to confirm the identification.

III. MATERIALS

A. Media

1. Primary media

a. Biphasic blood culture

(1) Septi-Chek (CHOC, MAC, and malt agar with BHI or Columbia broth) (BD Diagnostic Systems)

(2) PML biphasic media (CHOC and BHI agar with TSB) (PML Microbiologicals, Inc.)

(3) Castaneda bottles (TSB or brucella broth and agar) (2)

b. Automated blood culture system

◩ **NOTE:** Automated systems may require subculture to detect growth. The pediatric lysis-centrifugation method using a volume of 1.5 ml was not as sensitive as automated methods in one study (15).

2. Media for blind subculture or for positive cultures

a. BAP preferably with BHI base

b. CHOC

c. Brucella agar

III. MATERIALS *(continued)*

B. Reagents
1. Gram stain reagents
2. Catalase test (procedure 3.17.10)
3. Oxidase test (procedure 3.17.39)
4. Rapid urea-phenylalanine deaminase (PDA) disks (6) (Remel Inc.; Hardy Diagnostics) (procedures 3.17.40 and 3.17.48)
5. 10% Ferric chloride
6. Antiserum to *Brucella* from febrile agglutination test

C. Supplies
1. Incubator with 5 to 10% CO_2
2. Microscope slides

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that plate media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (formerly NCCLS) document M22. See procedure 14.2 and procedure 3.3.1 for further procedures.

B. For QC of blood culture broths, refer to procedure 3.4.1.

C. See individual tests in procedure 3.17 for biochemical test QC.

◩ **NOTE:** Specific QC using *Brucella* organisms is not recommended because of the danger to laboratory workers handling such cultures. The confirmation of the identification of suspicious isolates generally should be done in a reference laboratory to avoid exposure of laboratory personnel to the organism.

V. PROCEDURE

Observe standard precautions.

It is imperative that these cultures be handled in a biological safety cabinet.

A. Laboratory processing of blood cultures

When working with suspected *Brucella* isolates, all manipulations of the culture, including oxidase and catalase tests and Gram staining, should be performed in a biological safety cabinet to minimize the possibility of exposure to aerosols of this organism. Gloves should be worn to protect from skin exposure to the culture. Laboratory-acquired cases of *Brucella* are not uncommon and include infections resulting by absorption of culture droplets through intact skin (7, 9, 11).

1. For biphasic systems, invert twice daily for the first 2 days and then daily for the remaining days of incubation. Incubate for 21 days.
2. Automated broth systems
   - **a.** Incubate broths for a minimum of 7 days and a maximum of 10 days.
   - **b.** Subculture an aliquot at day 10 to BAP, CHOC, or brucella agar and incubate plates in 5 to 10% CO_2 for at least 72 h.

   ◩ **NOTE:** When using the BACTEC 9240 system in one study, no positives were detected by the system after an incubation time of 10 days (1). Subcultures were not done in this study. In a study using Peds Plus medium, all but 1 of 42 positive cultures were detected by the BACTEC instrument in 6 days; blind subculture yielded only the 1 additional positive (13). The success of this medium may be due to the decreased amount of sodium polyanethol sulfonate in the pediatric bottle. In another study with the BacT/Alert system, a terminal subculture at 10 days detected all but one of the 7 positive cultures that were not detected by the instrument prior to that time (13).

B. Laboratory processing of positive cultures from any site

1. *Brucella* is most often isolated from blood cultures and sterile fluids (e.g., synovial, cerebrospinal, and pleural fluids) and tissues (e.g., bone marrow, lymph nodes, and lung). Rarely is *Brucella* isolated from semen and urine cultures.
2. *Brucella* has been listed as a potential agent of bioterrorism by the CDC (*see* section 16). Sentinel laboratories (level A laboratories) in the CDC Laboratory Referral Network should follow the identification guidelines outlined below for the presumptive identification of this organism (3). *Brucella* iso-

V. PROCEDURE *(continued)*

lates from any clinical site or source should be immediately referred to a reference laboratory for further analysis.

3. Brucellae are class 3 organisms, and laboratory staff may acquire infections when working on positive cultures if the proper containment procedures are not followed. All cultures suspected or confirmed to contain *Brucella* spp. should be opened and handled in a BSL 2 cabinet. Culture plates should never be smelled. Staff should wear personal protective equipment, including gloves, gowns, and goggles. Figure 3.4.2–1 outlines the recommended identification procedures for suspected *Brucella* spp. from cultures of blood and other clinical specimens (4, 5).
4. Look for colonies typically showing "dust-like" growth after overnight incubation on BAP and CHOC. Colonies are tiny but smooth, glistening, raised with an entire edge, and white to cream color at 48 to 72 h.
   - **a.** Gram stain suspicious colonies or broth; brucellae are tiny, gram-negative coccobacilli.
   - **b.** Perform catalase, oxidase, and rapid urea tests. Brucellae are *positive* by all three tests.
   - **c.** Optional: if the urea test is positive and the disk or tablet contains phenylalanine, do the following.
     (1) Add 2 drops of 1 N HCl to acidify the alkaline urea reaction (optional).
     (2) Perform PDA test by adding ferric chloride to tube. Brucellae are negative for PDA.

   ◩ **NOTE:** If a microorganism having the above reactions is isolated from a normally sterile site and is growing on BAP throughout the inocula, rather than just satelliting around a colony of staphylococci, it is presumptively identified as *Brucella* at this point and the physician should be informed.
5. If the colony is growing only on CHOC, subculture to CHOC, MAC, and BAP with a spot of *Staphylococcus aureus* ATCC 25923 added after inoculation of BAP with the suspected colony. If equivalent growth is seen on CHOC and BAP, but *not* on MAC or EMB, and the organism does not satellite around the staphylococcus, it is likely to be *Brucella* (procedure 3.17.44).

   ◩ **NOTE:** The most common organisms misidentified as *Brucella* spp. are *Haemophilus* spp., which will satellite around the staphylococcus on the BAP. Brucellae grow on the entire BAP without inhibition.
6. Confirm identification with serologic test or send to a reference laboratory for serologic confirmation.
   - **a.** Prepare a suspension of the isolate in 1.0% formalinized saline to match a no. 2.0 McFarland standard.
   - **b.** Let sit overnight to kill the organisms.
   - **c.** Set up tube agglutination test using the positive control antiserum from the febrile agglutinin test and normal rabbit serum as a negative control.
   - **d.** As a positive control, test the positive control antiserum with the antigen in the febrile agglutinin test.

POSTANALYTICAL CONSIDERATIONS

VI. INTERPRETATION

A. If a gram-negative coccobacillus is isolated from a blood, lymph node, tissue, or joint culture and meets the following criteria, it is preliminarily identified as *Brucella*.

1. Catalase positive
2. Oxidase positive

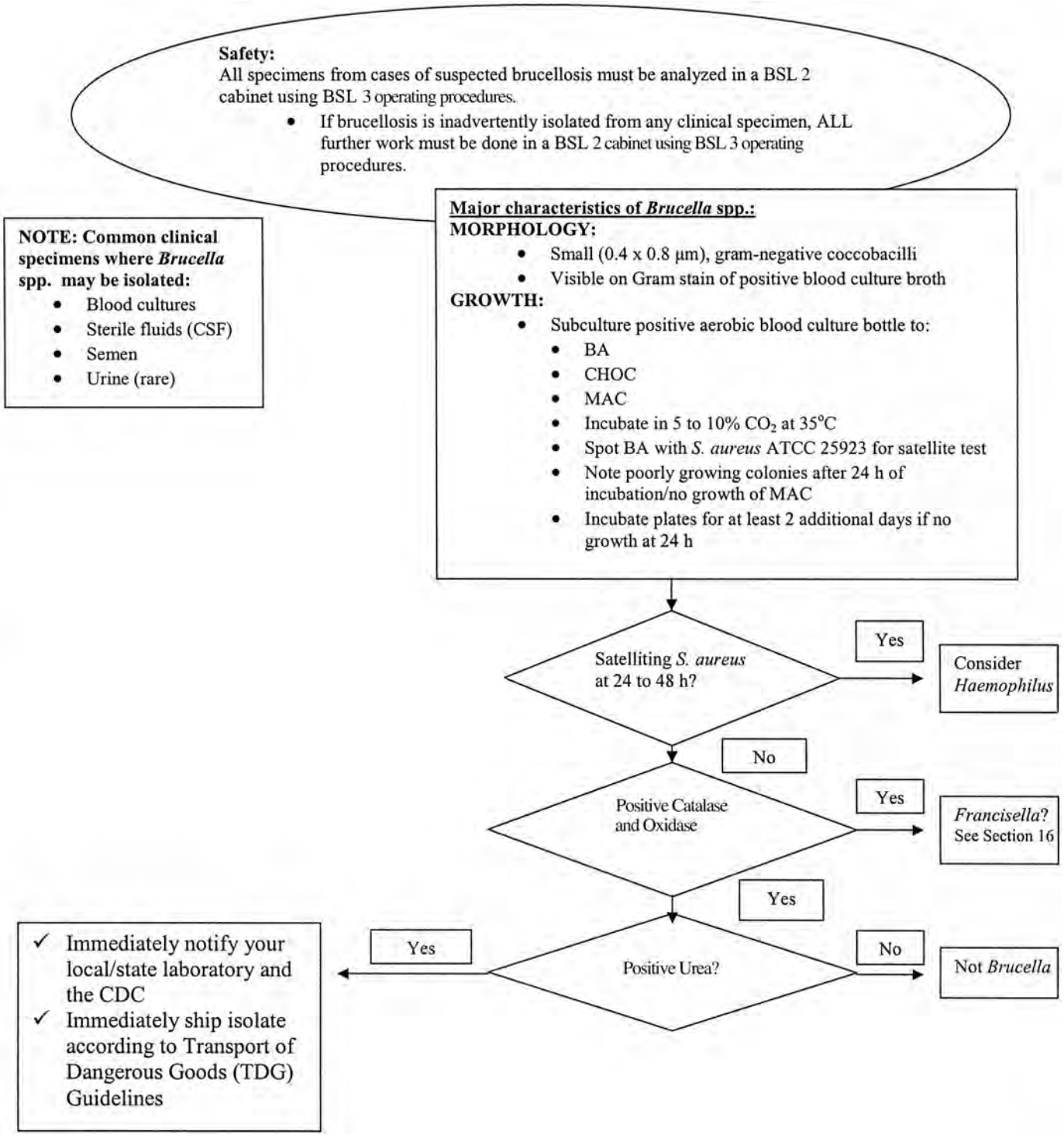

Safety:
All specimens from cases of suspected brucellosis must be analyzed in a BSL 2 cabinet using BSL 3 operating procedures.
If brucellosis is inadvertently isolated from any clinical specimen, ALL further work must be done in a BSL 2 cabinet using BSL 3 operating procedures.
NOTE: Common clinical specimens where Brucella spp. may be isolated:
Blood cultures
Sterile fluids (CSF)
Semen
Urine (rare)
Major characteristics of Brucella spp.:
MORPHOLOGY:
Small (0.4 x 0.8 µm), gram-negative coccobacilli
Visible on Gram stain of positive blood culture broth
GROWTH:
Subculture positive aerobic blood culture bottle to:
BA
CHOC
MAC
Incubate in 5 to 10% CO2 at 35°C
Spot BA with S. aureus ATCC 25923 for satellite test
Note poorly growing colonies after 24 h of incubation/no growth of MAC
Incubate plates for at least 2 additional days if no growth at 24 h
Satelliting S. aureus at 24 to 48 h?
Yes
Consider Haemophilus
No
Positive Catalase and Oxidase
Yes
Francisella? See Section 16
Yes
Positive Urea?
Yes
No
Not Brucella
✓ Immediately notify your local/state laboratory and the CDC
✓ Immediately ship isolate according to Transport of Dangerous Goods (TDG) Guidelines

Figure 3.4.2–1 Identification flowchart for *Brucella* spp. from cultures

Table 3.4.2–1 Urea-positive fastidious gram-negative coccobacilli similar to *Brucella*[a]

Characteristic or test	*Brucella*	EO-2, EO-4 *Psychrobacter immobilis*	*Psychrobacter phenylpyruvicus*	*Oligella ureolytica*[b]	*Actinobacillus* spp.[c]	*Bordetella bronchiseptica, Ralstonia paucula* (IV c2)	*Bordetella hinzii*	*Haemophilus* spp.[d]
Gram stain morphology	Tiny CCB, stains faintly	Small CCB, rods, EO-2 in packets	CCB	Tiny CCB	CCB, rods	CCB, rods	CCB, rods	CCB
Catalase	+	+	+	+	V	+	+	V
Oxidase	+	+	+	+	+	+	+	V
Urea[e]	+	V	+	+	+	+	14% Positive	V
Motility	−	−	−	+, delayed	−	+	+	−
PDA	−	−	+	+	−	V	−	−
Nitrate	+	V	68%	+	+	V	−	NA
Nitrite	−	V	−	+	−	−	−	NA
TSI[f]	Alkaline	Alkaline	Alkaline	Alkaline	Acid/acid	Alkaline	Alkaline	No growth
MAC, 48 h	−, poor	−, poor	−, poor	−, poor	−, poor	+	+	−

[a] Reactions extracted from references 9 and 12. NA, not applicable; V, variable; CCB, coccobacilli.

[b] *O. ureolytica* is primarily a uropathogen.

[c] *A. actinomycetemcomitans* is urea negative and rarely oxidase positive. Urea-positive *Actinobacillus* organisms are from animal sources.

[d] Grows only on CHOC, or on blood agar associated with staphylococcus colony.

[e] Use rapid urea test to increase sensitivity.

[f] TSI, triple sugar iron agar.

VI. INTERPRETATION *(continued)*

3. Urea positive (may be delayed), PDA negative
4. Grows on both BAP and CHOC without satelliting around staphylococcus
5. Does not grow on MAC

◩ **NOTE:** If the microorganism is not identified as *Brucella* by these tests, consider other fastidious gram-negative rods, such as *Haemophilus aphrophilus, Eikenella, Actinobacillus actinomycetemcomitans, Capnocytophaga,* and *Kingella kingae* (HACEK group), which are urea negative (Tables 3.18.2–4 and 3.18.2–5). However, some brucellae are urea negative.

B. The identification is confirmed if the isolate agglutinates with specific antiserum.

C. Organisms confused with *Brucella* (*see* Table 3.4.2–1)
1. If the isolate is from a urine culture, it could be *Oligella ureolytica,* a uropathogen with the same phenotypic characteristics as *Brucella.*
2. *Psychrobacter phenylpyruvicus* has the same phenotypic characteristics but is rare and is PDA positive.
3. *Haemophilus* does not grow on BAP without association with a staphylococcus colony.
4. Some *Bordetella* spp. are urea positive but grow on MAC and are motile.

VII. REPORTING RESULTS

A. For negative cultures, indicate the length of incubation: e.g., "No growth of *Brucella* after *x* days of incubation" for both preliminary and final reports, where "*x*" indicates the number of days of incubation.

B. Positive cultures
1. *Report suspected cases to the physician and to infection control.*
2. Report preliminary and final identification to the genus level as soon as available following the testing listed in this procedure.
3. Since brucellosis is a reportable disease in most states, notify the local or state health department of a positive culture (*also see* procedure 16.6).

VIII. LIMITATIONS

A. When evaluating patients, leukocyte counts are often within normal limits and liver function tests are not abnormal. Such a nonspecific presentation makes it difficult to consider this pathogen.

B. *Brucella* isolation is often delayed compared to that of other bloodstream pathogens, with peak isolation occurring at 3 to 4 days, compared to 6 to 36 h for most other pathogens.

C. The laboratory diagnosis of brucellosis is made primarily by recovery of organisms from blood cultures. Overall blood cultures are positive in 50 to 90% of cases, but the chances of recovery decrease over time. Joint and bone marrow specimens may also be a good source of *Brucella.*

D. In suspected cases, serologic testing of serum for *Brucella* agglutinins is a rapid method to diagnose brucellosis. Options include agglutination tests (Rose Bengal, Wright's tube, Wright's card, and Wright-Coombs), indirect immunofluorescence, complement fixation, and ELISAs. The standard with which all other methods are compared is Wright's tube agglutination test. A titer equal to or greater than 1/160 is considered significant (10). These tests can have variable sensitivity since there is no standard for antigen preparations and methodology, even for the "standard" Wright's tube agglutination test.

E. Antimicrobial susceptibility tests are not necessary and should not be reported, since they erroneously show susceptibility to some agents. The correct identification is sufficient information for appropriate therapy. Tetracyclines (generally doxycycline) are the most active drugs and should be used in combination with streptomycin (or gentamicin or rifampin, if streptomycin is unavailable) to prevent relapse.

REFERENCES

1. **Bannatyne, R. M., M. C. Jackson, and Z. Memish.** 1997. Rapid detection of *Brucella* bacteremia by using the BACTEC 9240 system. *J. Clin. Microbiol.* **35:**2673–2674.
2. **Castaneda, M. R.** 1947. A practical method for routine blood culture in brucellosis. *Proc. Soc. Biol. Med.* **64:**114.
3. **Centers for Disease Control and Prevention.** 2000. Biological and chemical terrorism: strategic plan for preparedness and response (recommendations of the CDC Strategic Planning Workgroup). *MMWR Morb. Mortal. Wkly. Rep.* **49**(RR-4)**:**1–14.
4. **Centers for Disease Control and Prevention.** *Basic Protocols for Level A Laboratories for the Presumptive Identification of* Brucella *spp.* Centers for Disease Control and Prevention, Atlanta, GA. http://www.cdc.gov.
5. **Centers for Disease Control and Prevention, Association of Public Health Laboratories.** 2002. *Presumptive* Brucella *spp. Identification and Similar Organisms.* Centers for Disease Control and Prevention, Association of Public Health Laboratories. http://www.bt.cdc.gov/agent/agentlist.asp.
6. **Ederer, G. M., J. H. Chu, and D. J. Blazevic.** 1971. Rapid test for urease and phenylalanine deaminase production. *Appl. Microbiol.* **21:**545.
7. **Fiori, P. L., S. Mastrandrea, P. Rappelli, and P. Cappuccenelli,** 2000. *Brucella abortus* infection acquired in microbiology laboratories. *J. Clin. Microbiol.* **38:**2005–2006.
8. **Gotuzzo, E., C. Carrillo, J. Guerra, and L. Llosa.** 1986. An evaluation of diagnostic methods for brucellosis—the value of bone marrow culture. *J. Infect. Dis.* **153:**122–125.
9. **Lindquist, D., M. C. Chu, and W. S. Probert.** 2007. *Francisella* and *Brucella*, p. 815–834. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
10. **Moyer, N. P., G. M. Evins, N. E. Pigott, J. D. Hudson, C. E. Farshy, J. C. Feeley, and W. J. Hausler, Jr.** 1987. Comparison of serologic screening tests for brucellosis. *J. Clin. Microbiol.* **25:**1969–1972.
11. **Staszkiewicz, J., C. M. Lewis, J. Colvile, M. Zervos, and J. Band.** 1991. Outbreak of *Brucella melitensis* among microbiology laboratory workers in a community hospital. *J. Clin. Microbiol.* **29:**287–290.
12. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed. Williams & Wilkins, Baltimore, MD.
13. **Yagupsky, P.** 1999. Detection of brucellae in blood cultures. *J. Clin. Microbiol.* **37:**3437–3442.
14. **Yagupsky, P., and E. J. Baron.** 2005. Laboratory exposures to brucellae and implications for bioterrorism. *Emerg. Infect. Dis.* **11:**1180–1185.
15. **Yagupsky, P., N. Peled, J. Press, O. Abramson, and M. Abu-Rashid.** 1997. Comparison of BACTEC 9240 Peds Plus medium and Isolator 1.5 microbial tube for detection of *Brucella melitensis* from blood cultures. *J. Clin. Microbiol.* **35:**1382–1384.

3.4.3 *Bartonella* Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The genus *Bartonella* comprises at least 19 species of gram-negative short (0.25 to 0.75 by 1 to 3 μm) rod-shaped bacteria, 7 of which have been implicated in human diseases (17). Arthropod vectors transmit the organism, and cats constitute the major reservoir of the species most commonly recognized *(Bartonella henselae). B. henselae* is the classic agent of cat scratch disease (CSD) and is globally endemic. It is also the agent of bacillary angiomatosis (BA) and peliosis (BP) in immunocompromised patients as well as in the immunocompetent (1, 7, 14). *Bartonella quintana* was historically responsible for outbreaks of trench fever in World War I, but it has more recently been found to cause BA and BP (2, 7). Both species have been associated with bacteremia and, not uncommonly, endocarditis. *Bartonella elizabethae, Bartonella clarridgeiae, Bartonella grahamii, Bartonella vinsonii* subsp. *arupensis,* and *Bartonella vinsonii* subsp. *berkhoffii* have been rarely associated with bacteremic or CSD-like illnesses (17).

Bartonellosis (or Carrión's disease), caused by *Bartonella bacilliformis,* can be acquired by persons traveling to the Andes mountains of Peru, Colombia, and Ecuador. It has been found only in humans and is associated with exposure to sand fly vectors found on the western slopes of the Andes at elevations of approximately 2,500 to 9,000 ft (3). South American bartonellosis occurs in two forms, i.e., Oroya fever and verruga peruana, or Peruvian wart. Oroya fever is characterized by a severe acute-onset anemia, at the peak of which a majority of RBCs are infected with one to six organisms each, and massed intracellular bartonellae in vascular endothelial cells produce a diagnostic lesion that bulges into the lumen of the vessel. Verruga peruana is characterized by cutaneous eruptions ranging from small disseminated elevations to distinctive red-purple "cranberries." Verruga peruana is visually alarming but is benign and self-limiting. Conversely, Oroya fever is often life-threatening, and if not treated (with oral chloramphenicol), all of the erythrocytes become infected by the bacteria (16). In survivors of Oroya fever, verruga peruana may occur after the anemia and bartonellemia have subsided. In areas where the organism is endemic, verruga peruana is common but Oroya fever is rarely seen. The converse is true in areas of nonendemicity (3). Asymptomatic human infection may account for the continued presence of the disease. This organism remains the biggest threat to human health of all the *Bartonella* species (3). The causative agent, *B. bacilliformis,* is found in or on RBCs and vascular endothelial cells of infected patients. Diagnosis is generally made by serology, Wright stain of the peripheral blood, or culture.

Isolation of all *Bartonella* species is usually from blood and requires special procedures and long incubation (7, 10, 14). Contamination of cultures is common, and efforts must be taken to avoid loss of cultures from overgrowth with molds. Diagnosis is more often made serologically (4) or by histologic examination of biopsy samples of nodes, skin, or liver. PCR is also helpful for diagnosis (11).

II. SPECIMENS

Observe standard precautions.

◩ **NOTE:** Cultures should only be performed by or submitted to a laboratory that is familiar with the culture and identification of the organism. Freshly prepared culture media optimize the chance for isolation of *Bartonella* spp.

A. Process aseptically collected tissues, such as lymph nodes or liver, by direct smear, by inoculation into endothelial tissue culture cells, or by plating on blood agar or CHOC. Direct detection of *Bartonella* using molecular methods is usually performed in a reference laboratory (11). Smears may also be stained with Wright or Giemsa stain. Excised verrugae can also be stained or examined with specific antiserum.

II. SPECIMENS *(continued)*

B. In cases of Oroya fever, collect thick and thin blood smears (preferably before antimicrobial therapy) from fresh drops of peripheral blood for staining with Wright or Giemsa stain (procedure 9.8.6 or 9.8.5, respectively).

C. Collect 10 ml of blood for culture into an ISOLATOR tube using sterile technique (*see* Appendix 3.4.1–2). Prior antimicrobial treatment negates the ability to culture the organism. Collection of more than one specimen may increase the yield of a culture.

◩ **NOTE:** Automated blood culture systems have yielded *Bartonella*, but the organism typically does not produce sufficient CO_2 to be detected by the instrument. On day 8, remove a small aliquot of blood culture broth and stain with acridine orange (procedure 3.2.2) or observe motile organisms by wet mount using phase-contrast microscopy. If growth is observed, subculture to CHOC (14). La Scola and Raoult successfully cultured 1 ml of blood collected into a Vacutainer tube containing lithium heparin (Becton Dickinson Systems, Rutherford, NJ) and plated it onto Columbia sheep blood agar (10). The ISOLATOR method in combination with rabbit blood agar has the best yield for most species (5, 8).

III. MATERIALS

A. ISOLATOR system (Appendix 3.4.1–2)

B. Anaerobic jar or bag and CO_2-generating system

C. Sterile M199 tissue culture medium (Gibco, Invitrogen, Carlsbad, CA) for dilutions

D. Media

1. Fresh CHOC (double poured [40 ml per plate] and less than 3 weeks old has higher yield)

2. Heart infusion agar with 5% rabbit blood

3. In addition to the media listed above, the following media have been used successfully for culture of *B. bacilliformis.*

a. Columbia agar with 10% whole horse, rabbit, or sheep blood (2, 3)

b. Biphasic media consisting of the following

(1) A solid medium of 10% defibrinated sheep blood, glucose, tryptose, NaCl, and agar

(2) A liquid RPMI 1640 medium (Mediatech, Fisher Scientific) enriched with HEPES buffer, sodium bicarbonate, and 10% fetal bovine serum (9)

◩ **NOTE:** In some cases successful culture of *Bartonella* can only be achieved by initial cultivation in an endothelial tissue culture cell line or in liquid RPMI 1640 medium (2).

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Test each medium with a known *Bartonella* species (e.g., *B. henselae* ATCC 49793, *B. quintana* ATCC 51694, *B. bacilliformis* ATCC 35685 or ATCC 35686) to verify the ability to grow the organism.

B. Check each medium for sterility by incubation of sample plates and animal blood products prior to culture.

C. Refer to procedure 3.3.1 for other QC requirements.

V. PROCEDURE

It is imperative that these cultures be handled in a biological safety cabinet.

A. Process ISOLATOR as in Appendix 3.4.1–2, with the following exceptions.
- **1.** Use gloves, and wash gloves with alcohol during each step to avoid contamination of the culture.
- **2.** Centrifuge ISOLATOR tube for 45 min at 3,000 × *g* in a fixed-angle rotor.
- **3.** Pop the top of the ISOLATOR tube using alcohol-soaked wipes with plastic backs for protection.
- **4.** Using the ISOLATOR supernatant pipette, remove all the supernatant blood without disturbing the Fluorinert. Discard blood in infectious-waste container.
- **5.** Add 0.2 ml (4 drops) of M199 to the pellet with a sterile pipette. Mix well with the pipette.
- **6.** Prior to inoculation, expose the plates and lids to UV light in a biological safety cabinet while drying completely.
- **7.** Inoculate the plates with the entire volume of Fluorinert. Use 0.3 ml/plate of chocolate and blood media. Streak for isolation.
- **8.** Place the plates in the anaerobe jar or bag, moisten with sterile water and sterile gauze, and activate a CO_2-generating system.
- **9.** Incubate bag or jar at 37°C for most species; however, incubate at 25 to 30°C without increased CO_2 for *B. bacilliformis* and *B. clarridgeiae.*

B. Examine plates for growth weekly for up to 5 weeks.
- **1.** Wear sterile gloves and swab often with alcohol. Work quickly and subculture if growth is observed or if contaminants are growing on the plate.
- **2.** Tiny colonies may be visible as early as day 4 under ×60 to ×100 magnification.
- **3.** *B. bacilliformis* colonies may be friable, small, dark, and round with an entire edge and may appear to have a halo under certain lighting conditions. A second colony type which may also be present is lighter, larger with an irregular edge, and not friable. Both cause an indentation of the agar under the center of the colonies (15).

C. If tiny colonies are observed, perform a Gram stain with extended exposure to safranin or using carbol fuchsin as a counterstain. Examine for the presence of small, gram-negative, curved bacilli. These will be suggestive of *Campylobacter* or *Helicobacter* in morphology. Colonies visualized with a stereoscope take two forms: irregular, dry, and cauliflower-like or small, circular, tan, moist colonies that pit the agar.

D. Perform wet mount motility and observe for twitching motility.

E. Perform catalase and oxidase tests, which are either negative or weakly positive. Perform urea test, which should be negative (14).

◩ **NOTE:** *Bartonella* organisms do not ferment or oxidize common carbohydrates or grow anaerobically. Other organisms, including *Mycobacterium tuberculosis,* could grow on the media used for culture. They are likely to be detected by Gram stain or wet mount and should be identified using charts and tables in other sections of this handbook.

F. Confirm identification, using specific genetic or immunofluorescence tests and cellular fatty acid analysis.

◩ **NOTE:** There are no Food and Drug Administration-cleared assays to identify this organism.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING AND INTERPRETATION OF RESULTS

A. Very slowly growing, small, gram-negative, curved bacilli with twitching motion, that are catalase and oxidase negative or weakly reacting but are urea negative, isolated from a blood culture are likely to be one of the *Bartonella* spp.

B. Confirm identification usually by submitting the isolate to a reference laboratory. Serologic testing of the patient may be useful pending confirmation of the isolate's identity.

C. For blood smears stained with Wright or Giemsa stain for *B. bacilliformis*, a presumptive diagnosis of bartonellosis can be made if all of the following three conditions are met.

1. The patient has lived in or visited an area in which the disease is endemic.

2. The patient's clinical signs are consistent with bartonellosis.

3. Large numbers of RBCs are infected with multiple organisms. The organisms stain reddish violet and are pleomorphic and may be seen as slender rods (straight or bent) or as oval to round forms; they may be found singly or in segmenting chains. Forms range from tiny dots to large irregular shapes and spindles. Vascular endothelial cells swollen with rounded masses of organisms are diagnostic of Oroya fever.

◩ **NOTE:** Excellent color plates are available in reference 16. The severe anemia is macrocytic and often hypochromic, with various aberrations; Howell-Jolly bodies and basophilic granules may be seen in addition to the bartonellae. In verruga peruana, the bartonellae are scattered among young cells and are definitive in the presence of the distinctive histology of the verrugae.

VII. LIMITATIONS

A. Culture is generally unsuccessful if the patient has been treated with macrolide or aminoglycoside antimicrobial agents.

B. Contamination of cultures is common, and every precaution should be taken to guard against mold growth. Exposure of plates and equipment to UV light and wearing gloves during all steps in the culture inoculation and observation of the cultures are important.

C. Caution must be used in the interpretation of growth on the media, since mycobacteria, which are gram positive, and the yeast *Histoplasma* may be isolated from patients with similar symptoms.

D. Many species of *Bartonella* are difficult to grow and identify. See reference 5.

E. *Bartonella*-like structures due to other pathological conditions and other agents (e.g., *Haemobartonella* and *Eperythrozoon* spp.) may cause confusion in the diagnosis of Oroya fever. These other causes of RBC inclusions should be suspected in patients who have had no contact with an area of endemicity.

F. Smears and cultures of specimens taken very early and late in the disease may be negative.

G. Asymptomatic individuals may yield positive cultures or test positive for *Bartonella* antibodies.

H. Reference laboratories offer serologic testing by either immunofluorescence assay (IFA) or EIA methods. Immunoglobulin G and M antibodies to crude and partially purified *Bartonella* antigens have been detected by EIAs. Antigens in both the IFA and EIA cross-react with *Chlamydia* and *Coxiella* antibodies, as well as with species within the *Bartonella* genus (6).

I. Reference laboratories offer molecular testing because *Bartonella* culture is very slow and has a low sensitivity. The most common types of clinical specimens referred for *Bartonella* detection include lymph node and skin tissue and pus or

VII. LIMITATIONS *(continued)*

fluid from abscesses. The reference nucleic acid amplification method targets the 16S rRNA gene using a universal primer set that is neither species nor genus specific. Amplified products are characterized either by nucleic acid sequencing or hybridization probes (12). Amplification of the *rpoB* gene followed by restriction analysis of the products with three enzymes has also been used to identify *Bartonella* species in clinical specimens with the same sensitivity as the reference method (13).

REFERENCES

1. **Adal, K. A., C. J. Cockerell, and W. A. Petri.** 1994. Cat scratch disease, bacillary angiomatosis, and other infections due to *Rochalimaea. N. Engl. J. Med.* **330:**1509–1515.
2. **Anderson, B. E., and M. A. Neuman.** 1997. *Bartonella* spp. as emerging human pathogens. *Clin. Microbiol. Rev.* **10:**203–219.
3. **Birtles, R. J., N. K. Fry, P. Ventosilla, A. G. Cáceres, E. Sánchez, H. Vizcarra, and D. Raoult.** 2002. Identification of *Bartonella bacilliformis* genotypes and their relevance to epidemiological investigations of human bartonellosis. *J. Clin. Microbiol.* **40:**3606–3612.
4. **Chamberlin, J., L. Laughlin, S. Gordon, S. Romero, N. Solorzano, and R. L. Regnery.** 2000. Serodiagnosis of *Bartonella bacilliformis* infection by indirect fluorescence antibody assay: test development and application to a population in an area of bartonellosis endemicity. *J. Clin. Microbiol.* **38:**4269–4271.
5. **Chomel, B. B., and J. M. Rolain.** 2007. *Bartonella,* p. 850–861. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
6. **Knobloch, J., L. Solano, O. Alvarez, and E. Delgado.** 1985. Antibodies to *Bartonella bacilliformis* as determined by fluorescence antibody test, indirect hemagglutination and ELISA. *Trop. Med. Parasitol.* **36:**183–185.
7. **Koehler, J. E.** 1996. *Bartonella* infections. *Adv. Pediatr. Infect. Dis.* **11:**1–27.
8. **Koehler, J. E., F. D. Quinn, T. G. Berger, P. E. LeBoit, and J. W. Tappero.** 1992. Isolation of *Rochalimaea* species from cutaneous and osseous lesions of bacillary angiomatosis. *N. Engl. J. Med.* **327:**1625–1631.
9. **Kosek, M., R. Lavarello, R. H. Gilman, J. Delgado, C. Maguiña, M. Verastegui, A. G. Lescano, V. Mallqui, J. C. Kosek, S. Recavarren, and L. Cabrera.** 2000. Natural history of infection with *Bartonella bacilliformis* in a non-endemic population. *J. Infect. Dis.* **182:**865–872.
10. **La Scola, B., and D. Raoult.** 1999. Culture of *Bartonella quintana* and *Bartonella henselae* from human samples: a 5-year experience (1993 to 1998). *J. Clin. Microbiol.* **37:**1899–1905.
11. **Matar, G. M., J. E. Koehler, G. Malcolm, M. A. Lambert-Fair, J. Tappero, S. B. Hunter, and B. Swaminathan.** 1999. Identification of *Bartonella* species directly in clinical specimens by PCR-restriction fragment length polymorphism analysis of a 16S rRNA gene fragment. *J. Clin. Microbiol.* **37:**4045–4047.
12. **Relman, D. A., J. S. Loutit, T. M. Schmidt, S. Falkow, and L. S. Tompkins.** 1990. The agent of bacillary angiomatosis. An approach to the identification of uncultured pathogens. *N. Engl. J. Med.* **323:**1573–1580.
13. **Renesto, P., J. Gouvernet, M. Drancourt, V. Roux, and D. Raoult.** 2001. Use of *rpoB* gene analysis for detection and identification of *Bartonella* species. *J. Clin. Microbiol.* **39:**430–437.
14. **Spach, D. H., A. S. Kanter, M. J. Dougherty, A. M. Larson, M. B. Coyle, D. J. Brenner, B. Swaminathan, G. M. Matar, D. F. Welch, R. K. Root, and W. E. Stamm.** 1995. *Bartonella (Rochalimaea) quintana* bacteremia in inner-city patients with chronic alcoholism. *N. Engl. J. Med.* **332:**424–428.
15. **Walker, T. S., and H. H. Winkler.** 1981. *Bartonella bacilliformis:* colonial types and erythrocyte adherence. *Infect. Immun.* **31:**480–486.
16. **Weinman, D.** 1981. Bartonellosis and anemias associated with bartonella-like structures, p. 235–248. *In* A. Balows and W. J. Hausler, Jr. (ed.), *Diagnostic Procedures for Bacterial, Mycotic and Parasitic Infections,* 6th ed. American Public Health Association, Washington, DC.
17. **Zeaiter, Z., Z. Liang, and D. Raoult.** 2002. Genetic classification and differentiation of *Bartonella* species based on comparison of partial *ftsZ* gene sequences. *J. Clin. Microbiol.* **40:**3641–3647.

SUPPLEMENTAL READING

Brouqui, P., and D. Raoult. 2001. Endocarditis due to rare and fastidious bacteria. *Clin. Microbiol. Rev.* **14:**177–207.

Daly, J. S., M. G. Worthington, D. J. Brenner, C. W. Moss, D. G. Hollis, R. S. Weyant, A. G. Steigerwalt, R. E. Weaver, M. I. Daneshvar, and S. P. O'Connor. 1993. *Rochalimaea elizabethae* sp. nov. isolated from a patient with endocarditis. *J. Clin. Microbiol.* **31:**872–881.

Ellis, B. A., L. D. Rotz, J. A. D. Leake, F. Samalvides, J. Bernable, G. Ventura, C. Padilla, P. Villaseca, L. Beati, R. Regnery, J. E. Childs, J. G. Olsen, and C. P. Carrillo. 1999. An outbreak of acute bartonellosis (Oroya fever) in the Urubamba region of Peru, 1998. *Am. J. Trop. Med. Hyg.* **61:**344–349.

3.5 Body Fluid Cultures (Excluding Blood, Cerebrospinal Fluid, and Urine)

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Infection of normally sterile body fluids often results in severe morbidity and mortality; therefore, rapid and accurate microbiological assessment of these samples is important to successful patient management. Most organisms infecting these sites are not difficult to culture, but determining the significance of low numbers of commensal cutaneous microorganisms does present a challenge (14). With the increased use of prostheses, immunosuppressive therapeutic regimens, and long-term care of individuals with chronic debilitating disease, the likelihood of true infection with commensal organisms has increased, making accurate diagnoses difficult. Care must be taken during specimen collection and transport to ensure that the specimen is not contaminated. Any microorganism found in a normally sterile site must be considered significant, and all isolates must be reported.

Culture of the specimen should include the most likely organisms to cause infection. Joint infections are commonly caused by *Staphylococcus aureus,* but *Streptococcus pyogenes, Neisseria gonorrhoeae,* anaerobes, *Kingella kingae,* and *Brucella* spp. may be likely causes of infectious arthritis (2, 6, 13–15, 24, 25). The lysis-centrifugation method (Appendix 3.4.1–2) is more sensitive than plate culture without lysis, but automated blood culture systems have been shown to be faster than lysis-centrifugation and have a higher yield (13, 22, 24–26). Pediatric bottles have the advantage of containing less sodium polyanethol sulfonate (SPS), which inhibits some organisms (24, 25). For prosthetic-joint infections, the importance of preoperative joint fluid cultures cannot be overemphasized. The diagnosis is increased by culture of large volumes, by culturing anaerobically, and by culturing more than one specimen (2, 5, 6, 10, 15, 22). Typically the Gram stain shows inflammation, without the presence of bacteria. In fact, a British collaborative study group has recommended that Gram stains be abandoned for evaluation of elective revision arthroplasty, since they are not helpful to diagnosis (2).

Peritoneal fluid can be contaminated with numerous mixed gastrointestinal microbiota in cases of ruptured intestine, but in patients with chronic ambulatory peritoneal dialysis (CAPD) or spontaneous bacterial peritonitis (SBP), the likely pathogen is usually present in very low numbers. In CAPD the pathogens are staphylococci; viridans group streptococci; non-glucose-fermenting, gram-negative rods; *Candida albicans* and other *Candida* spp.; and fungi (23). Numerous studies have shown that culture of large volumes of fluid in blood culture bottles, rather than concentration by centrifugation, will result in a higher yield (1, 4, 5, 10, 18, 21, 23). Blood culture bottles are superior to the lysis-centrifugation system for diagnosis of SBP (21).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. *Refer to Table 3.5–1 for commonly submitted body fluids and synonyms.*

◩ **NOTE:** Because the body may respond to infections with infiltration of fluid, some of these sites may have fluid accumulation only during infection.

B. There are many common drainage tubes and other devices that are used clinically. Table 3.5–2 outlines some of the most common types of drainage tubes in clinical use. All of these tubes and tips are not suitable for culture and should not be processed. A fresh sample of the fluid draining out of each of these devices should be collected and sent for culture. There are many commercial versions of these products, and each laboratory should include a list of the products used locally in their manual that includes the names for the systems used locally. Requests for drainage tube or device cultures can cause confusion and errors in processing because clinicians often use the person or company inventing or popularizing the drainage device (Axiom sump drain, Shirley sump

Table 3.5–1 Types of body fluids submitted for culture

Fluid	Location	Synonym	Definition of synonym
Joint	At the union of two bones	Synovial	Viscid fluid of the joint cavity
Pleural	Within the pleural space between the membrane surrounding the lungs and the chest wall	Empyema	Fluid with purulent exudate
		Thoracentesis	Fluid collected by aspiration of the pleural space following puncture of the chest wall
Peritoneal	Within the abdominal peritoneal cavity bounded by the peritoneum	Abdominal	Same as peritoneal
		Ascites	Abnormal accumulation of fluid in the cavity
		Paracentesis	Fluid collected by aspiration following puncture of the peritoneal cavity
		CAPD	Fluid from the peritoneal cavity of a patient undergoing continuous ambulatory peritoneal dialysis (i.e., may be a mixture of peritoneal and dialysate fluids)
		PV fluid	Fluid from the peritoneal cavity of a patient with a shunt tube inserted from the ventricles of the brain, under the skin into the peritoneal cavity
Pericardial	Within the pericardial sac between the pericardium and the heart		
Cul-de-sac	A blind pouch between the anterior wall of the rectum and the posterior wall of the uterus	Culdocentesis	Fluid obtained by transvaginal puncture and aspiration of the cul-de-sac
Amniotic	Within the amniotic cavity between the amniotic membrane and the fetus	Amniocentesis	Fluid obtained by puncture and aspiration of amniotic fluid

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

drain, Abramson drain, Chaffin drain, Lakey tube drain, Wheaton tube drain, Tenckhoff catheter, etc.) rather than the type or location of the device (e.g., Wheaton T-tube biliary drain). Calling the departments of surgery and invasive radiology and communicating with the hospital's distribution department would provide the proper names and descriptions of drainage devices used to collect specimens.

Table 3.5–2 Common drainage tubes in clinical use

Drainage tube	Collected fluid	Comments
Biliary tube (T-tube)	Bile	T-tube used to externally drain biliary ducts
Chest tube	Pleural fluid	Pleural space drainage tube
Duval catheter tip	Peritoneal (intra-abdominal fluid or pus)	Large-bore abdominal drainage tube
G-tube	Gastric fluid	Gastric tube used for feeding
Jackson-Pratt (JP)	Peritoneal (intra-abdominal fluid or pus)	Vacuum drainage placed at the time of surgery that may be used to access sterile sites
Penrose drain	Peritoneal (intra-abdominal fluid or pus)	Nonsterile drain placed into the abdominal cavity during surgery to drain fluid or pus
Reo (Entriflex Dobbhoff) tube	Gastric/upper small bowel fluid	Nonsterile nasogastric tube for feeding
RRC	Peritoneal (intra-abdominal fluid or pus)	Nonsterile red rubber catheter used for abdominal drainage
Sump drain	Peritoneal (intra-abdominal fluid or pus)	Large-bore abdominal drainage tube
Tenckhoff catheter	Peritoneal/dialysate	Sterile catheter used for continuous peritoneal dialysis access
T-tube	Bile	Sterile T-shaped tube put into the biliary tree during surgery to drain off bile and other fluids

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

C. Specimen collection: body fluid specimens collected by percutaneous aspiration for pleural, pericardial, peritoneal, amniotic, and synovial fluids

◩ **NOTE:** Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and other caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection. Use care to avoid contamination with commensal microbiota.

1. Clean the needle puncture site with alcohol, and disinfect it with an iodine solution (1 to 2% tincture of iodine or a 10% solution of povidone-iodine [1% free iodine]) to prevent introduction of specimen contamination or infection of patient. (If tincture of iodine is used, remove with 70% ethanol after the procedure to avoid burn.)

2. Aseptically perform percutaneous aspiration with syringe and needle to obtain pleural, pericardial, peritoneal, or synovial fluid. Use safety devices to protect from needle exposure.

3. Immediately place a portion of the joint fluid or peritoneal fluid collected from patients with CAPD or SBP into aerobic and anaerobic blood culture bottles, retaining some (0.5 ml) in syringe for Gram stain and direct plating.

a. Use the minimum and maximum volumes recommended by the bottle manufacturer (generally up to 10 ml is the maximum for each bottle).

b. Alternatively, inoculate the blood culture bottles after receipt in the laboratory.

4. Submit other fluids and the remainder of specimens placed in blood culture bottles in one of the following

a. A sterile, gassed-out tube or a sterile blood collection tube without preservative (e.g., sterile Vacutainer brand red-top tube); however, fluids in such tubes may clot during transport. Alternatively, fluids may be placed in a purple-top tube containing an anticoagulant such as heparin or SPS, although some fastidious organisms may be inhibited if the specimen is delayed in transport. EDTA and citrate anticoagulants are the most likely to inhibit microorganisms in fluid specimens and should not be used.

b. Anaerobic transport vial (for small-volume specimens)

c. Specimens received by the laboratory in a syringe with the needle still attached should be rejected because of the risk of a needless sharps exposure by laboratory staff. The physician should be immediately phoned to recollect the sample and send it in the proper container.

◩ **NOTE:** Establish a policy for the proper collection and transport of clinical specimens not collected on swabs. Educate the physicians that needles must be removed from the syringe and the syringe cap secured prior to transport to avoid leakage.

d. Syringes that have been capped with a Luer-Lok (with needle removed) prior to transport may be accepted for culture provided the specimen has not clotted inside the syringe and there is no leakage during transport which could result in contamination of the culture. The laboratory may reject specimens that have clotted in a capped syringe because they cannot be processed for culture without inadvertently contaminating the specimen.

D. Specimen transport

1. Submit to laboratory as soon as possible and, if from a normally sterile site, alert laboratory that specimen has been submitted.

2. Do not refrigerate.

3. Label specimens with patient demographics and date, time, and site of collection: e.g., left knee joint fluid.

4. Record the patient diagnosis for improved processing of specimen.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

E. Rejection criteria

1. If only blood culture bottles are received, a Gram stain cannot be performed.

2. Collect prior to antimicrobial therapy for greatest diagnostic sensitivity.

3. Do not submit specimens from drains after they have been infused with antimicrobial agents.

4. Call physician when fluid specimens are received on a swab.

◩ **NOTE:** Swabs afford the least desirable sample for culture of body fluids and should be discouraged as devices for transport, since the quantity of sample may not be sufficient to ensure recovery of a small number of organisms.

5. Contact physician if specimen is insufficient for the number of tests requested.

◩ **NOTE:** Routine bacterial culture is sufficient for culture for *Candida* species, if blood culture bottles are used or specimen is centrifuged. Fungal cultures of joint and abdominal specimens are occasionally indicated (especially for *Blastomyces dermatitidis* and *Histoplasma capsulatum*) but should be discouraged routinely. AFB cultures should not be routine but should be limited to those with a clinical indication.

6. Invasively collected specimens in leaky containers must be processed, but alert the physician of the possibility of contamination.

III. MATERIALS

A. Media

1. BAP

2. CHOC

3. MAC

4. Nonselective anaerobic medium, e.g., CDC anaerobic sheep blood agar. See section 4 for media and methods.

5. Special selective media for recovery of fastidious or unusual organisms (e.g., charcoal-yeast extract agar for *Legionella*). Refer to the table of contents for procedures for specific microorganisms.

6. Add media selective for gram-negative or gram-positive organisms (*see* Table 3.3.1–1) based on Gram-stained smear of the specimen, which shows multiple morphologies of microorganisms.

7. Broth known to support the growth of both fastidious aerobic organisms and anaerobes, to detect small numbers of organisms (6, 14, 15, 17, 19). Common broth culture media are as follows.

a. Blood culture bottles (added supplements have not been shown to be important [5, 10])

b. Anaerobic BHI or TSB with 0.1% agar with or without yeast extract

c. Fastidious anaerobe broth or fastidious broth (Quelab Laboratories, Inc., Montreal, Quebec, Canada; Remel, Inc., Lenexa, KS) (19)

d. Brucella broth

e. THIO is least desirable as a broth to grow low numbers of aerobic organisms and yeasts. It is excellent for anaerobic organisms (17, 19).

8. See procedure 3.17 for biochemical test reagents and procedure 3.3.1 for medium descriptions.

B. Gram stain reagents

C. Other supplies

1. Cytocentrifuge and holders

2. Cleaned glass slides

3. Sterile Pasteur pipettes

4. CO_2-generating system and 35°C incubator

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Verify that media meet expiration date and QC parameters per Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22-A3 (8). See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

B. For QC of blood culture broths, refer to procedure 3.4.1.

C. See individual tests in procedure 3.17 for biochemical test QC.

D. Periodically check laboratory processing to ensure there is no break in sterile technique and that media, stain reagents, collection tubes, slides, and other supplies are not contaminated with dead organisms.

1. Filter sterilize (through a 0.22-μm-pore-size membrane filter) fluid from saved specimens, and periodically process along with patient samples.

2. No organisms should be detected on Gram stain, and these cultures should yield no growth.

V. PROCEDURE

Observe standard precautions.

A. Inoculation

1. Process specimen as soon as it is received.

◩ **NOTE:** Use of biosafety cabinet will avoid contamination of the culture or specimen as well as protect laboratory processing personnel.

2. Record volume of specimen.

3. Record gross appearance, i.e., color, viscosity, light-transmitting properties, and presence of a clot.

4. Inoculate BAP and CHOC with 2 or 3 drops of specimen.

a. If only blood culture bottles were received, subculture immediately to CHOC to isolate microorganisms that prefer agar plates for growth (e.g., *N. gonorrhoeae*).

b. If little specimen is received (1 or 2 drops), inoculate only CHOC and rinse tube with broth culture medium. Omit Gram stain. Note on report the volume received.

5. If greater than 0.5 ml of specimen is received, do the following.

a. Inoculate up to 1 ml into 10 ml of broth culture media.

b. For greater than 2 ml of pericardial, peritoneal, amniotic, or synovial fluid specimens not received in blood culture bottles, inoculate aerobic and, if there is sufficient volume of specimen, anaerobic blood culture bottles. Do not add less than the amount recommended by the manufacturer, as the excess SPS may be inhibitory to growth of the infecting organism.

6. Inoculate anaerobic medium with 2 or 3 drops of specimen, *if* an anaerobic broth or blood culture bottle was not inoculated. For pleural fluid or drainage fluid, perform only on request and if specimen was transported anaerobically,

7. For peritoneal fluid contaminated with bowel contents, add MAC or EMB, Columbia colistin-nalidixic acid (CNA) or phenylethyl alcohol agar, and selective media for anaerobic isolation of mixed anaerobic microbiota, and omit broth cultures.

8. For requests to culture for microorganisms that do not grow on routine laboratory media (e.g., *Legionella*), inoculate special media as appropriate (refer to Appendix 3.4.1–1 for details on isolation and detection of these microorganisms).

V. PROCEDURE *(continued)*

9. Dispersion of clots
- **a.** Pour the sediment containing clotted material into a sterile tissue grinder.
- **b.** Add a small volume (<0.5 ml) of sterile broth to the grinder, and gently homogenize this mixture to disperse the clots and release any trapped bacteria.

◩ **NOTE:** Do not grind the clots if a fungal culture is also requested for the sample. Tease the clots apart. Vigorous grinding can kill hyphal filaments.

B. Prepare Gram stain by placing 1 or 2 drops of fluid specimen on an alcohol-rinsed slide.
- **1.** Allow the drop(s) to form one large drop. Do not spread the fluid.
- **2.** Air dry the slide in a biosafety cabinet or covered on a slide warmer.
- **3.** Fix smear with methanol and stain (see procedure 3.2.1 for staining details).

◩ **NOTE:** The use of a cytocentrifuge for preparation of the Gram stain from clear aspirates or nonviscous body fluids is highly recommended (20).

C. Incubation
- **1.** Incubate plates at 35 to 37°C in 5% CO_2. Alternatively, use a CO_2-generating system to provide the proper atmosphere if a CO_2 incubator is unavailable.
- **2.** Incubate broths at 35 to 37°C in ambient air.

D. Save some specimen at 4°C for 1 week for further testing.

E. Gram stain
- **1.** Interpret Gram stains immediately.
- **2.** If positive
  - **a.** Notify the physician immediately.
  - **b.** For invasively collected specimens, refer to positive blood culture workup (Table 3.4.1–2) for additional tests that can be performed on the direct specimen.
  - **c.** *If mixed morphologies are seen in Gram stain, retrieve the specimen and inoculate selective aerobic (e.g., MAC and CNA) and anaerobic plates to select for mixed pathogens.*

F. Culture examination
- **1.** Examine all plated and broth media for macroscopic evidence of growth at 24 h.
- **2.** If no visible growth is observed on the culture media, reincubate.
  - **a.** Read aerobic plates daily for 4 days for invasively collected specimens and 2 days for drainage cultures.

    ◩ **NOTE:** For specific requests or where patient history or clinical state suggests a slow-growing pathogen (e.g., *Brucella*), additional incubation time is appropriate.
  - **b.** For broth cultures, refer to procedure 3.3.2 for general guidelines and incubation.
  - **c.** Incubate blood culture bottles for 5 to 7 days. Extend incubation if *Brucella* culture is requested.
- **3.** Cultures with growth
  - **a.** Notify physician of positive culture findings.
  - **b.** Correlate culture results with those of the direct Gram stain.
  - **c.** Identify all organisms, using the rapid tests listed in Table 3.3.2–5. For less common pathogens refer to procedures 3.18.1 and 3.18.2.
  - **d.** Do not perform complete identification if the physician indicates that the organism is a probable contaminant or that the isolate is one or two colonies of a coagulase-negative staphylococcus on one plate medium with no growth in the broth.

V. PROCEDURE *(continued)*

e. For peritoneal specimens that contain mixed gastrointestinal microbiota and no predominant organism, generally group organisms into "enteric," "anaerobic," and "skin" microbiota and do not identify further. However, screen the culture for the usual fecal pathogens (*see* procedures 3.8.1 and 3.8.2). Detection of yeasts, *S. aureus*, *Pseudomonas aeruginosa*, or, possibly, vancomycin-resistant enterococci may represent etiologies not covered by empiric regimens and should be listed individually.

◩ **NOTE:** Empirical antimicrobial therapies are selected for the treatment of gastrointestinal tract microbiota, including anaerobes, enteric gram-negative bacilli, and enterococci. To attempt to isolate and report each of these agents is labor-intensive and does not add to the requirement to treat the patient with agents that are effective against all the usual microbiota.

f. Perform antimicrobial susceptibility testing as appropriate per CLSI standards (7).

g. Hold positive culture plates or tubes for at least 7 days or, preferably, freeze isolates for long-term retrieval.

h. Isolates from broth cultures may be contaminants. Analysis should be directed by the clinical picture.

G. Broad-range PCR

Culture results for sterile body fluids may be falsely negative when fastidious or noncultivable bacteria are involved or when the patient has been treated prior to the start of antimicrobial therapy. Broad-range PCR that detects the 16S rRNA gene (i.e., conserved bacterial sequence) followed by nucleic acid sequencing for identification of the pathogen to the genus or species level may be helpful in these cases. Several broad-range PCR assays, including real-time quantitative assays, have been reported (11, 12, 16, 27). Real-time quantitative assays for broad-range PCR may improve the lower limits of molecular detection to the threshold required by this approach (i.e., sterile fluids may contain only 1 to 2 CFU/ml). In order to perform a broad-range PCR assay if a sterile fluid proves culture negative, a minimum 1- to 2-ml aliquot of the freshly collected sample should be aseptically pipetted into a sterile container at the time of specimen processing and frozen at −86°C.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Refer to procedure 3.3.2 for general reporting procedure.

B. When reporting negative results, indicate the incubation time in the report.

C. If a plate contaminant is suspected, add a notation: "Unable to differentiate contamination of plate media from true infection; suggest repeat culture of appropriately collected specimen."

D. Report the probable genus and species as soon as preliminary tests are completed. For mixed abdominal microbiota, a general statement listing the groups of organisms may be sufficient, e.g., "Numerous enteric rods, numerous mixed anaerobic microbiota, including *Clostridium perfringens* and *Staphylococcus aureus*, present."

E. Document notification of positive findings.

VII. INTERPRETATION

A. Generally, a positive culture indicates infection with the organism.

B. WBCs are usually present with infections of body fluids.

VIII. LIMITATIONS

A. False-positive cultures can result from contamination of the specimen with skin microbiota.

B. False-negative results can be caused by low numbers of organisms, prior antimicrobial treatment, or the fastidious nature of the infective organism.

C. Many organisms that cannot be easily cultured cause arthritis. These include *Borrelia burgdorferi*, the agent of Lyme disease.

REFERENCES

1. **Alfa, M. J., P. Degagne, N. Olson, and G. K. M. Harding.** 1997. Improved detection of bacterial growth in continuous ambulatory peritoneal dialysis effluent by the use of BacT/Alert FAN bottles. *J. Clin. Microbiol.* **35:**862–866.
2. **Atkins, B. L., N. Athanasou, J. J. Deeks, D. W. M. Crook, H. Simpson, T. E. A. Peto, P. Mclardy-Smith, A. R. Berendt, and the Osiris Collaborative Study Group.** 1998. Prospective evaluation of criteria for microbiological diagnosis of prosthetic-joint infection at revision arthroplasty. *J. Clin. Microbiol.* **36:**2932–2939.
3. **Bernard, L., B. Pron, A. Vuagnat, V. Gleizes, F. Signoret, P. Denormandie, A. Si-Ali, C. Perrone, J. M. Feron, J. L. Gaillard, and the Groupe d'Etude sur l'Ostéite.** 2002. The value of suction drainage fluid culture during aseptic and septic orthopedic surgery: a prospective study of 901 patients. *Clin. Infect. Dis.* **34:**46–49.
4. **Blondeau, J. M., G. B. Pylypchuk, J. E. Kappel, R. B. Baltzan, Y. Yaschuk, and A. J. Adolph.** 1995. Evaluation of aerobic Bactec 6A non-resin- and 16A resin-containing media for the recovery of microorganisms causing peritonitis. *Diagn. Microbiol. Infect. Dis.* **22:**361–368.
5. **Bourbeau, P., J. Riley, B. J. Heiter, R. Master, C. Young, and C. Pierson.** 1998. Use of the BacT/Alert blood culture system for culture of sterile body fluids other than blood. *J. Clin. Microbiol.* **36:**3273–3277.
6. **Brook, I., and E. H. Frazier.** 1993. Anaerobic osteomyelitis and arthritis in a military hospital: a 10-year experience. *Am. J. Med.* **94:**21–28.
7. **Clinical and Laboratory Standards Institute.** 2008. *Performance Standards for Antimicrobial Susceptibility Testing.* Eighteenth informational supplement. Approved standard M100-S18. Clinical and Laboratory Standards Institute, Wayne, PA.
8. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
9. **Everts, R. J., J. P. Heneghan, P. O. Adholla, and L. B. Reller.** 2001. Validity of cultures of fluid collected through drainage catheters versus those obtained by direct aspiration. *J. Clin. Microbiol.* **39:**66–68.
10. **Fuller, D. D., T. E. Davis, P. C. Kibsey, L. Rosmus, L. W. Ayers, M. Ott, M. A. Saubolle, and D. L. Sewell.** 1994. Comparison of BACTEC Plus 26 and 27 media with and without fastidious organism supplement with conventional methods for culture of sterile body fluids. *J. Clin. Microbiol.* **32:**1488–1491.
11. **Goldenberger, D., A. Kunzli, P. Vogt, R. Zbinden, and M. Altwegg.** 1997. Molecular diagnosis of bacterial endocarditis by broad-range PCR amplification and direct sequencing. *J. Clin. Microbiol.* **35:**4759–4764.
12. **Harris, K. A., and J. C. Hartley.** 2003. Development of broad-range 16S rRNA PCR for use in the routine diagnostic clinical microbiology service. *J. Med. Microbiol.* **52:**685–691.
13. **Lejbkowicz, F., L. Cohn, N. Hashman, and I. Kassis.** 1999. Recovery of *Kingella kingae* from blood and synovial fluid of two pediatric patients by using the BacT/Alert system. *J. Clin. Microbiol.* **37:**878. (Letter.)
14. **Morris, A. J., S. J. Wilson, C. E. Marx, M. L. Wilson, S. Mirrett, and L. B. Reller.** 1995. Clinical impact of bacteria and fungi recovered only from broth cultures. *J. Clin. Microbiol.* **33:**161–165.
15. **Nakata, M. M., and R. P. Lewis.** 1984. Anaerobic bacteria in bone and joint infections. *Rev. Infect. Dis.* **6**(Suppl. 1)**:**S165–S170.
16. **Rantakokko-Jalava, K., S. Nikkari, J. Jalava, E. Eerola, M. Skurnik, O. Meurman, O. Ruuskanen, A. Alanen, E. Kotilainen, P. Toivanen, and P. Kotilainen.** 2000. Direct amplification of rRNA genes in diagnosis of bacterial infection. *J. Clin. Microbiol.* **38:**32–39.
17. **Rinehold, C. E., D. J. Nickolai, T. E. Piccinni, B. A. Byford, M. K. York, and G. F. Brooks.** 1988. Evaluation of broth media for routine culture of cerebrospinal and joint fluid specimens. *Am. J. Clin. Pathol.* **89:**671–674.
18. **Runyon, B. A., M. R. Antillon, E. A. Akriviadis, and J. G. McHutchison.** 1990. Bedside inoculation of blood culture bottles with ascitic fluid is superior to delayed inoculation in the detection of spontaneous bacterial peritonitis. *J. Clin. Microbiol.* **28:**2811–2812.
19. **Scythes, K. D., M. Louis, and A. E. Simor.** 1996. Evaluation of nutritive capacities of 10 broth media. *J. Clin. Microbiol.* **34:**1804–1807.
20. **Shanholtzer, C. J., P. J. Schaper, and L. R. Peterson.** 1982. Concentrated Gram stain smears prepared with a cytospin centrifuge. *J. Clin. Microbiol.* **16:**1052–1056.

REFERENCES *(continued)*

21. **Siersema, P. D., S. de Marie, J. H. van Zeijl, D. J. Bac, and J. H. Wilson.** 1992. Blood culture bottles are superior to lysis-centrifugation tubes for bacteriological diagnosis of spontaneous bacterial peritonitis. *J. Clin. Microbiol.* **30:**667–669.
22. **von Essen, R., and A. Holtta.** 1986. Improved method of isolating bacteria from joint fluids by the use of blood culture bottles. *Ann. Rheum. Dis.* **45:**454–457.
23. **von Graevenitz, A., and D. Amsterdam.** 1992. Microbiological aspects of peritonitis associated with continuous ambulatory peritoneal dialysis. *Clin. Microbiol. Rev.* **5:**36–48.
24. **Yagupsky, P.** 1999. Use of blood culture systems for isolation of *Kingella kingae* from synovial fluid. *J. Clin. Microbiol.* **37:**3785. (Letter.)
25. **Yagupsky, P., N. Peled, and J. Press.** 2001. Use of BACTEC 9240 blood culture system for detection of *Brucella melitensis* in synovial fluid. *J. Clin. Microbiol.* **39:**738–739.
26. **Yagupsky, P., and J. Press.** 1997. Use of the Isolator 1.5 microbial tube for culture of synovial fluid from patients with septic arthritis. *J. Clin. Microbiol.* **35:**2410–2412.
27. **Zucol, F., R. A. Ammann, C. Berger, C. Aebi, M. Altwegg, F. K. Niggli, and D. Nadal.** 2006. Real-time quantitative broad-range PCR assay for detection of the 16S rRNA gene followed by sequencing for species identification. *J. Clin. Microbiol.* **44:**2750–2759.

SUPPLEMENTAL READING

Smith, J. W., and E. A. Pierey. 1995. Infectious arthritis. *Clin. Infect. Dis.* **20:**225–231.

3.6 Catheter Tip Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Intravascular (intra-arterial or intravenous) catheter insertions cause a break in the skin barrier amenable to infection. Table 3.6–1 lists the most common types of vascular and hemodialysis access catheters used for patient care that the laboratory may encounter. The continued presence of this foreign body predisposes further to infection, which can result from either colonization of the catheter by the cutaneous microbiota or, less commonly, hematogenous seeding due to hub contamination. Since infected catheters are usually exposed directly to sterile spaces, there is a risk that the infection will result in bacteremia. Intravascular catheter-related infections are a major cause of morbidity and mortality in the United States. The Infectious Disease Society of America practice guidelines for management of these infections include culture of both catheters and blood (9). Central catheter infection may manifest as infection at the skin insertion site, as cellulitis along the soft tissues overlying the tunneled portion, or as bacteremia without evidence of external infection at either of these superficial sites. Bacteremia occurs secondary to infection of the central catheter or as a manifestation of more serious complications, including septic thrombophlebitis or endocarditis. Laboratory confirmation of central catheter infection requires recovery of the same organism from a patient's blood and from cultures of the catheter tip and/or intracutaneous catheter segment. Clinical policy should instruct physicians to submit both catheter segments and blood cultures to the laboratory. The most common infecting organisms are *Staphylococcus aureus,* enterococci, *Candida* spp., *Pseudomonas aeruginosa, Enterobacteriaceae,* and resident skin organisms, such as coagulase-negative staphylococci and *Corynebacterium* spp. The significance of this last group of organisms is not always clear, since the catheter is removed through the skin.

Several methods have been used to diagnose catheter-related sepsis. Semiquantitative (roll plate) cultures, catheter flush cultures, and quantitative (sonication) cultures are more reliable diagnostic methods than qualitative cultures, where the tip is cultured in broth and a single contaminating microbe can give a positive result (9, 14). The semiquantitative technique is reported to distinguish infection from contamination, with counts of $\geq$15 CFU considered significant (7). The quantitative method and flush culture method use $\geq 10^2$ CFU as the significant count for most organisms. However, lower counts of particular organisms, including *S. aureus* and *Candida albicans,* may be clinically significant if there is associated bacteremia (13). If such counts are accompanied by signs of local or systemic infection, they are indicative of catheter-related infection (9). Quantitative and semiquantitative methods have high specificity in the diagnosis of catheter-related septicemia, but the quantitative sonication method is reported to be 20% more sensitive than the semiquantitative culture method in the diagnosis of catheter-related bloodstream infection (12). Quantitation with sonication (*see* Appendix 3.6–1) is thought to be more sensitive because it is able to detect lumen colonization. At this time it is unclear whether the greater sensitivity of the quantitative method is clinically significant (9). Most laboratories perform the semiquantitative method, because of its ease of use and because there is a lack of scientific studies showing that the quantitative method offers added benefit.

The submission of two blood cultures (one peripheral and one through the catheter) is an alternate method to diagnose catheter-related bacteremia without removal of the catheter. A blood culture from a peripheral site and a blood culture through the catheter are submitted simultaneously, for comparison of time to positivity. If the culture through the catheter is positive $>$2 h before the peripheral culture is positive with the same microorganism, it is likely that the infection is catheter related (1, 8). Quantitative blood cultures can also be performed using the ISOLATOR system (Appendix 3.4.1–2). If the blood collected through the catheter has a count that is 5- to 10-fold greater than the count of the same microorganism from the peripheral vein, there is evidence of catheter-related sepsis (9). Alternatively, for staphylococci, comparison of the clone from the catheter and the peripheral site can also predict catheter-related sepsis. (*See* procedure 13.12 for further information on catheter cultures from an infection control perspective.)

Table 3.6–1 Common types of vascular and hemodialysis access catheters[a]

Vascular access devices	Comments
Arterial line	Catheter usually inserted into the radial artery for continuous blood pressure monitoring in ICU; other arteries may be used, including femoral, pedal, or carotid. Arterial lines are used for blood draws for arterial blood gas monitoring.
Broviac catheter	A type of tunnelled CVC that is positioned just above the heart but tunnelled under the skin and brought out on the chest and thigh away from the site where it enters the vein. A small cuff is located around the catheter about 1 in. inside the skin at the catheter exit site; it helps to anchor the catheter during long-term use and prevents infections. Broviac and Hickman catheters are similar types of CVCs. Commonly used for oncology patients receiving chemotherapy.
CVC	A central venous access catheter placed into a large vein in the neck (internal jugular), chest (subclavian vein), or groin (femoral vein) and used to give transfusions, draw blood for tests, and administer medications, fluids, and TPN solutions. A single line may provide one or more of these treatment options depending upon the number of lumens available. Specialized large-bore CVC lines also provide venous access for hemodialysis.
Cook catheter	A single-lumen PICC line that is made of minocycline/rifampin-impregnated silicone (http://www.cookmedical.com)
Cordis	Term may be used interchangeably for (i) a CVC line holder device that sits in the vein and comes out through the skin through which the line is threaded and (ii) Cordis (Johnson & Johnson) (http://www.cordis.com) makes many types of stents, including coronary artery stents.
Double lumen	A type of central venous line with two channels used for blood draws and administration of medications
Fogarty catheter	Arterial embolectomy catheter used to remove fresh, soft emboli and thrombi from arteries
Gortex graft	Gortex stretch vascular grafts are used in many vascular surgery procedures to replace parts of an artery or create a prosthetic connection between an artery and vein (e.g., arteriovenous fistula for hemodialysis).
Heparin lock for CVC	Central venous line lumens must be heparin sodium locked when not in use or after each intermittent medication administration to ensure patency. The lumen extension (pigtail) is heparin flushed (sodium heparin, 50 U/50 ml per pigtail), and the lumen is capped with a yellow Luer-lok cap after each use.
Hickman catheter	A type of tunnelled CVC that is positioned just above the heart but tunnelled under the skin and brought out on the chest and thigh away from the site where it enters the vein. A small cuff is located around the catheter about 1 in. inside the skin at the catheter exit site; it helps to anchor the catheter during long-term use and prevents infections. Hickman and Broviac catheters are similar types of CVCs. Commonly used for oncology patients receiving chemotherapy.
IJ line	A CVC that is inserted into the internal jugular vein
PICC	A PICC (or PICC line) is used for prolonged intravenous access (e.g., chemotherapy, extended antimicrobial therapy, or TPN). PICC lines are inserted into a peripheral vein such as the cephalic, basilic, or brachial veins and advanced through increasingly larger veins toward the heart until the tip rests in the distal superior vena cava or cavoatrial junction. Used as an alternative to central venous lines, which have a higher rate of infection. "Power" PICC lines are designed to withstand the high pressures associated with radiocontrast studies and are used in radiology.
Port-A-Cath	A type of CVC where the port is implanted entirely under the skin, usually on the upper chest above the heart. Some implanted ports contain a small reservoir that can be refilled by injection through the skin into the catheter. Medications are then slowly released into the bloodstream from the reservoir. Port-A-Caths have the same function as a PICC or tunnelled CVC.
Scribner shunt	An artificial Teflon arterial-venous U-shaped connector used for hemodialysis access
Sheldon catheter	A large-bore CVC inserted for hemodialysis access or access for large-volume leukapharesis
Swan-Ganz catheter	Pulmonary artery catheters inserted in ICU patients to directly and simultaneously measure the pressures of the right atrium, right ventricle, and pulmonary artery and the filling or "wedge" pressure of the left atrium. Those measurements allow an accurate clinical monitoring of heart failure and allow fluid and drug therapy of a variety of conditions that cause shock (sepsis, hypovolemia from acute trauma, etc.).
Triple lumen	A type of central venous line with three channels used for transfusions, blood draws, and administration of medications and TPN
Udall catheter	A large-bore CVC placed for hemodialysis access
UVC	Single-, double-, and triple-lumen catheters (ARGYLE) (http://www.kendall-ltp.com) inserted into the umbilical vein of premature infants in neonatal ICUs

[a] ICU, intensive care unit; CVC, central venous catheter; TPN, total parenteral nutrition; PICC, peripherally inserted central catheter; IJ, internal jugular; UVC, umbilical vein catheter.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

◩ **NOTE:** Refer to procedure 3.3.1 for additional details. This portion can be copied for preparation of a specimen collection instruction manual for caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

A. Collect two blood cultures, one through the catheter and one from a peripheral site at the time the catheter is submitted for culture.

B. Clean the skin with 70% alcohol prior to catheter removal.

C. Observing aseptic technique, hold the exposed end of the catheter and carefully remove the catheter from the patient with a sterile instrument, taking care to avoid contact with exposed skin. Holding the distal end over a sterile container, cut the tip with a sterile scissors, dropping the last 2 to 3 in. into the container.

D. Avoid drying by sealing the tube and submit to the laboratory as soon as possible.

E. Rejection criteria

1. Do not accept urinary Foley catheter tips, chest tube tips, or abdominal drainage tips.

2. Catheter tips should not be placed in saline or transport medium.

3. Smears of catheter tips add little to the diagnosis of catheter-related sepsis.

4. Submit catheter tips for culture only if there are signs of infection, i.e., inflammation at the insertion site, fever, signs of sepsis, or documented bacteremia in which the source is not apparent. Patients suspected of having a central line infection should also have blood cultures done (3).

5. For ventricular-peritoneal shunts, peritoneal or spinal fluid is preferred to the catheter tip. If tip is submitted, culture it, but request submission of fluid. Refer to procedure 3.7 for workup of such cultures.

6. If there is evidence of pus and local tissue infection at the central catheter insertion site, the skin surface should be cleansed of debris and pus collected from the deep wound using a sterile swab. There is rarely enough pus at the catheter exit site to collect an adequate sample by aspiration. Aspirates of pus or fluid expressed from an infected subcutaneous catheter tunnel track may be sent.

III. MATERIALS

A. BAP

B. MAC or EMB (optional)

C. Forceps

D. Olive oil
Decant olive oil into a sterile container for use, but there is no need to sterilize the oil.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Verify that media meet expiration date and QC parameters per Clinical and Laboratory Standards Institute (formerly NCCLS) document M22-A3 (4). See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

V. PROCEDURE

Observe standard precautions.

Semiquantitative method

A. Using a sterile forceps, remove catheter tip from transport tube.

B. Lay catheter tip on BAP.

C. Roll the tip back and forth across the entire surface of a BAP (and, optionally, either MAC or EMB, in addition to the BAP) using sterile forceps and exerting slight downward pressure.

D. If the tip is too long, using sterile scissors, cut the end closest to the top of the tube (proximal end) prior to rolling on the plates (Fig. 3.6–1). The proximal end may be rolled on a second plate, if desired.

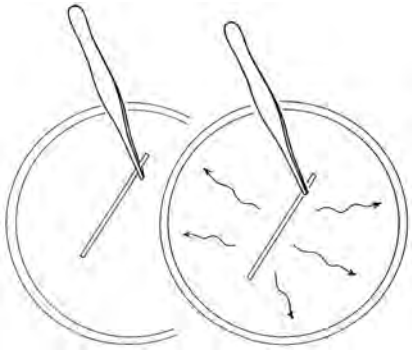


Figure 3.6–1 Inoculation of catheter tip to agar plate.

V. PROCEDURE *(continued)*

E. If the specimen is from the total parenteral nutrition or is the catheter tip from a hyperalimentation line, culture also for *Malassezia furfur*.
  1. Using a sterile pipette, add a small drop of olive oil to the initial area of roll after inoculation of the blood plate.
  2. Do not allow the oil to spread beyond a small area of the plate.

◩ **NOTE:** The diagnosis of catheter infection with *M. furfur* is difficult with most blood culture systems. However, the organism load in the catheter is high and a Gram stain of a drop of blood from the catheter hub will generally demonstrate the infecting organisms, without removal of the catheter.

F. Incubate plates at 35°C in CO_2.

G. Certain types of catheter tips require prolonged incubation. Cultures from central line and vascular catheter tips should be held for up to 4 days, mainly to look for yeasts, including *M. furfur*, which grow as pinpoint colonies in 3 days. Cultures from central nervous system shunt tips (e.g., ventriculoperitoneal and ventriculoatrial shunts) should be incubated for up to 14 days in order to recover important slow-growing pathogens such as *Propionibacterium acnes*.

H. Read the semiquantitative plates at 24, 48, 72, and 96 h.
  1. Count each type of colony isolated, comparing growth on each medium. Only enumerate the growth on the BAP; MAC or EMB is used only to provide separation of colony types.
  2. Identify to at least the genus level any of the following.
    a. Each organism present from vascular catheter tips with colony counts of >15 CFU, including gram-positive rods.
    b. For counts of <15 CFU, identify only significant pathogens (e.g., *Candida albicans*, group A streptococci, and gram-negative rods).
  3. Save plates with growth for 1 week for comparison in case blood cultures become positive.
  4. Antimicrobial susceptibility testing should be performed on all significant isolates from vascular catheters or grafts and central nervous system shunts even when the same organism is identified from the patient's blood. Since catheter tip infections are due to the formation of a biofilm on the device, different morphotypes of the same organism (e.g., two or more species of coagulase-negative *Staphylococcus*) may be recovered from the blood and the device. These morphotypes may have different antimicrobial susceptibility profiles, and antimicrobial therapy should be directed against the most resistant isolate.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. For any morphotype with a count of >15 CFU
   1. Enter the number of CFU isolated for each organism; i.e., 18 CFU followed by the organism name, at least to the genus level.
   ◩ **NOTE:** Organisms are *not* enumerated in catheter tip cultures as few, moderate, and numerous.
   2. For gram-positive rods, report as "Coryneform rods; not otherwise specified" if reactions are appropriate per Table 3.3.2–5.
   3. If different morphologies of coagulase-negative staphylococci are present, report "[number] CFU of coagulase-negative staphylococci (mixed morphologies)."

B. If any morphotype has <15 CFU
   1. Report significant pathogens by name.
   2. Group together mixed skin microbiota organisms (i.e., coagulase-negative *Staphylococcus*, diphtheroids, non-*Candida albicans* yeasts, *Acinetobacter*, or viridans group streptococci) as, e.g., "25 CFU of mixed skin microbiota."
   3. Report minimal identification of pure cultures of skin microbiota, e.g., staphylococci or gram-positive rods.

C. If organisms are too numerous to count, report as ">100 CFU."

D. Report preliminary negative cultures as "No growth at *x* days," where "*x*" is the number of days of incubation. Report final negative cultures as, e.g., "No growth at 4 days."

E. If gram-negative rods or *S. aureus* is isolated and no blood culture was submitted, add the following note to the report: "Submit blood cultures to diagnose catheter-related sepsis."

VII. INTERPRETATION

A. Antimicrobial susceptibility testing should be performed on isolates both from blood and from the catheter tip and other segments submitted to the laboratory for culture. Different bacterial morphotypes isolated from the blood and the catheter may only be recognized by variations in their antibiogram profiles. If a central catheter cannot be immediately removed for clinical reasons (e.g., risk of bleeding), then antimicrobial therapy is directed against the antimicrobial susceptibility profile of the blood isolate. In cases where the antimicrobial profiles of the blood and catheter isolate(s) are different, then antimicrobial therapy is directed against the most resistant morphotype isolated from either site.

B. The presence of >15 CFU suggests the catheter as a potential source of bacteremia, which occurs with about 10% of colonized catheters.

VIII. LIMITATIONS

A. Semiquantitative catheter tip cultures are estimated to have a sensitivity of 85% in diagnosis of catheter-related bacteremia, but the specificity to diagnose catheter-related sepsis is low (14). Blood cultures collected from peripheral sites are helpful in confirmation of the diagnosis of catheter-related sepsis (1, 5, 7).

B. Infections of the catheter hub lumen may be missed by culture of only the tip.

C. Efforts to diagnose catheter-related sepsis using unpaired quantitative blood cultures drawn from the catheter are less sensitive than tip cultures (14).

D. Catheter cultures can be helpful in determination of the cause of fever.

E. Some authors have shown that quantitative catheter tip cultures have a greater sensitivity and specificity than the semiquantitative method of Maki et al. (7) in the diagnosis of catheter-related sepsis; however, these methods are more labor-intensive (2, 6, 10, 12–14). See Appendix 3.6–1 for method.

VIII. LIMITATIONS *(continued)*

F. Siegman-Igra et al. (14) suggest that antimicrobial treatment of patients with positive catheter tip cultures but negative blood cultures increases medical costs unnecessarily, because of the low specificity of catheter tip cultures. For this reason, susceptibility testing of catheter tip isolates is not indicated. If concomitant blood cultures are positive, antimicrobial testing of the blood isolate is appropriate. Exceptions include performing antimicrobial testing when specifically requested by a physician and when testing the catheter tip isolate would provide more rapid results than waiting for the companion blood culture isolate to be tested (9).

G. In simulated studies, rolling catheters, impregnated with antiseptics, on a blood plate inhibited the ability of the organisms to grow on the agar (11). This suggests that alternative methods must be found to diagnose infections in antiseptic-impregnated catheters.

REFERENCES

1. **Blot, F., G. Nitenberg, E. Chachaty, B. Raynard, N. Germann, S. Antoun, A. Laplanche, C. Brun-Buisson, and C. Tancrede.** 1999. Diagnosis of catheter-related bacteremia: a prospective comparison of the time to positivity of hub-blood versus peripheral-blood cultures. *Lancet* **354:**1071–1077.
2. **Brun-Buisson, C., F. Abrouk, P. Legrand, Y. Huet, S. Larabi, and M. Rapin.** 1987. Diagnosis of central venous catheter-related sepsis. Critical level of quantitative tip cultures. *Arch. Intern. Med.* **147:**873–877.
3. **Centers for Disease Control and Prevention.** 2002. Guidelines for the prevention of intravascular catheter-related infections. *MMWR Morb. Mortal. Wkly. Rep.* **51**(RR-10)**:**1–26.
4. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Dooley, D. P., A. Garcia, J. W. Kelly, R. N. Longfield, and L. Harrison.** 1996. Validation of catheter semiquantitative culture technique for nonstaphylococcal organisms. *J. Clin. Microbiol.* **34:**409–412.
6. **Gutierrez, J., C. Leon, R. Matamoros, C. Nogales, and E. Martin.** 1992. Catheter-related bacteremia and fungemia. Reliability of two methods for catheter culture. *Diagn. Microbiol. Infect. Dis.* **15:**575–578.
7. **Maki, D. G., C. E. Weise, and H. W. Sarafin.** 1977. A semiquantitative culture method for identifying intravenous catheter-related infection. *N. Engl. J. Med.* **296:**1305–1309.
8. **Malgrange, V. B., M. C. Escande, and S. Theobald.** 2001. Validity of earlier positivity of central venous blood cultures in comparison with peripheral blood cultures for diagnosing catheter-related bacteremia in cancer patients. *J. Clin. Microbiol.* **39:**274–278.
9. **Mermel, L. A., B. M. Farr, R. J. Sherertz, I. I. Raad, N. O'Grady, J. S. Harris, and D. E. Craven.** 2001. Guidelines for the management of intravascular catheter related infections. *Clin. Infect. Dis.* **32:**1249–1272.
10. **Raad, I. I., M. F. Sabbagh, K. H. Rand, and R. J. Sherertz.** 1992. Quantitative tip culture methods and the diagnosis of central venous catheter-related infections. *Diagn. Microbiol. Infect. Dis.* **15:**13–20.
11. **Schmitt, S. K., C. Knapp, G. S. Hall, D. L. Longworth, J. T. McMahon, and J. A. Washington.** 1996. Impact of chlorhexidine-silver sulfadiazine-impregnated central venous catheters on in vitro quantitation of catheter-associated bacteria. *J. Clin. Microbiol.* **34:**508–511.
12. **Sherertz, R. J., S. O. Heard, and I. I. Raad.** 1997. Diagnosis of triple-lumen catheter infection: comparison of roll plate, sonication, and flushing methodologies. *J. Clin. Microbiol.* **35:**641–646.
13. **Sherertz, R. J., I. I. Raad, A. Belani, L. C. Koo, K. H. Rand, D. L. Pickett, S. A. Straub, and L. L. Fauerbach.** 1990. Three-year experience with sonicated vascular catheter cultures in a clinical microbiology laboratory. *J. Clin. Microbiol.* **28:**76–82.
14. **Siegman-Igra, Y., A. M. Anglim, D. E. Shapiro, K. A. Adal, B. A. Strain, and B. M. Farr.** 1997. Diagnosis of vascular catheter-related bloodstream infection: a meta-analysis. *J. Clin. Microbiol.* **35:**928–936.

APPENDIX 3.6–1

Sonication Method for Culture of Catheter Tips (1)

I. PROCEDURE

A. Place catheter tip in 10 ml of TSB.
B. Sonicate for 1 min at 55,000 Hz and 125 W.
C. Vortex for 15 s.
D. Add 0.1 ml of broth to 9.9 ml of saline. Vortex.
E. Drop 0.1 ml of broth and 0.1 ml of saline suspension onto separate BAP and MAC (or EMB). Spread with spreader (Excel Scientific, Rightwood, CA).
F. Incubate for 48 h in 5% CO_2 and count colonies.

II. INTERPRETATION

A. Multiply the number of colonies on the broth culture plate by 10^2 CFU.
B. If too numerous to count, multiply the number of colonies on the saline culture plate by 10^4 CFU.
C. A count of greater than 10^2 CFU is considered significant for catheter-related infection.

III. REPORTING RESULTS

A. Report the genus and species of organisms present preceded by their count in CFU. If organisms are too numerous to count on the higher dilution plate, report as "Greater than 10^6 CFU." See item VI in procedure 3.6 for further details.
B. If no organisms are present, report "No growth at 1:100 dilution."

IV. LIMITATION

Although this method is more reliable than the roll plate method (2), it is not clear whether the difference is clinically significant. Blood cultures remain a very important part of detection of catheter-related sepsis.

References

1. **Sherertz, R. J., I. I. Raad, A. Belani, L. C. Koo, K. H. Rand, D. L. Pickett, S. A. Straub, and L. L. Fauerbach.** 1990. Three-year experience with sonicated vascular catheter cultures in a clinical microbiology laboratory. *J. Clin. Microbiol.* **28:**76–82.
2. **Siegman-Igra, Y., A. M. Anglim, D. E. Shapiro, K. A. Adal, B. A. Strain, and B. M. Farr.** 1997. Diagnosis of vascular catheter-related bloodstream infection: a meta-analysis. *J. Clin. Microbiol.* **35:**928–936.

3.7 Cerebrospinal Fluid Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Bacterial meningitis is the result of infection of the meninges. Identification of the infecting agents is one of the most important functions of the diagnostic microbiology laboratory because acute meningitis is life-threatening. *CSF from a patient suspected of meningitis is an emergency specimen that requires immediate processing to determine the etiologic agent.*

CSF is obtained by transcutaneous aspiration, and therefore, all organisms recovered from the culture are potential pathogens and must be reported to the physician immediately. Because the number of organisms in the CSF can be as low as 10^3 CFU/ml (1, 8), concentration of the Gram stain by cytocentrifugation is important for rapid diagnosis. Cytospin concentration can increase the sensitivity up to 100-fold compared with both uncentrifuged and conventional centrifuged fluid (14). Concentration for culture is unnecessary, since the plate inoculum is sufficient to detect the usual low numbers of organisms (5, 8, 11).

Aerobic bacteria commonly cause bacterial meningitis (Table 3.7–1), but anaerobes may be present in CSF when a meningeal abscess or a similar infectious process is adjacent to the meninges. These include traumatic head injury or prostheses, such as metal cranial plates and shunt drains. Inoculation of anaerobic media is not recommended for diagnosis of community-acquired meningitis. Because slow-growing anaerobic organisms such as *Propionibacterium acnes* are common pathogens of central nervous system shunt infections, anaerobic cultures of CSF should be incubated for a minimum of 7 days; the addition of a broth culture that will grow aerobes and anaerobes may enhance isolation in low-number infections (5, 10). However, growth of low-number contaminants is also enhanced by CSF broth culture, so specific comments should be appended to laboratory reports for isolates from CSF in broth only to aid the physician in interpreting the clinical relevance of such isolates (10).

Direct antigen testing is rarely performed because the cost-benefit is low. The sensitivity of testing for certain serogroups of *Neisseria meningitidis* is poor. While the sensitivity of testing for *Haemophilus influenzae* serogroup B is high, the disease has virtually disappeared in countries with active neonatal vaccination programs. For streptococcal and *Escherichia coli* K-12 infections, the Gram stain is usually positive, except possibly in cases of partially treated meningitis. To maintain reagents for the latter indication is of low benefit. In a study of 103 episodes of meningitis, antigen tests had a sensitivity of 9% for Gram stain-negative specimens, with a 33% sensitivity overall (7).

Table 3.7–1 Common bacterial organisms causing acute meningitis by age or condition

Age or condition	Organism(s)
Neonate	*E. coli*, *S. agalactiae* (group B streptococci), *Listeria monocytogenes*
<2 mo	*S. agalactiae*, *L. monocytogenes*, *E. coli*
<10 yr	*H. influenzae*, *S. pneumoniae*, *N. meningitidis*
Young adult	*N. meningitidis*
Adult	*S. pneumoniae*, *N. meningitidis*
Elderly	*S. pneumoniae*, gram-negative bacilli, *L. monocytogenes*
Central nervous system shunt infection/ventriculitis	CoNS, *S. aureus*, *Corynebacterium* spp., *Propionibacterium acnes*, aerobic gram-negative bacilli, other anaerobes

PCR is becoming the method of choice for rapid, sensitive diagnosis of meningitis, especially for organisms that are present in low numbers or for organisms that are difficult to grow, particularly when the patient is partially treated with antimicrobial agents, e.g., *Borrelia, Ehrlichia, Mycobacterium tuberculosis, Mycoplasma,* and *Streptococcus pneumoniae* (4, 6, 9). Unfortunately, many of these tests are not available commercially.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

NOTE: Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and other caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

Observe standard precautions.

A. Specimen collection

NOTE: This is a medical procedure that is performed by a physician guided by appropriate precautions.

1. Lumbar puncture

- **a.** Disinfect the puncture site with antiseptic solution and alcohol in a manner identical to phebotomy skin preparation for blood culture to prevent specimen contamination and introduction of infection.
- **b.** Insert a needle with stylet at the L3-L4, L4-L5, or L5-S1 interspace. When the subarachnoid space is reached, remove the stylet; spinal fluid will appear in the needle hub.
- **c.** Measure the hydrostatic pressure with a manometer.
- **d.** Sequentially collect the CSF into five calibrated sterile tubes labeled no. 1 to no. 5.
- **e.** Physicians should be instructed to sequentially collect 2.0 ml of CSF into three sterile calibrated tubes if only routine chemistry (total protein and glucose), bacteriology (C&S), and hematology (cell count) are required.
- **f.** Table 3.7–2 outlines the collection and test ordering guidelines that should be used by physicians who collect CSF samples from adults, children, and neonates. The laboratory should include specific collection guidelines for CSF samples in its guide to services, including the minimal volume of samples required to perform specific tests, including those that will be referred out. Up to five sequentially collected sterile calibrated tubes should be collected depending on the extent of CSF testing required. Physicians should be instructed to collect CSF into a fifth tube if a portion of the sample needs to be sent to a reference laboratory for specialized microbiology tests as outlined in Table 3.7–2.

2. Ommaya reservoir fluid or ventricular shunt fluid

- **a.** See Table 3.7–3 for a listing of central nervous system shunts.
- **b.** Clean the reservoir site with antiseptic solution and alcohol prior to removal of fluid to prevent introduction of infection.
- **c.** Remove fluid by aspiration of CSF from the Ommaya reservoir or by collection from the ventricular drain or shunt. Sequentially collect CSF into a minimum of three sterile calibrated tubes if only routine chemistry (total protein and glucose) (tube no. 1), bacteriology (C&S) (tube no. 2), and hematology (cell count) (tube no. 3) are required.

3. An initial CSF sample should be collected prior to antimicrobial therapy for highest diagnostic sensitivity. Subsequent CSF samples are then collected every 2 to 3 days once antimicrobial therapy is started to monitor for resolution of the infection.

Table 3.7–2 Collection and test ordering guidelines for CSF specimens

Collection tube no.	Laboratory tests performed	Minimum CSF vol (ml)/test for adults and children	Minimum CSF vol (ml)/test for neonates	Comments
1 (chemistry)	Total protein	0.2	0.1	Chemistry immediately performs total protein and glucose tests and splits the remaining CSF sample in tube no. 1 as required for the other tests.
	Glucose	0.2	0.1	
	Immunoglobulin index	0.2	NA[a]	
	Oligoclonal banding (electrophoresis)	3.0	NA	
2 (microbiology)	Bacterial culture and sensitivity (includes Gram stain)	1.0	0.5	Tube no. 2 should be transported STAT to the clinical microbiology laboratory. A minimum of 3.0 ml of CSF should be cultured for optimal recovery of fungi from this site. Up to three separate CSF samples may need to be submitted for fungal culture from patients suspected of chronic meningitis to definitively rule out a fungal etiology.
	Viral culture	0.5	0.2	
	Cryptococcal antigen	1.0	NA	
	Fungal culture (includes calcofluor white stain)	3.0	NA	
3 (microbiology)	AFB culture (includes special stains)	3.0	NA	AFB culture is usually done by a reference laboratory. A minimum of 3.0 ml of CSF should be cultured for optimal recovery of AFB from this site. Up to three separate CSF samples may need to be submitted for AFB culture from patients suspected of chronic meningitis to definitively rule out an AFB infection.
4 (hematology)	Cell count	1.0	0.5	Hematology immediately performs the cell count and splits the remaining CSF for distribution to the flow cytometry and cytology laboratories. CSF samples for flow cytometry and/or cytology testing must be transported STAT and be received by the laboratory within 1 h after collection. The CSF cytology sample is immediately placed in a sterile container with fixative (e.g., Cytolyt) if transport has been delayed. A separate portion of the CSF sample is placed into RPMI/TTM solution for flow cytometry.
	Flow cytometry	2.0–10.0	NA	
	Cytology	2.0		
5 (special pathogens)	Creutzfeldt-Jakob disease[b] (14-3-3 protein)	3.0	NA	These tests are usually performed by a reference laboratory. CSF samples for *Acanthamoeba* testing should be placed into a separate sterile calibrated tube containing Pages Amoeba saline and transported to the laboratory within 1 h of collection. CSF for anaerobic culture should be placed into a tube containing anaerobic transport medium (e.g., prereduced anaerobically sterilized media). Specialized viral tests should not be done unless the patient CSF total protein and the CSF WBC count are increased. CSF samples for Creutzfeldt-Jakob disease, West Nile virus, or viral PCR testing should each be collected into separate tubes. Common viral pathogens detected by PCR include herpesviruses (primarily herpes simplex virus) and enteroviruses.
	Viral PCR	1.0		
	West Nile virus	1.0		
	Acanthamoeba	10.0		
	Anaerobic culture (includes Gram stain)	1.0		

[a] NA, not applicable.

[b] See Appendix 3.7–2.

Table 3.7–3 Common types of central nervous system devices[a]

Type of CNS device	Comments
ICP monitor	An intraventricular catheter inserted into the ventricle that monitors intracranial pressure measurements that are recorded by an external device.
Ommaya reservoir	An intraventricular catheter system used to directly deliver drugs (e.g., chemotherapy) into the CNS
VA shunt	A catheter is placed into the ventricles, and CSF is removed (shunted) from the brain and drains into the heart to relieve elevated intracranial pressure in patients with hydrocephalus. CNS shunts are rarely placed in this manner.
VP shunt	A catheter is placed into the ventricles, and CSF is removed (shunted) from the brain and drains into the abdominal cavity to relieve elevated intracranial pressure in patients with hydrocephalus. Most CNS shunts are placed in this manner.

[a] CNS, central nervous system; ICP, intracranial pressure; VA, ventricular-atrial; VP, ventricular-peritoneal.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

B. Specimen transport

1. Submit to laboratory as soon as possible and alert laboratory that specimen is in transit.
2. Do not refrigerate.
3. Each sterile calibrated tube containing CSF must be properly labeled with the patient's name, birthdate, and/or unique identification number, and the date and time of collection. The laboratory requisition or electronic order entry system should also request physicians to indicate the site of collections including lumbar puncture, Ommaya reservoir, ventricular shunt, ventricular drain, or ventricular puncture.
4. Complete requisition with demographic and specimen collection information. Record the patient diagnosis for improved processing of specimen.
5. Limit requests to those reflecting patient's condition.

C. Rejection criteria

1. Call physician to prioritize requests if there is insufficient volume.

   ◩ **NOTE:** Fungal and acid-fast bacillus (AFB) cultures of the CSF are infrequently indicated in acute community-acquired meningitis. Refer to Appendix 3.7–1 for a sample laboratory policy to communicate to caregivers. Since the fungal pathogens in CSF *(Cryptococcus neoformans, Coccidioides immitis,* and *Histoplasma capsulatum)* are best and most rapidly diagnosed by serologic methods or cultures of other sites, fungal CSF culture should be discouraged. However, the fungi that cause CSF disease grow well on the media inoculated for routine culture. For fungal requests, incubate the routine culture plates for a longer period and inoculate a fungal broth (Sabouraud) with a large volume of specimen to increase the yield of *Cryptococcus* and *Coccidioides. M. tuberculosis* is best diagnosed by PCR. Refer to sections 7 and 8 of this handbook.
2. Specimens in leaky containers must be processed, but alert the physician of the possibility of contamination.
3. Direct antigen testing is not recommended.

III. MATERIALS

A. Media
- **1.** BAP
- **2.** CHOC
- **3.** MAC
- **4.** Broth known to support the growth of both fastidious aerobic organisms and anaerobes, such as anaerobic BHI or TSB with 0.1% agar and 5% sheep blood or fastidious anaerobe broth or fastidious broth (Quelab Laboratories, Inc., Montreal, Quebec, Canada; Remel, Inc., Lenexa, KS) (2, 12, 13).

  ◩ **NOTE:** Since THIO is mainly a broth for anaerobes and does not support the growth of the most common pathogens in CSF, it cannot be recommended for CSF culture (12, 13).
- **5.** See procedure 3.3.2 for common biochemical tests and media and procedure 3.17 for detailed procedures for biochemical tests.

B. Gram stain reagents

C. Other supplies
- **1.** Cytocentrifuge
- **2.** Sterile Pasteur pipettes

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that plate media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (formerly NCCLS) document M22. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

B. Periodically check laboratory processing to ensure that there is no break in sterile technique and that media, stain reagents, collection tubes, slides, and other supplies are not contaminated with dead organisms.

V. PROCEDURE

Observe standard precautions.

A. Inoculation
- **1.** Process specimen as soon as received.

  ◩ **NOTE:** Use of a biosafety cabinet will avoid contamination of the culture or specimen as well as protect laboratory processing personnel.
- **2.** Verify patient name on both label and requisition.
- **3.** Record the following.
  - **a.** Volume of CSF
  - **b.** Gross appearance of CSF, i.e., clear, bloody, cloudy, xanthochromic
- **4.** Inoculate media.
  - **a.** Using a sterile pipette, aspirate fluid from the bottom of the collection tube.
  - **b.** Place 2 or 3 drops each onto BAP, CHOC, and MAC. Streak in quadrants using a separate loop or flamed loop for streaking each plate.
  - **c.** If greater than 1 ml is available for routine culture from a ventricular source or shunt, inoculate 1 ml to broth. If the site of the collection is unknown, use the patient location as an aid to determination of the need for anaerobic culture or use of a backup broth.
    - (1) Specimens from the emergency department usually are from community-acquired meningitis.
    - (2) Specimens from the neurology service likely represent those requiring broth culture.
    - (3) If large volumes of specimen are received, they usually are from shunts or reservoirs.

V. PROCEDURE *(continued)*

5. If a CSF specimen tube appears to be empty or contains only 1 to 5 drops of fluid and if more specimen cannot be obtained sterilely from other laboratory departments, proceed as follows.
   a. If 1 drop is available, use a sterile Pasteur pipette and prepare a smear for Gram stain from a portion of the specimen.
   b. Using a sterile Pasteur pipette, place about 0.5 ml of broth medium into the specimen tube. Recap the tube, and invert it to mix the contents.
   c. Use all the broth to inoculate media and do smear.
   d. Note on report the volume received.

B. Gram stain
1. Place 5 or 6 drops of sample plus 1 drop of 37% formalin into a cytospin specimen chamber. Follow procedure for operation of centrifuge from manufacturer.
2. As an alternative, when the specimen is cloudy or the quantity is not sufficient for concentration, prepare the smear by placing 1 or 2 drops of CSF on an alcohol-rinsed slide.
   a. Allow the drop(s) to form one large drop. Do not spread the fluid.
   b. Air dry the slide in a biosafety cabinet or covered on a slide warmer.
3. Fix smear with methanol. (See procedure 3.2.1 for staining and reading smear.)
4. Interpret CSF Gram stains immediately.
5. Any bacteria seen are considered significant. However, confirm low numbers only seen in one or two fields with a second smear. If positive, do the following.
   a. Notify the physician immediately.
   b. If sufficient specimen is left, refer to blood culture workup of positive broths (Table 3.4.1–2) for additional tests that can be performed on the direct specimen.

C. Save some CSF at 4°C and/or at −20°C for 1 week for PCR or other subsequent requests.

D. Incubation
1. Incubate plates at 35 to 37°C in 5% CO_2. Alternatively, use a CO_2-generating system to provide the proper atmosphere if a CO_2 incubator is unavailable.
2. Incubate broths at 35 to 37°C in ambient air.

E. Culture examination
1. Examine all plate and broth media for macroscopic evidence of growth at 24 h.
2. If no visible growth is observed on the culture media, reincubate.
   a. Read aerobic plates daily for 4 days.
   b. If the Gram stain is positive and there is no growth on the plates or a fungal culture was ordered, hold all plates for at least 1 week.
   c. Examine broth media daily for 4 days and hold for 7 days before discarding.
   d. Refer to procedure 3.3.2 for general guidelines.
3. Cultures with growth
   a. Notify physician of positive culture findings.
   b. Identify all organisms, using the rapid tests listed in Table 3.3.2–5.
      (1) Perform rapid bile solubility spot test (procedure 3.17.6) on all alpha-hemolytic streptococci to identify *S. pneumoniae.* If positive, report *S. pneumoniae.*
      (2) Perform catalase and Gram stain of organisms growing on BAP and/or CHOC. Identify further according to Gram stain and rapid tests in Table 3.3.2–5. *Listeria* and group B streptococci are significant CSF pathogens.

V. PROCEDURE *(continued)*

It is imperative that these cultures be handled in a biological safety cabinet.

(3) Perform oxidase test on gram-negative diplococci. If positive and colony is grayish to white, perform commercial kit containing δ-glutamyl-aminopeptidase (*see* Table 3.18.2–1). If positive, report *N. meningitidis.*

(4) For all staphylococci, perform the tube coagulase for confirmation of slide or latex coagulase results.

(5) For less common pathogens, refer to procedures 3.18.1 and 3.18.2.

(6) *Generally determine the probable genus and usually the species identification of most CSF pathogens within 2 h of visible growth on the plates.*

c. Do not perform complete identification or antimicrobial susceptibility testing (AST) if the isolate is clearly a plate contaminant or the isolate is a coagulase-negative staphylococcus (CoNS) isolated from broth only.

◩ **NOTE:** Isolates of CoNS and *Corynebacterium* are probably contaminants in community-acquired infection but may or may not be a cause of infection in shunt infections and those with head injuries (5, 10, 11). A few colonies of catalase-positive, gram-positive rods growing only on CHOC should be subcultured to BAP to check hemolysis and rule out *Listeria* before being reported as corynebacteria.

d. Perform AST on enteric and nonfermenting gram-negative rods, enterococci, *S. pneumoniae, Staphylococcus aureus,* and other significant staphylococci.

(1) For *H. influenzae,* perform beta-lactamase test. Perform AST to penicillin or ampicillin if beta-lactamase test is negative and either agent will be used for therapy.

(2) For *Listeria, Streptococcus agalactiae,* and *N. meningitidis,* do not perform AST or beta-lactamase testing, which can lead to erroneous results. Resistance to penicillin in these isolates is rare; confirm any non-penicillin-susceptible isolates in a reference laboratory (3). For the penicillin-allergic patient, consult with the physician to guide AST.

e. Hold positive culture plates for at least 7 days or, preferably, freeze isolates for long-term retrieval.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report the Gram stain results as soon as possible, usually within 1 h of receipt.
B. Report probable genus and species as soon as preliminary tests are completed.
C. Refer to procedure 3.3.2 for general reporting.
D. Document notification of physician of positive findings.

VII. INTERPRETATION

A. Generally a positive culture indicates infection with the organism.
B. Lack of WBCs in CSF does not rule out infection, especially in listeriosis (7).
C. The most common cause of community-acquired bacterial meningitis is *S. pneumoniae.* Performance of the rapid bile solubility spot test (procedure 3.17.6) or the Quellung test (procedure 3.17.42) on all alpha-hemolytic streptococci seen in Gram stain is key to rapid diagnosis.
D. Isolation of enterococci from CSF is always a cause for concern. The presence of the organism may be an indication of strongyloidiasis.

VIII. LIMITATIONS

A. False-positive results can result from contamination of the specimen or the culture with skin microbiota.

B. False-negative results can be caused by low numbers of organisms, prior antimicrobial treatment, or the fastidious nature of the infective organism.

REFERENCES

1. **Bingen, E., N. Lambert-Zechovsky, P. Mariani-Kurkdjian, C. Doit, Y. Aujard, F. Fournieris, and H. Mathieu.** 1990. Bacterial counts in cerebrospinal fluid of children with meningitis. *Eur. J. Clin. Microbiol. Infect. Dis.* **9:**278–281.
2. **Cartwright, C. P., F. Stock, and V. J. Gill.** 1994. Improved enrichment broth for cultivation of fastidious organisms. *J. Clin. Microbiol.* **32:**1825–1826.
3. **Clinical and Laboratory Standards Institute.** 2008. *Performance Standards for Antimicrobial Susceptibility Testing.* Eighteenth informational supplement. Approved standard M100-18. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Dumler, J. S.** 2003. Algorithms for identification of *Mycoplasma, Ureaplasma,* and obligate intracellular bacteria, p. 349–353. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology,* 8th ed. ASM Press, Washington, DC.
5. **Dunbar, S. A., R. A. Eason, D. M. Musher, and J. E. Clarridge.** 1998. Microscopic examination and broth culture of cerebrospinal fluid in diagnosis of meningitis. *J. Clin. Microbiol.* **36:**1617–1620.
6. **Gillespie, S. H.** 1999. The role of the molecular laboratory in the investigation of *Streptococcus pneumoniae* infections. *Semin. Respir. Infect.* **14:**269–275.
7. **Hussein, A. S., and S. D. Shafran.** 2000. Acute bacterial meningitis in adults. A 12-year review. *Medicine* (Baltimore) **79:**360–368.
8. **La Scolea, L. J., Jr., and D. Dryja.** 1984. Quantitation of bacteria in cerebrospinal fluid and blood of children with meningitis and its diagnostic significance. *J. Clin. Microbiol.* **19:**187–190.
9. **Luft, B. J., C. R. Steinman, H. C. Neimark, B. Muralidhar, T. Rush, M. F. Finkel, M. Kunkel, and R. J. Dattwyler.** 1992. Invasion of the central nervous system by *Borrelia burgdorferi* in acute disseminated infection. *JAMA* **267:**1364–1367.
10. **Meredith, F. T., H. K. Phillips, and L. B. Reller.** 1997. Clinical utility of broth cultures of cerebrospinal fluid from patients at risk for shunt infections. *J. Clin. Microbiol.* **35:**3109–3111.
11. **Morris, A. J., S. J. Wilson, C. E. Marx, M. L. Wilson, S. Mirrett, and L. B. Reller.** 1995. Clinical impact of bacteria and fungi recovered only from broth cultures. *J. Clin. Microbiol.* **33:**161–165.
12. **Rinehold, C. E., D. J. Nickolai, T. E. Piccinni, B. A. Byford, M. K. York, and G. F. Brooks.** 1988. Evaluation of broth media for routine culture of cerebrospinal and joint fluid specimens. *Am. J. Clin. Pathol.* **89:**671–674.
13. **Scythes, K. D., M. Louis, and A. E. Simor.** 1996. Evaluation of nutritive capacities of 10 broth media. *J. Clin. Microbiol.* **34:**1804–1807.
14. **Shanholtzer, C. J., P. J. Schaper, and L. R. Peterson.** 1982. Concentrated gram stain smears prepared with a cytospin centrifuge. *J. Clin. Microbiol.* **16:**1052–1056.

SUPPLEMENTAL READING

Gray, L. D., and D. P. Fedorko. 1992. Laboratory diagnosis of bacterial meningitis. *Clin. Microbiol. Rev.* **5:**130–145.

Mandell, G. 1995. *Principles and Practice of Infectious Diseases,* 4th ed., p. 950–1136. Churchill Livingstone Inc., New York, NY.

Ray, C. G., J. A. Smith, B. L. Wasilauskas, and R. J. Zabransky. 1993. *Cumitech 14A, Laboratory Diagnosis of Central Nervous System Infections.* Coordinating ed., A. J. Smith. American Society for Microbiology, Washington, DC.

APPENDIX 3.7–1

Suggested Policy for Caregivers for Microbiological Examination of Cerebrospinal Fluid

I. ROUTINE MICROBIOLOGY CSF TESTS

The following tests should be routinely ordered to detect bacterial meningitis.

A. Total protein and glucose (chemistry)—tube no. 1

B. Bacterial C&S—the laboratory routinely performs a Gram stain using cytospin concentration when a bacterial culture is ordered—tube no. 2

C. All CSF samples will be held at 4°C for 7 days after receipt by the laboratory for further testing as clinically required—tube no. 3.

II. RATIONALE FOR SPECIALIZED MICROBIOLOGY CSF TESTS

A. Acute bacterial meningitis is a medically urgent condition that requires prompt institution of appropriate antimicrobial therapy. CSF abnormalities associated with this condition typically include an elevated total protein, decreased glucose compared to

APPENDIX 3.7–1 *(continued)*

the level in serum, an elevated count of WBCs that are predominantly polymorphonuclear cells, and a positive Gram stain and bacterial culture of the CSF. Since this is the most common type of infection, routine microbiology CSF tests should always include a bacterial culture. Although anaerobes rarely cause acute bacterial meningitis, anaerobic culture of the CSF should be done for patients with a parameningeal infection, particularly in cases where there has been leak or rupture into the cerebrospinal space (e.g., brain abscess, suppurative thrombophlebitis, or mycotic aneurysm). CSF should also be cultured for anaerobes, particularly *P. acnes,* for patients with central nervous system shunt infections.

B. Patients with acute aseptic meningitis due to a viral infection may present with a picture similar to that of patients with a bacterial infection. Viral tests should be done on the CSF if the total protein and WBC count are elevated and the CSF Gram stain and bacterial culture are negative. Enterovirus infection occurs during the late summer and fall months in most temperate climates. CSF viral culture or PCR for enteroviruses should be done during this period. West Nile virus is a flavivirus that is seasonally transmitted by infected mosquitoes that has recently been introduced to North America and causes acute meningitis or encephalitis in susceptible patients during the summer and early fall months. West Nile virus may be detected in the CSF using a combination of ELISA and PCR methods. Herpes simplex virus (HSV) encephalitis may produce hemorrhagic necrosis of the temporal lobe resulting in an increased CSF RBC count. PCR for HSV should be done for patients suspected of having meningitis or encephalitis.

C. Chronic meningitis due to yeast or fungal infection of the central nervous system is uncommon, and requests for specialized tests should be restricted to patients with exposure to systemic fungal pathogens (*C. immitis, H. capsulatum,* or *Blastomyces dermatitis*) and those who are immunocompromised. Cryptococcal meningitis is an opportunistic infection that indicates that a human immunodeficiency virus-infected patient has AIDS. Microbiology laboratories should be able to detect the presence of *Cryptococcus* on a CSF Gram stain, and a cryptococcal antigen (CRAG) test should routinely be done in this case and on CSF samples collected from immunocompromised patients. CRAG testing should also be routinely done whenever a yeast or fungal culture is ordered. The sensitivity of CRAG testing approaches 100% from serum and 98% from CSF in diagnosing disseminated cryptococcal infection. The sensitivity of the CSF India ink wet mount stain is only 50%, so laboratories should be performing CRAG tests. Although CRAG testing may not be performed routinely as a STAT procedure, it should be urgently done when *Cryptococcus* meningitis is clinically suspected in an immunocompromised patient and when *Cryptococcus* infection is suspected on the basis of CSF Gram stain or India ink wet mount stain. The sensitivity of CSF fungal culture for systemic mycosis agents is only ~30% even when large volumes of specimen (e.g., 10 ml) are cultured. Serology should be performed on the CSF for *C. immitis* since it has a sensitivity of 100%.

D. Chronic meningitis due to *Mycobacterium tuberculosis* infection of the central nervous system is also uncommon, and requests for AFB testing should be restricted to patients with a history of exposure or risk of *M. tuberculosis* infection and those with a positive purified protein derivation skin test, those with miliary tuberculosis (TB), and immunocompromised patients. The clinical symptoms that suggest TB meningitis include mental status changes over a period of time, chronic headache and neck stiffness, and cranial nerve palsy since the infection is mainly located along the base of the brain. Characteristic CSF abnormalities include an elevated total protein to the extent that CSF will form a protein precipitate of pellet in a tube, a decreased glucose compared to levels in serum, and an elevated WBC count that is predominantly lymphocytic. The sensitivity of AFB culture is only ~30% even if 10 ml of CSF is submitted for culture, and a result is not available for 4 to 8 weeks. The current recommendation is to perform a PCR assay if TB meningitis is suspected. The PCR TB assay has a sensitivity that approaches 100%, it requires a small sample volume (0.5 ml), and it can be performed within 2 to 4 days of receipt of the sample.

APPENDIX 3.7–1 *(continued)*

E. Acanthamoebic meningitis is a life-threatening condition with a high mortality rate. CSF tests for detection of *Acanthamoeba* should be done for patients who develop acute meningitis and who have a recent history of swimming in freshwater. Differentiation between *Naegleria fowleri* infection and *Acanthamoeba* infection requires wet mount examination and culture of a large volume of CSF.

APPENDIX 3.7–2

Laboratory Safety Practices for Handling CSF Samples from Patients Suspected or Confirmed To Have Creutzfeldt-Jakob Disease

Creutzfeldt-Jakob disease (CJD) is a transmissible spongiform encephalopathy (TME) caused by a prion (1). TME agents are highly resistant to heat, radiation, and chemical inactivation, so sterilization and disinfection procedures for prion-contaminated instruments and equipment are far more stringent than those for other types of infection. As yet there are no confirmed cases of laboratory-acquired infection, but the long incubation period for CJD makes it difficult to accurately associate exposure with disease. Due to the transmissible nature of prions, particularly in infected central nervous system tissues and fluids, special biosafety precautions should be taken in the handling and analysis of CSF or central nervous system tissue specimens from patients suspected or confirmed to have CJD (i.e., positive CSF test for 14-3-3 protein) as recommended by ISO 15190, the World Health Organization, and the CDC (2–4). The following precautions are recommended.

A. All handling and analysis procedures should be performed in a BSL 2 cabinet using BSL 3 procedures.
B. Aerosol production should be minimized. All tests ordered on CSF samples should be reviewed, and tests that would potentially require extensive manual manipulation (e.g., CSF immunoelectrophoresis) should not be done pending review of the CJD test result.
C. PPE should be worn by staff, including eye protection, gowns, and gloves.
D. Formalin-fixed tissues should be regarded as potentially infectious even after prolonged exposure.
E. Disposable instruments and equipment should be used because of the problems with disinfection. Central nervous system and neural or leptomeningeal tissue should not be ground.
F. All materials, equipment, and waste must be collected and decontaminated, by either autoclaving or treatment with hypochlorite to achieve a final concentration of 20,000 rpm. Decontamination is done with hypochlorite (20,000 rpm) for at least 1 h, and repeated wetting with the disinfectant is necessary over the entire decontamination period. Autoclaving should be carried out at 134°C for 18 min as a single cycle, or as six separate cycles of 3 min each at this temperature.

References

1. **Aguzzi, A., and M. Glatzel.** 2007. Transmissible spongiform encephalopathies, p. 1660–1666. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed., ASM Press, Washington, DC.
2. **Centers for Disease Control and Prevention and National Institutes of Health.** 2007. *Biosafety in Microbiological and Biomedical Laboratories*, 5th ed. U.S. Government Printing Office, Washington, DC.
3. **International Organization for Standardization.** 2003. *ISO 15190:2003. Medical Laboratories—Requirements for Safety*. International Organization for Standardization, Geneva, Switzerland. http://www.iso.org.
4. **World Health Organization.** 2003. *Laboratory Safety Manual.* World Health Organization, Geneva, Switzerland. http://www.who.int/publications.

3.8.1 Fecal Culture for Aerobic Pathogens of Gastroenteritis

◩ **NOTE:** For isolation of *Campylobacter jejuni/coli,* see procedure 3.8.2.

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Gastroenteritis can be caused by bacteria, parasites, or viruses. With such a wide array of pathogens and the need for cost containment, physician input and practice guidelines (13) can help the laboratory determine which tests are appropriate for detecting the etiological agent of diarrhea. Microbiology laboratories should review the local epidemiology of bacterial enterocolitis and implement routine stool culture methods that will allow recovery and detection of all of the major pathogens causing most of the cases in their geographic area. All microbiology laboratories should routinely test for the presence of *Salmonella* spp., *Shigella* spp., and *Campylobacter* spp. on all stool cultures. Other major pathogens, such as Shiga-toxin-producing *Escherichia coli,* particularly *E. coli* O157 or enterohemorrhagic *E. coli* (EHEC), should also be routinely tested for on bloody stool samples during the spring, summer, and early fall months in geographic areas where the prevalence of these strains has been shown to be increased. Microbiology laboratories situated in or near coastal communities may also test for *Aeromonas* and *Vibrio* spp. since the prevalence of these types of infections is increased with exposure to water or contaminated food such as shellfish.

Salmonella and *Shigella* are screened by using differential and selective direct plating media (Table 3.8.1–1), which are based on lactose fermentation and H_2S production (31). However, the use of CHROMagar for *Salmonella* spp. improves detection while decreasing the number of subcultures and biochemical tests that need to be done using other nonspecialized media (32). Since some nonpathogenic, gram-negative rods found in normal feces may give the same reactions as enteric pathogens, biochemical screening and agglutination are necessary for identification. Routinely, culture for *Campylobacter jejuni* is performed, but because the method for isolation is different from the method to detect other pathogens, details are presented in a separate procedure (*see* procedure 3.8.2).

Other pathogens, such as *Yersinia enterocolitica, Aeromonas* spp., *Plesiomonas shigelloides, Vibrio cholerae,* and other *Vibrio* spp., including *Vibrio parahaemolyticus,* may be detected in routine culture, but there is a better chance of detection if the physician specifies the suspect organism. For many of these cultures, selective media for these agents are inoculated. If requests for culture for these organisms are infrequent, a reference or public health laboratory may be used for testing. In addition, several organisms produce toxin-mediated disease. While the organism may be detected in culture, detection of the toxin that is responsible requires other laboratory tests. These organisms include verocytotoxin-producing *E. coli* (VTEC), enterotoxigenic *E. coli, Clostridium botulinum, Clostridium difficile, Bacillus cereus,* and *Staphylococcus aureus.* Commercial tests are available for VTEC and for *C. difficile.* For other toxins, testing is available only from public health or reference laboratories. In addition, other genera of gram-negative rods *(Hafnia, Edwardsiella,* DF-3, and even *Klebsiella)* have been implicated in gastroenteritis and may be isolated in culture.

VTEC, also known as Shiga toxin-producing or EHEC, produces a toxin mediated by a bacteriophage, which has been implicated in hemorrhagic colitis (HC) and has been associated with hemolytic-uremic syndrome (HUS) (18). VTEC is the fourth leading cause of diarrhea in the United States, with severe complications from HUS and HC, especially in children. Over 60 *E. coli* serotypes produce the toxin, but *E. coli* O157:H7 is responsible for at least half of the cases (12, 19, 23). Since it has a unique biotype, *E. coli* O157 can easily be detected on selective medium (14). A direct assay for the verocytotoxin can detect the presence of toxin in stools infected with other strains or confirm that an *E. coli* O157 strain is a toxin producer (*see* procedure 11.8). At a minimum, testing for *E. coli* O157 or, preferably, verocytotoxin should be performed routinely on bloody stools from all patients, especially children. Testing on all stools sent for diagnosis of diarrhea is preferred from a public health standpoint but may not be cost-effective for hospital laboratories if the prevalence of disease is low (30). Use of CHROMagar improves detection and work flow efficiency of the routine detection of *E. coli* O157 (6).

Fecal cultures should not be performed for patients being treated with broad-spectrum antimicrobial agents, because it is likely that the antimicrobial therapy is re-

Table 3.8.1–1 Commonly used primary plating and broth media for isolation of *Salmonella* and *Shigella*[a]

Medium (abbreviation)	Type	Expected isolates	Inhibitors or indicators	Reactions of lactose fermenters	Reactions of pathogens	Comments
Hektoen enteric agar (HEK) (21)	D, S plate	*Salmonella* and *Shigella* spp. (especially for *Shigella* spp.)	Bile salts, ferric ammonium citrate, sodium thiosulfate, lactose, sucrose, salicin, bromthymol blue, fuchsin	Yellow-orange or salmon pink; pink precipitate around colonies, may have black centers.	*Shigella* is green. *Salmonella* is blue or green; may have black centers.	Inhibits *Citrobacter* but is small and blue-green, if present. *Proteus* and *Providencia* are yellow or green; may have black centers. Detects H_2S.
MacConkey agar (MAC)	D, S plate	Gram-negative enteric bacilli	Bile salts, crystal violet, lactose, neutral red	Pink	Colorless or transparent	5% Agar will inhibit swarming of *Proteus* spp.
Salmonella-shigella agar (SS)	D, highly S plate	*Salmonella* and *Shigella* spp. (*S. sonnei* inhibited)	Bile salts, lactose, citrate, thiosulfate, ferric citrate, brilliant green, neutral red	Pink, red; may have black centers.	Colorless or transparent; may have black centers.	Detects H_2S.
Xylose, lysine, deoxycholate agar (XLD) (31)	D, S plate	*Salmonella* and *Shigella* spp. (especially for *Shigella* spp.)	Deoxycholate, thiosulfate, ferric ammonium citrate, xylose, lactose, sucrose, lysine, phenol red	Yellow; may have black centers.	*Salmonella* and *Shigella* are red. *Edwardsiella* and *Salmonella* may be red with black centers.	*Providencia rettgeri, Morganella morganii,* and *Proteus* spp. are yellow even though they are lactose negative. Detects H_2S.
CHROMagar Salmonella	D, S	*Salmonella* spp.	Chromogenic substrates and inhibitory agents	Light blue-green colony	*Salmonella* spp. produce mauve (rose to purple) colonies.	Improved detection with decreased subculture and biochemical tests compared to nonchromogenic agars
Gram-negative (GN) broth	E broth	*Shigella* and possibly *Salmonella* spp.	Deoxycholate, citrate, dextrose, mannitol	Initially enhances growth of mannitol-fermenting rods		Subculture at 6–8 h.
Selenite-F	E broth	*Salmonella* and *Shigella* spp. (some shigellae may be inhibited)	Selenite, lactose	Selenite is toxic to *Escherichia coli* and some other enteric bacteria.		Subculture at 18–24 h. Selenite broth with cystine may inhibit some salmonellae.

[a] Either bile salts, deoxycholate, or Selenite is present in each medium to inhibit gram-positive microbiota. Abbreviations: D, differential; E, enriched; S, selective. Ferric ammonium citrate reacts with hydrogen sulfide (H_2S) from organism to produce black color of colony.

sponsible for the diarrhea (13). They may have overgrowth with other bacteria, including *Pseudomonas aeruginosa*, and *Candida* spp., the role of which in disease production is not clear. Their presence may be reported, along with a statement indicating that the organism was the predominant organism recovered and that expected enteric organisms were not present, suggesting antimicrobial inhibition. In such cases, the *C. difficile* toxin assay (procedure 3.8.3) is more meaningful. Generally these patients have been hospitalized for more than 3 days; after that time requests for routine stool cultures should be rejected.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. Have patient obtain stool specimen.

a. Patient should pass the stool into a clean, dry pan or special container mounted on the toilet for this purpose.

b. Transfer at least 5 ml of diarrheal stool, 1 g of material or a walnut-sized portion of stool, or the amount of fecal specimen that displaces to the line of commercial transport vials to one of the following:

(1) Clean, leakproof container with a tight-fitting lid *or*

(2) Buffered glycerol saline (preferred for *Shigella* spp. but inhibits *Campylobacter*)

(3) Modified Cary-Blair medium (modified to contain 1.6 g of agar per liter rather than 5 g/liter)

(a) Purchase from most commercial medium sources *or*

(b) Prepare as follows.

Dispense the following ingredients into 991 ml of H_2O.

sodium thioglycolate ..... 1.5 g
disodium phosphate ..... 1.1 g
sodium chloride ..... 5.0 g
phenol red ..... 0.003 g
agar ..... 1.6 g

Heat to dissolve and cool to 50°C. Then add 9 ml of 1% $CaCl_2$. Adjust pH to 8.4; dispense in vials and steam for 15 min. Cool and tighten caps.

(c) Whether purchased or prepared in-house, QC transport medium to determine if it will support the viability of *Shigella* and *Campylobacter*. See section 14, Table 14.2–2, for details; medium is listed under "Transport."

◩ **NOTE:** Do not fill commercial transport vials above indicator line. Overfilling of transport vial results in improper specimen preservation.

(4) Stool enrichment broths (*see* Table 3.8.1–1)

◩ **NOTE:** Generally, fecal specimens are not placed directly into these broths at collection, but it can be done.

(5) Anaerobic transport tube for *C. difficile* culture, not toxin assay (culture is only for epidemiological or nosocomial studies)

◩ **NOTE:** Do not use toilet paper to collect stool, because it may be impregnated with barium salts, which are inhibitory to some fecal pathogens. The specimen should not be mixed with urine, but semisolid to solid feces can be scooped out of urine, if necessary.

2. Rectal swabs

a. Pass the tip of a sterile swab approximately 1 in. beyond the anal sphincter.

b. Carefully rotate the swab to sample the anal crypts, and withdraw the swab.

c. Send the swab in Cary-Blair medium or buffered glycerol saline.

3. Submit duodenal, colostomy, or ileostomy contents in leakproof cup or transport vial.

B. Timing and transport

1. Submit specimen during the acute stage of infection (usually 5 to 7 days), because pathogens decrease in number with time.

2. If fresh stool is submitted for culture that is not in transport medium, the specimen should be transported to laboratory and processed within 2 h after collection.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

3. If fresh stool is submitted for culture in transport medium, the specimen may be refrigerated at 4°C and transported to the laboratory within 24 h for the best recovery of pathogens.
4. Studies with adult and pediatric patients have shown that culture of a single stool specimen has a sensitivity of >95% for detection of the enteric bacterial pathogen causing enterocolitis provided the laboratory culture method detects all of the major pathogens prevalent in a given geographic area. If the initial stool culture is negative, then additional fecal samples may be submitted for testing provided the patient collects them from different defecations on successive days.

C. Labeling

1. Provide a place on laboratory order form or computer entry screen to indicate if the stool is bloody.
2. Information on the laboratory requisition and specimen label must match. In both places provide the patient's full name, birthdate, a unique patient identifier number (i.e., hospital number), physician's name, type of sample, and the date and time of collection.
3. Document when specimen is received; if it is liquid, formed, or solid; or if it contains mucus.

D. Rejection criteria

1. Reject stools not in transport medium received >2 h after collection, as changes occur that are detrimental to most *Shigella* spp. If recollection is not possible and the doctor requests that the stool be processed, set up the culture and enter a note: "Delay in specimen receipt by laboratory may compromise recovery of pathogens."
2. If specimen in transport medium is delayed for more than 48 h at 4°C or is delayed more than 24 h at 25°C, request immediate recollection since the specimen may be compromised. If another sample cannot be submitted, then proceed to culture the sample but place a comment on the report stating, "Culture may be falsely negative because of a delay in transport."
3. Multiple stool samples should not be cultured from the same patient that were submitted on the same day.
4. Reject fecal cultures received from adults and pediatric patients hospitalized for >3 to 4 days, unless the patient is known to be human immunodeficiency virus positive or in cases of a cluster epidemic within the institution. Do not reject stool samples from infants and toddlers until after the fourth day of hospitalization since studies have shown that it may take longer to collect a stool sample from pediatric patients admitted with gastroenteritis that are placed on bowel rest and are not eating a normal diet (5).
5. Notify caregiver if transport tube is filled above line, indicating that too much specimen was submitted in vial.
6. If transport vial indicator has turned yellow, refrigerate it at 4°C and notify the physician that a fresh stool sample should be recollected. If another sample cannot be submitted, then proceed to culture the sample but place a comment on the report stating, "Culture may be falsely negative because of a delay in transport."
7. Do not process hard, solid stools that cannot be sampled for inoculation.
8. Do not process dry swabs.
9. Do not process stools with barium.
10. Reject more than three stools from the same patient in a 3-week period or multiple specimens received on the same day.
11. Do not use specimens submitted in bacteriology transport tubes for parasitology examination.

III. MATERIALS

A. Media for routine stool culture (*see* Table 3.8.1–1 for descriptions and abbreviations)

1. BAP

2. MAC

3. Choose one or more (to increase yield) of the following to select for *Salmonella* and *Shigella.*

a. HEK

b. XLD

c. CHROMagar Salmonella

d. SS (do not use as only selective medium)

4. Enrichment broth (choose one)

a. Selenite-F (generally for *Salmonella* enrichment)

b. GN broth (for both *Salmonella* and *Shigella* enrichment, but requires subculture at 6 to 8 h)

◩ **NOTE:** The use of enrichment broths for detecting small numbers of *Salmonella* or *Shigella* spp. can be justified for culturing stools of workers in sensitive occupations, such as day care workers and food service employees. Laboratories that have historical data showing very poor recovery of additional pathogens not seen on initial plates can make a case for abandoning routine use of enrichment broths. Generally enrichment broth increases the yield of *Salmonella* and *Shigella* by 10%.

5. If selected other pathogens are requested or indicated, choose additional selective media from Table 3.8.1–2 to enhance recovery of these stool pathogens. Also use these media based on prevalence of disease and public health requirements in specific geographic areas (e.g., use bismuth sulfite agar for cluster epidemics of *Salmonella*).

B. Biochemical tests

1. For screening and detection of *Salmonella, Shigella,* and *Yersinia*

a. Spot indole reagent (procedure 3.17.23)

b. Oxidase reagent (procedure 3.17.39)

◩ **NOTE:** False-negative results are common if indole or oxidase test is done from selective medium. Test only from BAP.

c. Urea agar slants or disks (procedure 3.17.48)

d. Kligler's iron agar (KIA) or triple sugar iron agar (TSI) slants (procedures 3.17.22 and 3.17.25)

e. Andrade's glucose with a Durham tube for gas production (optional) (procedure 3.17.9)

◩ **NOTE:** This medium allows easy reading of gas production and can be used for motility and Voges-Proskauer (VP) reaction.

f. Pyrrolidonyl-β-naphthylamide (PYR) (procedure 3.17.41)

g. Semisolid motility medium (procedure 3.17.31)

h. Kit identification system (API 20E, IDS panel, Vitek GNI Plus card [bioMérieux, Inc.]; MicroScan gram-negative identification panels [Dade Behring MicroScan Microbiology Systems]; BD CRYSTAL [BD Diagnostic Systems]; etc.)

i. Acetate for problematic *Shigella* identifications (procedure 3.17.2)

j. Antisera for grouping (*see* Appendix 3.8.1–1)

(1) *Salmonella* polyvalent and Vi (optional, A, B, C1, C2, and D)

(2) *Shigella* groups A, B, C, and D *or*

(3) *Salmonella* and *Shigella* colored latex agglutination reagents (Wellcolex; Remel Inc., Lenexa, KS)

2. For *Vibrio* and *Aeromonas*

a. O/129 disks–150 µg (procedure 3.17.36)

b. Mueller-Hinton agar (MH) with and without 4% salt

c. Esculin slants or broth (optional for identification of *Aeromonas* to species level) (procedure 3.17.5)

d. O1 antiserum and toxigenicity kit (optional)

◩ **NOTE:** *Vibrio* toxin testing is performed by latex test, DNA probe, EIA, or tissue culture. One source is Unipath Co., Oxoid, Ogdenburg, NY (*V. cholerae* toxigenicity kit, VET-RPLA).

3. For *E. coli* O157

a. 4-Methylumbelliferyl-β-D-glucuronide test (MUG) (procedure 3.17.34)

Table 3.8.1–2 Special highly selective media for specific pathogen requests

Medium	Abbreviation	Inhibitors or indicators	Pathogen selected and colony description	Notes[a]
BAP with ampicillin (20)	BAP-A	20 μg of ampicillin/ml	*Aeromonas* is oxidase positive and may be hemolytic	Increases the yield of isolation of pathogen.
Bismuth sulfite		Brilliant green, bismuth sulfite, ferrous sulfate, dextrose	*Salmonella* (especially serovar Typhi) is black, with or without brownish-black zones, or green with no zones.	Especially good for *Salmonella* serovar Typhi outbreaks; *Morganella* is not inhibited but appears green.
Brilliant green agar		Brilliant green, lactose, sucrose, phenol red	*Salmonella* is red, pink, or white surrounded by red zone.	Fermenters are yellow to yellow-green; agar is inhibitory to *Salmonella* serovars Typhi and Paratyphi.
Inositol-brilliant green-bile salt agar	IBB	Brilliant green, bile salts, inositol	*P. shigelloides* forms white to pink colonies (7).	Coliforms are either green or pink.
Cefsulodin-Irgasan-novobiocin (20, 27)	CIN	Deoxycholate, crystal violet, cefsulodin, Irgasan, novobiocin, mannitol, neutral red	*Y. enterocolitica*, *Aeromonas* spp., and other yersiniae have deep red center with a transparent margin, or "bull's-eye" appearance.	Incubate at 25°C for 48 h. *Citrobacter*, *Pantoea agglomerans*, and *Serratia liquefaciens* are red. *Enterobacter cloacae* and *S. marcescens* colonies are raised and mucoid, with diffuse, pink coloration.
Cycloserine-cefoxitin-egg yolk agar[b]	CCEY[b]	Cycloserine, cefoxitin, cholic acid, egg yolk, *p*-hydroxyphenylacetic acid, lysed horse or sheep blood	*Clostridium difficile* colonies are large (4 mm) and gray with whitish centers; they fluoresce chartreuse under long-wave UV light on anaerobic BAP or CCEY; typical barnyard odor.	Incubate under anaerobic conditions for 48–72 h; gram-positive rod with rare spores.
CHROMagar O157	CHROM-O157	Chromogenic substrates	*E. coli* O157 produces a mauve colony.	Improves detection and work flow (6)
MAC-sorbitol with cefixime and tellurite	CT-SMAC	MAC with sorbitol (rather than lactose), cefixime, and tellurite	*E. coli* O157:H7 is colorless; other coliforms are pink or inhibited.	Better selection than SMAC, which can also be used (12).
MAC broth	MAC broth	MAC without agar	VTEC, especially non-O157 strains	Use 50 μl of overnight culture to perform EHEC EIA. May incubate at 25°C to enrich for *Yersinia* (28).
Thiosulfate citrate bile salts[c]	TCBS[c]	Bile salts, citrate, thiosulfate, 1% NaCl, sucrose, bromthymol blue	*V. cholerae* is yellow; *V. parahaemolyticus* is green or blue. Some *Vibrio* spp. are inhibited.	*Proteus* is yellow; other enteric bacteria are inhibited but blue to translucent if they grow. Enterococci may also grow.
Alkaline peptone water	APW	1% NaCl, pH 8.5	Vibrios are selected.	Subculture to TCBS at 24 h (22).

[a] Incubate at 37°C in O_2 unless otherwise stated.

[b] Since as many as 20% of asymptomatic hospitalized patients may be colonized with *C. difficile*, tests for the presence of toxin in stool are more specific for diagnosis of *C. difficile*-associated diarrhea. Isolation of the organism should only be done for epidemiological studies, with confirmation that the isolated strain is a toxin producer. CCFA (containing cycloserine, cefoxitin, fructose, egg yolk, and neutral red) can also be used for isolation. Do not use a medium with neutral red to demonstrate colonial fluorescence (3).

[c] Prepare TCBS fresh for use from powder or by melting previously prepared or purchased "deeps." If made from powder, boil but do not autoclave prior to use.

III. MATERIALS *(continued)*

b. *E. coli* O157 (and H7) antiserum or latex antibody test (*see* Appendix 3.8.1–2)

4. For *E. coli* O157 and non-O157 VTEC
- **a.** MAC broth
- **b.** EHEC EIA (*see* procedure 11.8)

C. Supplies

1. Incubator at 35°C

2. Petri dishes, slides, or disposable black cards for agglutination tests

3. Sticks and loops

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that media meet expiration date and QC parameters per Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22-A3 (8). See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

1. Visual inspection
- **a.** Inspect for cracks in media or plastic petri dishes, unequal fill, hemolysis, evidence of freezing, bubbles, and contamination.
- **b.** Check XLD; it should be reddish and relatively clear prior to inoculation. Excessive heating causes precipitation.
- **c.** Record and report deficiencies to manufacturer.

2. Most agar media that are purchased from commercial sources are not required to have additional user control with microorganisms expected to give appropriate reactions.
- **a.** These media currently include BAP, CIN, MAC, HEK, XLD, SS, Selenite-F, and GN broth
- **b.** For QC of these media, if prepared in-house, refer to section 14 of this handbook, except for CIN.
- **c.** For QC of CHROMagar Salmonella, see Table 3.8.1–3.
- **d.** For QC of CIN, see Table 3.8.1–3.
- **e.** Refer to CLSI document M22-A3 (8) or section 14 for instructions on preparation of QC microorganisms for testing.

3. Each lot of media for specialized organisms must have QC testing with appropriate microorganisms, regardless of whether it is prepared in-house or purchased from a commercial source.
- **a.** These media include BAP-A, brilliant green agar, bismuth sulfate, IGBB, campylobacter agars, CT-SMAC, CHROMagar O157, CHROMagar Salmonella, CCFA, and TCBS (*see* Table 3.8.1–2).
- **b.** Refer to Table 3.8.1–3 for list of QC microorganisms and their reactions.

4. QC each lot of transport media with *Campylobacter jejuni* ATCC 33291, *Shigella flexneri* ATCC 12022, and *Y. enterocolitica* ATCC 9610 by following the method outlined in Table 14.2–2 for QC listed under "Transport."

Include QC information on reagent container and in QC records.

B. Test antisera with known positively and negatively reacting organisms or commercial somatic O or H antigens prior to use of each new lot and every 6 months thereafter. See Appendix 3.8.1–1.

C. For QC of specific reagents or biochemical media, refer to individual procedures.

Table 3.8.1–3 QC of specialized media for detection of fecal pathogens

Test medium	Test organism	Inoculation method[a]	Incubation			Result
			Time (h)	Temp (°C)	Atmosphere	
BAP-A *Note:* Proposed in 2003 for exemption from user QC for commercially prepared media (9)	*Aeromonas hydrophila* ATCC 7965	A	18–24	35	Aerobic	Growth
	Escherichia coli ATCC 25922	B	18–24	35	Aerobic	Partial to complete inhibition
Bismuth sulfate	*Salmonella enterica* serovar Enteritidis ATCC 13076	A	18–24	35	Aerobic	Growth; black with metallic sheen
	Escherichia coli ATCC 25922	B	18–24	35	Aerobic	Partial inhibition; small brown to yellow-green colonies
Brilliant green	*Salmonella enterica* serovar Enteritidis ATCC 13076	A	18–24	35	Aerobic	Growth; colorless to light pink
	Escherichia coli ATCC 25922	B	18–24	35	Aerobic	Partial inhibition; yellow-green colonies
	Staphylococcus aureus ATCC 25923	B	18–24	35	Aerobic	Inhibition
Campylobacter agars	*Campylobacter jejuni* ATCC 33291	A	24–48	42	Microaerobic	Growth
	Escherichia coli ATCC 25922	B	24–48	42	Microaerobic	Partial to complete inhibition
CCEY or CCFA	*Clostridium difficile* ATCC 9689	A	24–48	35	Anaerobic	Growth; large, gray (CCEY) or yellow (CCFA) colonies that fluoresce chartreuse under UV light (CCEY only)
CHROMagar O157	*E. coli* O157 ATCC 35150, *E. coli* ATCC 25922, and *E. cloacae* ATCC 13047	B	24–48 h	35°C	Aerobic	*E. coli* O157 grows as a mauve colony, *E. coli* (non-O157) strain growth is inhibited (partial or complete), and *E. cloacae* grows as blue-green to blue colonies.
CHROMagar Salmonella	*S. enterica* serovar Typhimurium ATCC 14028, *C. freundii* ATCC 8090, and *S. aureus* ATCC 25923	B	24–28 h	35°C	Aerobic	*Salmonella* grows as a mauve colony, while *C. freundii* grows as a light blue-green colony; *S. aureus* growth is inhibited (partial or complete).
	Clostridium perfringens ATCC 13124	B	24–48	35	Anaerobic	Partial to complete inhibition
	Bacteroides fragilis ATCC 25285	B	24–48	35	Anaerobic	Partial to complete inhibition
	Escherichia coli ATCC 25922	B	24–48	35	Aerobic	Partial to complete inhibition
	Staphylococcus aureus ATCC 25923	B	24–48	35	Aerobic	Partial to complete inhibition
CIN *Note:* Exempt from user QC for commercially prepared media (8)	*Yersinia enterocolitica* ATCC 9610	A	24–48	25	Aerobic	Growth; red center, transparent border
	Aeromonas hydrophila ATCC 7965	A	24–48	35	Aerobic	Growth; red center, transparent border
	Enterococcus faecalis ATCC 29212	B	24–48	35	Aerobic	Partial to complete inhibition
	Pseudomonas aeruginosa ATCC 27853	B	24–48	35	Aerobic	Partial to complete inhibition
	Escherichia coli ATCC 25922	B	24–48	35	Aerobic	Partial to complete inhibition

(continued)

Table 3.8.1–3 QC of specialized media for detection of fecal pathogens *(continued)*

Test medium	Test organism	Inoculation method[a]	Incubation			Result
			Time (h)	Temp (°C)	Atmosphere	
CT-SMAC	*Escherichia coli* O157:H7 ATCC 35150	A	24	35	Aerobic	Clear colonies seen; no fermentation of sorbitol
	Escherichia coli ATCC 25922	B	24	35	Aerobic	Partial to complete inhibition. Pink colonies seen;
	Proteus mirabilis ATCC 12453	B	24	35	Aerobic	fermentation of sorbitol Partial to complete inhibition
TCBS *Note:* Proposed in 2003 for exemption from user QC for commercially prepared media (9)	*Vibrio parahaemolyticus* ATCC 17802	A	18–24	35	Aerobic	Growth; blue-green-centered colonies
	Vibrio alginolyticus ATCC 17749	A	18–24	35	Aerobic	Growth, yellow colonies
	Escherichia coli ATCC 25922	B	18–24	35	Aerobic	Partial inhibition; small, clear colonies

[a] Abbreviations are as follows. A, for testing nutritive properties. Inoculate each medium with 10 µl of a 1:100 dilution of standardized cell suspension (0.5 McFarland). If isolated colonies are not obtained, use a 10-fold-lighter inoculum. B, for testing selective properties. Inoculate each medium with 10 µl of a 1:10 dilution of standardized cell suspension (0.5 McFarland). Although ATCC strains are listed, any organism that will yield the identical result is acceptable. For medium abbreviations, refer to Table 3.8.1–2.

V. PROCEDURE

Observe standard precautions.

A. Perform wet prep for fecal leukocytes from fresh stools on request from outpatient specimens (*see* procedure 3.2.3).

NOTE: The presence of WBCs is one factor suggestive of invasive infection in cases of community-acquired gastroenteritis (*see* Table 3.8.1–4 for expected results). They are not helpful for diagnosis from hospitalized inpatients (26). Placement into transport medium destroys the laboratory's ability to determine relative numbers of WBCs or the presence of blood in the stool.

B. Culture methods

1. Inoculation of media

a. Inoculate plates using a swab dipped into the transport tube.

b. Roll swab over one small area of the BAP and MAC media and streak in quadrants for isolated colonies.

c. Use larger amounts of the specimen for HEK, XLD, and SS agars and streak with a heavier-handed method to account for the inhibitory activity of the media.

Table 3.8.1–4 Microscopic and gross observations of fecal specimens associated with various infections[a]

Organism or toxin	Other observations	Cells seen in smear	
		PMNs	RBCs
Campylobacter	Darting motile rods	Yes	Yes
Clostridium difficile toxin		Yes	Yes
Escherichia coli O157:H7, enterohemorrhagic	Watery	No	Yes
Escherichia coli, enteroinvasive	Mucous	Yes	Yes
Escherichia coli, enterotoxigenic	Watery	No	No
Salmonella spp.	Motile rods	Few	Yes
Shigella spp.	Lack of motile rods	Yes	No
Vibrio cholerae	Rice water	No	No
Staphylococcus toxin		No	No
Viruses		No	No

[a] Data are only a guideline, and in any infection, observations are variable. For example, only 50% of *C. difficile*-associated cases of diarrhea demonstrate the presence of PMNs.

V. PROCEDURE *(continued)*

d. Inoculate Selenite-F or GN broth with the swab that has been heavily saturated with stool from the transport vial.

e. For bloody stools and stools from pediatric patients, inoculate CT-SMAC or CHROMagar O157 plate to detect *E. coli* O157 and/or MAC broth to detect non-O157 VTEC.

f. Refer to Table 3.8.1–2 for selective media and enrichment broths for specific organism requests.

2. Incubate all agars, except CIN, in ambient air at 35 to 37°C for 24 h.

a. Incubate CIN and MAC broth for *Yersinia* requests in ambient air at 25°C for 48 h.

b. Incubate Selenite-F for 18 to 24 h in ambient air at 35 to 37°C. Then subculture to selective media, e.g., MAC and HEK or XLD.

c. Incubate GN broth for 6 to 8 h. Then subculture broth to MAC and HEK or XLD.

◩ **NOTE:** If enrichment broths are incubated for excessive periods, the nonpathogenic enteric bacteria can overgrow the pathogens and negate the value of the enrichment procedure. If it is not possible to subculture broths at the appropriate time interval, subculture as soon as feasible, and closely monitor recovery rates. Alternatively, test enrichment broth directly for the presence of *Salmonella* and *Shigella* using commercial colored latex agglutination reagents.

d. Incubate MAC broth for 24 h at 35 to 37°C for VTEC assay (procedure 11.8).

3. Save all stools in transport vial for 24 to 48 h at 4°C in case a toxin assay is later requested.

C. Examination of culture media

1. BAP and BAP-A

a. Sweep through colonies to test for oxidase production. Pursue all oxidase-positive colonies, unless colony smells and looks like *P. aeruginosa.*

◩ **NOTE:** Most *Vibrio, Plesiomonas,* and *Aeromonas* organisms, but not all, are indole positive and beta-hemolytic (15–17). Despite their salt requirement, most vibrios grow on BAP and MAC.

(1) Subculture any oxidase-positive colonies to BAP and either TSI or KIA.

(2) Identify further all those with TSI or KIA reactions of acid/acid or alkaline/acid (*see* Fig. 3.8.1–1).

(3) Set up kit identification (use saline as diluent, if needed, for strains that require salt for growth).

(4) Inoculate O/129 disks to both MH with and without 4% salt, as needed to verify identification.

◩ **NOTE:** Kits can misidentify *Vibrio vulnificus* as *V. parahaemolyticus, Aeromonas* as *Vibrio fluvialis,* and *Vibrio damselae* as *V. cholerae* (1). Use O/129 disk tests and salt tolerance to prevent initial misidentifications and potential public health consequences (*see* Fig. 3.8.1–1 and Table 3.18.2–8). *Aeromonas* organisms need not be identified to the species level, since it is difficult and may not be clinically important. For complete identifications, see references 15, 16, and 17.

(5) Unless reagents are available, submit *V. cholerae* to the health department for O1 serogrouping and toxin testing.

(6) Submit other *Vibrio* spp. if identification is questionable. Not all *Vibrio* spp. grow on TCBS or are indole positive.

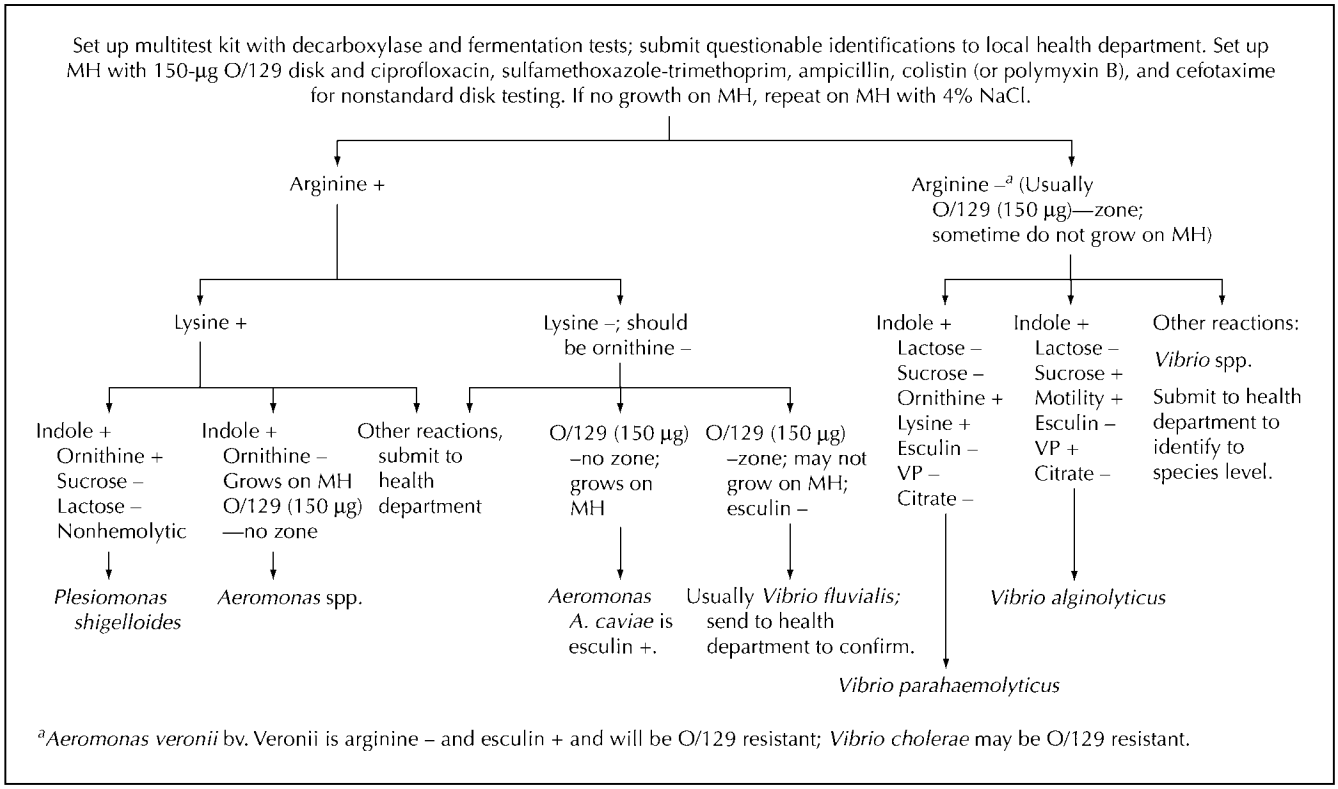

Set up multitest kit with decarboxylase and fermentation tests; submit questionable identifications to local health department. Set up MH with 150-µg O/129 disk and ciprofloxacin, sulfamethoxazole-trimethoprim, ampicillin, colistin (or polymyxin B), and cefotaxime for nonstandard disk testing. If no growth on MH, repeat on MH with 4% NaCl.
Arginine +
Arginine –[a] (Usually O/129 (150 µg)—zone; sometime do not grow on MH)
Lysine +
Lysine –; should be ornithine –
Indole + Lactose – Sucrose – Ornithine + Lysine + Esculin – VP – Citrate –
Indole + Lactose – Sucrose + Motility + Esculin – VP + Citrate –
Other reactions: *Vibrio* spp. Submit to health department to identify to species level.
Indole + Ornithine + Sucrose – Lactose – Nonhemolytic
Indole + Ornithine – Grows on MH O/129 (150 µg) —no zone
Other reactions, submit to health department
O/129 (150 µg) –no zone; grows on MH
O/129 (150 µg) –zone; may not grow on MH; esculin –
Plesiomonas shigelloides
Aeromonas spp.
Aeromonas A. caviae is esculin +.
Usually *Vibrio fluvialis;* send to health department to confirm.
Vibrio alginolyticus
Vibrio parahaemolyticus
[a]*Aeromonas veronii* bv. Veronii is arginine – and esculin + and will be O/129 resistant; *Vibrio cholerae* may be O/129 resistant.

Figure 3.8.1–1 Flowchart for the identification of oxidase-positive stool pathogens from BAP or from either TCBS or CIN. Most are also indole positive. Biochemical reactions for species identification are available on many commercial kits. *Note:* Growth on TCBS implies that the organism is a *Vibrio* sp., but not all *Vibrio* spp. grow on TCBS. Abbreviations: MH, Mueller-Hinton agar; ID, identification; K, alkaline; A, acid; r/o, rule out.

V. PROCEDURE *(continued)*

(7) Perform antimicrobial susceptibility testing (AST) on isolates, since results are variable. Use disk method with MH with salt if isolate will not grow in routine method.

b. Identify numerous *P. aeruginosa* and *S. aureus* organisms; do not perform AST.

c. Identify and report any amount of *B. cereus* organisms, if present.

(1) Screen beta-hemolytic colonies for large spore-forming, gram-positive rods.

(2) *B. cereus* is also catalase positive, motile, lecithinase positive, and penicillin resistant.

(3) *B. anthracis* may cause gastrointestinal disease when ingested, and any amount of a *Bacillus* sp. recovered from stool cultures that is nonhemolytic and nonmotile should immediately be sent to the local or state public health laboratory for further testing (*see* section 16 and procedure 3.18.1) (29).

d. Look for overgrowth of numerous or pure yeast, or other unusual findings (e.g., DF-3), and report without AST.

◩ **NOTE:** DF-3 has been associated with diarrheal disease (2). It is a nonhemolytic, catalase-negative, oxidase-negative, indole-negative, nonmotile, gram-negative coccobacillus that does not grow on MAC. It ferments glucose without gas and is nitrate reductase negative.

V. PROCEDURE *(continued)*

e. Note the absence of enteric gram-negative rods.

f. Do not report enterococci in stool.

2. HEK, XLD, CHROMagar Salmonella, SS, and MAC

a. Work up potential pathogens directly from HEK, XLD, CHROMagar Salmonella, SS, and MAC plates at 24 h and from broth subculture plates at 36 to 48 h (18 to 24 h of incubation). *Screen plates for lactose-negative and/or H_2S-positive colonies.* Refer to Table 3.8.1–1 for colony morphologies of stool pathogens.

◩ **NOTE:** On days when stool cultures are not examined, place plates in refrigerator until they can be examined. After 24 h, alkaline reversion of colonies may make them appear lactose negative. Do not pick colonies after 24 h.

b. MAC or SS: pick one representative colony of each morphological type of colorless colonies.

c. XLD: pick one representative colony of each morphological type of red to red-orange colony and any colony with a black center.

◩ **NOTE:** Species of *Edwardsiella* and *Salmonella* decarboxylate lysine and ferment xylose but not sucrose or lactose. The xylose/lysine ratio in XLD permits these organisms to exhaust the xylose and then attack the lysine, causing a reversion to an alkaline pH, and thus produce red colonies (31).

d. HEK: pick one representative colony of each morphological type of green and blue colonies that are not pinpoint and any colony with a black center.

◩ **NOTE:** *Salmonella* and *Shigella* spp., when surrounded by many bright fermenting *Enterobacteriaceae,* may appear to be faint pink with a green tinge, but there is usually a clear halo around the colonies in the area of precipitated bile. This halo is most apparent when the plates are held up to a light. The colonies are more translucent than those of other *Enterobacteriaceae* (21).

e. CHROMagar Salmonella: pick mauve colonies directly off the plate at 24 h for identification using biochemical typing and serotyping. If the plate demonstrates only a small amount of growth or is negative at 24 h, the plate should be reincubated for another day before being read as negative. Fecal cultures with a low colony count of *Salmonella* may require 48 h of incubation to demonstrate growth on CHROMagar.

f. Subculture each colony picked to BAP, KIA or TSI, urea agar (optional; may use rapid urea disks instead), and Andrade's glucose (optional).

◩ **NOTE:** For laboratories with few stool culture requests or when the colony morphology indicates typical *Salmonella* or *Shigella,* use of kit identifications initially, rather than screening, may be more economical.

g. Next day, read biochemical tests. Follow the flowchart in Fig. 3.8.1–2.

h. Test for agglutination for *Salmonella* and *Shigella* somatic (O) antigens when the screening biochemical tests fit. See Appendix 3.8.1–1 for procedure. Alternatively, use Wellcolex latex kit or send isolate to reference laboratory for typing.

◩ **NOTE:** *Shigella* spp. are genetically *E. coli.* If there is a question between *E. coli* and *Shigella,* 80% of *E. coli* organisms are acetate positive; shigellae are 100% negative (11). *Shigella* spp. are also negative for motility, citrate, and lysine.

i. If the kit gives a genus and species name, be sure that it corresponds to the serologic grouping.

j. For *Shigella* spp., identification to the species level is performed using the grouping sera, in combination with the kit biochemical tests.

(1) Group A—*Shigella dysenteriae*

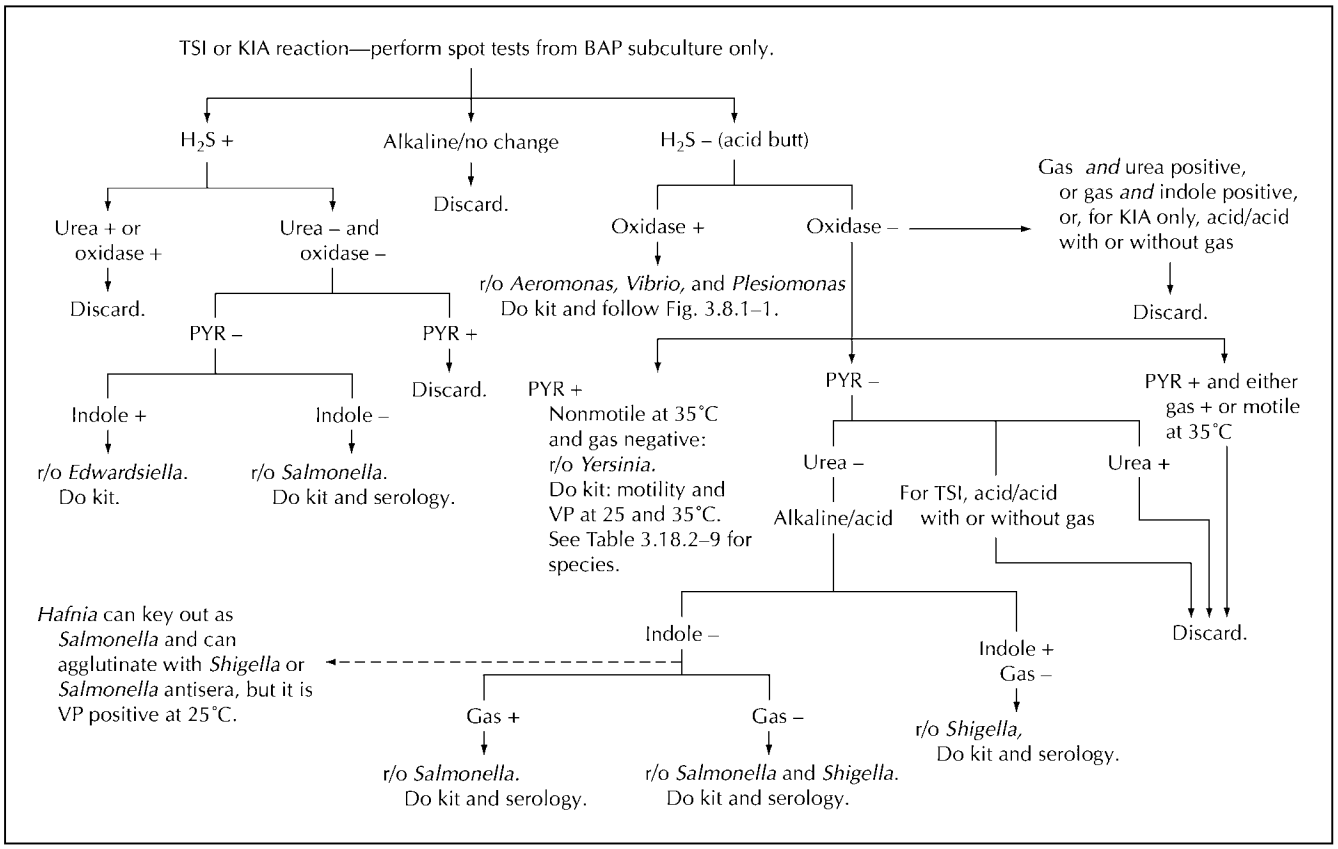

TSI or KIA reaction—perform spot tests from BAP subculture only.
H2S +
Alkaline/no change
H2S – (acid butt)
Discard.
Urea + or oxidase +
Urea – and oxidase –
Oxidase +
Oxidase –
Gas and urea positive, or gas and indole positive, or, for KIA only, acid/acid with or without gas
Discard.
r/o Aeromonas, Vibrio, and Plesiomonas Do kit and follow Fig. 3.8.1–1.
Discard.
PYR –
PYR +
Discard.
PYR –
PYR + and either gas + or motile at 35°C
PYR + Nonmotile at 35°C and gas negative: r/o Yersinia. Do kit: motility and VP at 25 and 35°C. See Table 3.18.2–9 for species.
Indole +
Indole –
Urea –
Urea +
For TSI, acid/acid with or without gas
Alkaline/acid
r/o Edwardsiella. Do kit.
r/o Salmonella. Do kit and serology.
Hafnia can key out as Salmonella and can agglutinate with Shigella or Salmonella antisera, but it is VP positive at 25°C.
Indole –
Indole + Gas –
Discard.
Gas +
Gas –
r/o Shigella, Do kit and serology.
r/o Salmonella. Do kit and serology.
r/o Salmonella and Shigella. Do kit and serology.

Figure 3.8.1–2 Flowchart for identification of stool pathogens from routine stool cultures. Set up either TSI or KIA, BAP, and urea agar (or rapid urea tube) from all lactose-negative or H_2S-positive colonies on enteric selective agars. Reactions of the slant are listed with a slash before the butt reaction. Optionally for H_2S-negative colonies, Andrade's glucose tube with Durham tube for gas will eliminate most questionable production of gas and provide a broth for VP testing. *Perform spot tests (indole, oxidase, PYR) only from BAP.* r/o, rule out.

V. PROCEDURE *(continued)*

(2) Group B—*S. flexneri* (often indole positive)
(3) Group C—*Shigella boydii*
(4) Group D—*Shigella sonnei* (indole negative and *o*-nitrophenyl-β-D-galactopyranoside positive)

k. If the kit identifies the isolate as *Salmonella* or *Shigella*, but it fails to serogroup, repeat typing, using a boiled suspension of the organism in saline.

l. Check the following.
(1) Mannitol-negative *S. flexneri* may be identified as *S. dysenteriae* but type as group B.
(2) If kit codes as *Salmonella* serovar Pullorum or *Salmonella* serovar Gallinarium, perform additional biochemical tests, including VP at 25°C, as these are often *Hafnia* spp. Serovar Pullorum agglutinates with *Salmonella* group D antiserum.

m. Check biochemical reactions and serogroup to determine species identification of *Salmonella* serovar Typhi, *Salmonella* serovar Choleraesuis, or *Salmonella* serovar Paratyphi A. Confirm with appropriate tests as indicated in Table 3.8.1–5.

Table 3.8.1–5 Biochemical differentiation of selected members of the *Salmonella* group[a]

Test	Serogroup Choleraesuis	Serogroup Paratyphi A	Serogroup Typhi	Other
Salmonella group	C	A	D	A–E
Arabinose fermentation	−	+	−	+
Citrate utilization	V	−	−	+
Glucose gas production	+	+	−	+
Lysine decarboxylase	+	−	+	+
Ornithine decarboxylase	+	+	−	+
Rhamnose fermentation	+	+	−	+
Trehalose fermentation	−	+	+	+

[a] Symbols: −, ≤9% of strains positive; V, 10 to 89% of strains positive; +, ≥90% of strains positive.

V. PROCEDURE *(continued)*

(1) Only serovar Typhi will type in both group D and in Vi antisera, is ornithine decarboxylase negative, and produces no gas and little H_2S from carbohydrates in KIA or TSI.

◩ **NOTE:** Serovar Paratyphi C will also agglutinate in Vi antiserum.

(2) Serovar Paratyphi A will type in group A antiserum and is citrate and lysine decarboxylase negative.

(3) Serovar Choleraesuis will type in group C antiserum and is arabinose and trehalose negative.

n. Submit all *Salmonella* (and *Shigella,* if typing is not available or if submission is required by local policy) isolates to the health department for confirmatory identification and for typing.

o. Perform AST on all enteric pathogens except *E. coli* O157, because treatment has been shown to predispose children to HUS (24, 25). Since enteric bacterial gastroenteritis is a self-limited illness in otherwise healthy children and adults, AST results should not be routinely reported except for in the following clinical situations:

(1) At the request of the physician

(2) Patient has disseminated infection with positive urine, blood, or other sterile fluid cultures.

(3) *Salmonella* serovar Typhi infection regardless of patient's age

(4) *Shigella* infection

(5) *Salmonella* infection in a child <1 year of age

(6) *Salmonella* infection in an adult >65 years of age

(7) Immunocompromised patients, including human immunodeficiency virus/AIDS

3. Screen CIN at 24 and 48 h.

a. Look for any colonies with a deep red center with a sharp border surrounded by a translucent zone.

b. Inoculate BAP and identification kit.

c. Check oxidase reaction. If positive, follow Fig. 3.8.1–1 to identify *Aeromonas.*

d. If oxidase negative, inoculate Andrade's glucose (or methyl red-VP [MR-VP] broth) and motility semisolid medium at both 25 and 35°C.

e. Read VP and motility at 24 h to confirm kit identification.

(1) *Y. enterocolitica* and *Yersinia pseudotuberculosis* are nonmotile and VP negative at 35°C and motile at 25°C (10).

(2) *Y. enterocolitica* is VP positive at 25°C, but *Y. pseudotuberculosis* is VP negative.

(3) Both are usually urease positive.

V. PROCEDURE *(continued)*

(4) See Table 3.18.2–9 for biochemical reactions of significant members of this genus.
(5) Confirm atypical reactions by submitting species to reference laboratory or health department.

f. Perform AST on *Aeromonas* and *Yersinia* spp.

4. Screen TCBS at 24 and 48 h.
a. Look for yellow colonies (potential *V. cholerae*, *V. fluvialis*, and *Vibrio furnissii*) and blue colonies (potential *V. parahaemolyticus, Vibrio mimicus*, and *Vibrio hollisae*). Pinpoint colonies should also be examined.
b. Subculture one representative colony of each morphologic type of any colony to BAP and KIA or TSI.
c. Follow Fig. 3.8.1–1 for identification of oxidase-positive colonies that ferment glucose.
d. Expediently send cultures with suspected *V. cholerae* to the local public health laboratory for typing and toxin testing, unless reagents are available.
e. Refer to item V.C.1 for other details of testing.

◩ **NOTE:** For outbreaks, TCBS, in conjunction with enrichment in alkaline peptone water (APW), incubated for 24 h, followed by subculture onto TCBS significantly increased the yield of *V. cholerae.* Subculture of APW at 5 h is not necessary or practical (22).

5. Screen CT-SMAC or CHROMagar O157 plate for *E. coli* O157 at 24 h of incubation.
a. Pick at least four sorbitol-negative (transparent or colorless) colonies.
b. Subculture to BAP.
c. If isolate is oxidase negative and indole positive, perform MUG test.
d. If isolate is MUG negative, screen using the *E. coli* O157 latex agglutination kit (*see* Appendix 3.8.1–2).
e. If agglutination positive, confirm as *E. coli* by kit identification.
◩ **NOTE:** *E. coli* O157 may be sorbitol positive by the kit tests. Isolation of *E. coli* O157 from a diarrhea stool, particularly with the presence of blood or symptoms typical of HC or HUS, is indicative of a verocytotoxin-producing strain.
f. Confirm with H7 flagellum antiserum and/or toxin testing, if symptoms are not typical of HUS or HC. For toxin testing, submit to the health department or reference laboratory or see procedure 11.8.
g. Pick mauve colonies off the CHROMagar O157 plate and screen using *E. coli* O157 latex agglutination kit (*see* Appendix 3.8.1–2). If agglutination positive, confirm with H7 flagellum antiserum and/or toxin testing by submitting to the health department or reference laboratory, or see procedure 11.8.

6. Test 50 µl of MAC broth or plate sweep of culture with one of the EHEC serologic assays 24 h after inoculation with stool. See procedure 11.8.

7. Depending on state requirements, submit *V. cholerae*, *Salmonella* spp., *Shigella* spp., *E. coli* O157, or other stool pathogens to the health department. Send repeat isolates of *Salmonella* spp. per local health department policy.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Record biochemical results on colonies being screened on appropriate worksheets, noting media sampled and enumeration.

B. Negative cultures

1. For all stool cultures, report a pathogen-negative comment that specifies all of the enteric bacterial pathogens the laboratory has tested for. For example, report "No *Salmonella, Shigella,* or *Campylobacter* sp. isolate" if these are the only pathogens routinely tested.

2. If additional pathogens have been ruled out, the comment should be expanded to also list other culture-negative results. Include a comment such as "No *E. coli* O157, *Yersinia, Aeromonas,* or *Vibrio* detected."

3. Add "to date" for preliminary reports and delete for final reports.

C. Additional comments

1. If gram-negative enteric microbiota are not present in the culture, add a comment: "No normal enteric gram-negative rods isolated."

2. Report overgrowth of predominant *S. aureus* and *P. aeruginosa* as "Predominating or pure culture of [organism name]."

◩ **NOTE:** The presence of *S. aureus* may or may not indicate gastrointestinal disease or food poisoning. Toxin must be detected in stool or food for disease, which is rarely done.

3. Report yeast, if found in pure or predominating culture, without genus or species identification.

4. In the setting of diarrhea and no other likely cause, report a pure culture of unusual microbiota, such as DF-3 or *Klebsiella* spp.

D. Positive cultures

1. Report presumptive presence of any enteric pathogens listed in Table 3.8.1–6 with or without quantitation, depending on laboratory's policy to track positive cultures from enrichment broth only.

2. Report the pathogen with the preliminary designation as "probable" until the identification is confirmed by both biochemical testing and serology.

3. Enter preliminary note indicating that "Isolate has been sent for confirmatory identification to [name and location]" when isolate has been sent for serologic testing.

4. *Salmonella* reporting

◩ **NOTE:** *Salmonella* nomenclature is a topic of worldwide debate at the present time (4). There is only one species of *Salmonella* that infects humans; some call this species *Salmonella enterica* and others refer to it as *Salmonella choleraesuis.* In addition, the species is divided into six subspecies and over 2,000 serotypes. Serotypes are grouped into several serogroups noted alphabetically as A, B, C_1, C_2, etc. Serotype names are called either serovars or serotypes and are not in italics. Some texts capitalize the serotype name and others do not. Because the long names are cumbersome and laboratory computer systems do not italicize, *Salmonella enterica* subsp. *enterica* serotype Typhimurium is usually called *Salmonella* typhimurium or *Salmonella* serovar Typhimurium on laboratory reports.

a. Report the following to the serotype level: *Salmonella* serovar Paratyphi A, *Salmonella* serovar Choleraesuis, or *Salmonella* serovar Typhi, using Table 3.8.1–5.

b. Report isolates that are serologically A, C, or D, but are *not* biochemically serovar Paratyphi A, serovar Choleraesuis, or serovar Typhi, as follows.

(1) *Salmonella* serogroup A—not Paratyphi A

(2) *Salmonella* serogroup C—not Choleraesuis

(3) *Salmonella* serogroup D—not Typhi

Table 3.8.1–6 Summary of detection media and identification methods for fecal pathogens[a]

Pathogen(s)	Media	Initial observation	Initial testing	Confirmatory	Comments
Salmonella and *Shigella*	MAC, HEK, XLD	See Table 3.8.1–1	Follow Fig. 3.8.1–2	Serology	
Yersinia enterocolitica	MAC, CIN	Red on CIN, colorless on MAC	Kit	Motility and VP at 25 and 35°C	
Aeromonas spp.	BAP, BAP-A, MAC, CIN	Oxidase positive, usually indole positive, red on CIN	Kit; follow Fig. 3.8.1–1	O/129 disk Esculin (optional)	Lactose positive or negative
Plesiomonas shigelloides	BAP, MAC	Oxidase positive, indole positive	Kit; follow Fig. 3.8.1–1		Lactose negative
Vibrio	BAP, MAC, TCBS	Oxidase positive, may be colorless on MAC, yellow or blue on TCBS	Follow Fig. 3.8.1–1; kit; may need to add salt to inoculum.	O/129 disk, salt tolerance	*V. mimicus* is VP negative, colistin susceptible; *V. cholerae* is VP positive, colistin resistant.
Edwardsiella tarda	MAC, HEK, XLD	Colorless on MAC, black center on HEK or XLD	Kit; indole positive	None	
Bacillus anthracis	BAP	Nonhemolytic	Nonmotile	Immediate referral to state laboratory or CDC	Potential agent of bioterrorism (*see* section 16 and procedure 3.18.1).
Bacillus cereus	BAP	Hemolytic	Large gram-positive rods	Catalase positive, motile, lecithinase positive, and penicillin resistant	Toxin testing may be done by health department.
EHEC	CT-SMAC	Colorless	Kit; MUG negative	Serotyping	Toxin assay more sensitive to detect other strains.
Other *E. coli* strains Enteropathogenic Enteroinvasive Adherent Enterotoxigenic	NA	NA	NA	NA	Send stool to reference laboratory or health department for toxin, invasion, or adherence testing.
Clostridium difficile	CCEY or CCFA	Large, gray (CCEY) or opalescent yellow (CCFA) colonies that fluoresce chartreuse on anaerobic BAP or CCEY	Gram stain showing large gram-positive rods, "horse barn" odor	None	Perform toxin testing. (*See* procedure 3.8.3.)
Clostridium botulinum	NA	NA	NA	NA	Send stool and serum to public health department.

[a] For *Campylobacter* species, see procedure 3.8.2. NA, not applicable.

VI. REPORTING RESULTS
(continued)

c. Report other *Salmonella* isolates by group, if tested, or by genus only until the species name is available from the reference laboratory.

d. By accepted convention, report final identifications of *Salmonella,* followed by the serovar name only (4).

5. When confirmation is obtained, report *V. cholerae,* including the serogroup (O1 or non-O1), serotype of O1 strains (Inaba, Ogawa, and Hikojima), biotype (e.g., classical or El Tor), and presence of toxin.

6. Report sorbitol-negative colonies which show positive agglutination with O157 latex test reagent and a positive identification of *E. coli* as "*E. coli* serotype O157." If tested for toxin, report as, e.g., "*E. coli* O157, toxin producer."

7. Other pathogens listed in Table 3.8.1–6 are generally reported without confirmation by a reference laboratory, unless there is a question.

E. Notification

1. For hospitalized patients, notify infection control and the nursing care unit immediately by telephone with presumptive identification (agglutination positive without biochemical confirmation) of suspicious enteric pathogens so that proper patient isolation protocols can be instituted.

◩ **NOTE:** Patients shedding pathogens in their stool are placed on both standard precautions and contact isolation.

2. For patients who have been discharged from the hospital or whose specimens were received as outpatient specimens, communicate the final result of the presence of any of the enteric pathogens to the person responsible for the patient's care.

3. Because of its life-threatening consequences, contact the physician expediently if *V. cholerae, E. coli* O157, *Salmonella* serovar Typhi, or *Salmonella* serovar Paratyphi C is isolated.

4. For hospitalized patients, notify the physician if there will be a delay in reporting a highly suspicious culture (e.g., problems with agglutination, etc.).

5. Report all reportable enteric pathogens to the appropriate public health agency as required by the local regulatory and government jurisdictions. Usually these include *Salmonella, Shigella, Yersinia, Vibrio, E. coli* O157, and *Campylobacter.*

a. Check with local regulations to determine which agents are reportable in your state.

b. Because of their epidemiological consequences, *immediately* notify the local health department of suspected *V. cholerae, Salmonella* serovar Typhi, *Salmonella* serovar Paratyphi A, or *Salmonella* serovar Choleraesuis and potential cases of gastrointestinal anthrax (i.e., *Bacillus anthracis*).

F. AST

1. Since enterocolitis is a self-limited infection in otherwise healthy children and adults, AST should be performed on enteric pathogens but only selectively reported. Patients should not be routinely treated with antimicrobial agents unless they are <1 year of age, elderly, or immunocompromised.

2. For *Shigella, Salmonella, Aeromonas, Plesiomonas, Edwardsiella, Vibrio,* and *Yersinia,* report only ampicillin, sulfamethoxazole-trimethoprim, and a quinolone. For patients with invasive infection (i.e., positive blood cultures) and those with multiply resistant strains, a third-generation cephalosporin should also be reported. A comment should be placed on reports of *Salmonella* and *Shigella* that first- and second-generation cephalosporins and aminoglycosides are not appropriate treatment of these pathogens.

3. Suppress quinolone results for children under the age of 14 years, since these drugs inhibit bone growth.

4. For *V. cholerae,* also report doxycycline or tetracycline.

VI. REPORTING RESULTS *(continued)*

5. Susceptibility testing is contraindicated for *E. coli* O157 and other EHEC organisms, since treatment of enterohemorrhagic colitis with antimicrobial agents can induce bacterial cell lysis with the release of toxin, which may increase the risk of developing HUS. Add a comment to the report such as, "Antimicrobial therapy is not indicated for this pathogen."

VII. INTERPRETATION

A. The isolation of a stool pathogen may not identify the cause of the disease. For example, *Salmonella* is present in the carrier state, without disease, and *Plesiomonas* is a questionable pathogen.

B. Isolation of an organism whose toxin is responsible for disease may not indicate that the organism possesses the genetic ability to produce the toxin or that the toxin was produced in the patient.

VIII. LIMITATIONS

A. Because the media used for culture are highly selective, some pathogens that should grow may be inhibited. The more media inoculated, the more likely a pathogen will be isolated.

B. Use of enrichment broth or the use of both XLD and HEK will detect more pathogens, but the cost-benefit may not make the expense and time practical.

C. Without a specific request for *Vibrio* or *Yersinia,* selective medium will not be inoculated and these pathogens may or may not be detected in culture.

D. Pathogens present in small numbers may not be detected. Testing of two separate specimens increases the yield.

E. Many pathogenic *E. coli* organisms are difficult to detect, since there is no medium for their selection.

F. CT-SMAC and CHROMagar O157 will not detect VTEC strains other than *E. coli* O157.

G. The agent that is causing the patient's diarrheal disease may not have yet been recognized as a stool pathogen and will thus go undetected.

H. The physician must consider other agents, such as viruses and parasites, that can be responsible for disease.

REFERENCES

1. **Abbott, S. L., L. S. Seli, M. Catino, Jr., M. A. Hartley, and J. M. Janda.** 1998. Misidentification of unusual *Aeromonas* species as members of the genus *Vibrio:* a continuing problem. *J. Clin. Microbiol.* **36:**1103–1104.
2. **Blum, R. N., C. D. Berry, M. G. Phillips, D. L. Hamilos, and E. W. Koneman.** 1992. Clinical illnesses associated with isolation of dysgonic fermenter 3 from stool samples. *J. Clin. Microbiol.* **30:**396–400.
3. **Brazier, J. S.** 1993. Role of the laboratory in investigations of *Clostridium difficile* diarrhea. *Clin. Infect. Dis.* **16**(S4):228–233.
4. **Brenner, F. W., R. G. Villar, F. J. Angulo, R. Tauxe, and B. Swaminathan.** 2000. *Salmonella* nomenclature. *J. Clin. Microbiol.* **38:**2465–2467.
5. **Church, D. L., G. Cadrain, A. Kabani, T. Javaji, and C. Trevenen.** 1995. Practice guideline for ordering stool cultures in a pediatric population. Alberta Children's Hospital, Calgary, Alberta, Canada. *Am. J. Clin. Pathol.* **103:**149–153.
6. **Church, D. L., D. Emshey, H. Semeniuk, T. Lloyd, and J. Pitout.** 2007. Evaluation of BBL CHROMagar O157 versus sorbitol-MacConkey medium for routine detection of *Escherichia coli* O157 in a centralized regional microbiology laboratory. *J. Clin. Microbiol.* **45:**3098–3100.
7. **Clark, R. B., and J. M. Janda.** 1991. *Plesiomonas* and human disease. *Clin. Microbiol. Newsl.* **13:**49–52.
8. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
9. **Clinical and Laboratory Standards Institute.** 2008. *Performance Standards for Antimicrobial Susceptibility Testing.* Eighteenth informational supplement. Approved standard M100-S18. Clinical and Laboratory Standards Institute, Wayne, PA.

REFERENCES *(continued)*

10. **Darland, G., W. H. Ewing, and B. R. Davis.** 1974. *The Biochemical Characteristics of* Yersinia enterocolitica *and* Yersinia pseudotuberculosis. Center for Disease Control, Atlanta, GA.
11. **Farmer, J. J., III, B. R. Davis, F. W. Hickman-Brenner, A. McWhorter, G. P. Huntley-Carter, M. A. Asbury, C. Riddle, H. G. Wathen-Grady, C. Elias, G. R. Fanning, A. G. Steigerwalt, C. M. O'Hara, G. K. Morris, P. B. Smith, and D. J. Brenner.** 1985. Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. *J. Clin. Microbiol.* **21:**46–76.
12. **Fey, P. D., R. S. Wickert, M. E. Rupp, T. J. Safranek, and S. H. Hinrichs.** 2000. Prevalence of non-O157:H7 Shiga toxin-producing *Escherichia coli* in diarrheal stool samples from Nebraska. *Emerg. Infect. Dis.* **6:**530–533.
13. **Guerrant, R. L., T. Van Gilder, T. S. Steiner, N. M. Thielman, L. Slutsker, R. V. Tauxe, T. Hennessy, P. M. Griffin, H. DuPont, R. B. Sack, P. Tarr, M. Neill, I. Nachamkin, L. B. Reller, M. T. Osterholm, M. L. Bennish, and L. K. Pickering.** 2001. Practice guidelines for the management of infectious diarrhea. *Clin. Infect. Dis.* **32:**331–351.
14. **Hayes, P. S., K. Blom, P. Feng, J. Lewis, N. A. Strockbine, and B. Swaminathan.** 1995. Isolation and characterization of a β-D-glucuronidase-producing strain of *Escherichia coli* serotype O157:H7 in the United States. *J. Clin. Microbiol.* **33:**3347–3348.
15. **Huys, G., P. Kampfer, M. Altwegg, I. Kersters, A. Lamb, R. Coopman, J. Luthy-Hottenstein, M. Vancanneyt, P. Janssen, and K. Kersters.** 1997. *Aeromonas popoffii* sp. nov., a mesophilic bacterium isolated from drinking water production plants and reservoirs. *Int. J. Syst. Bacteriol.* **47:**1165–1171.
16. **Janda, J. M., C. Powers, R. G. Bryant, and S. L. Abbott.** 1988. Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. *Clin. Microbiol. Rev.* **1:**245–267.
17. **Janda, J. M.** 1991. Recent advances in the study of the taxonomy, pathogenicity, and infectious syndromes associated with the genus *Aeromonas. Clin. Microbiol. Rev.* **4:**397–410.
18. **Karmali, M.** 1989. Infection by verocytotoxin-producing *Escherichia coli. Clin. Microbiol. Rev.* **2:**15–38.
19. **Kehl, K. S., P. Havens, C. E. Behnke, and D. W. Acheson.** 1997. Evaluation of the Premier EHEC assay for detection of Shiga toxin-producing *Escherichia coli. J. Clin. Microbiol.* **35:**2051–2054.
20. **Kelly, M. T., E. M. Stroh, and J. Jessop.** 1988. Comparison of blood agar, ampicillin blood agar, MacConkey-ampicillin-Tween agar, and modified cefsulodin-Irgasan-novobiocin agar for isolation of *Aeromonas* spp. from stool specimens. *J. Clin. Microbiol.* **26:**1738–1740.
21. **King, S., and W. I. Metzger.** 1968. A new plating medium for the isolation of enteric pathogens. I. Hektoen enteric agar. *Appl. Microbiol.* **16:**577–578.
22. **Lesmana, M., E. Richie, D. Subekti, C. Simanjuntak, and S. E. Walz.** 1997. Comparison of direct-plating and enrichment methods for isolation of *Vibrio cholerae* from diarrhea patients. *J. Clin. Microbiol.* **35:**1856–1858.
23. **Mackenzie, A. M. R., P. Lebel, E. Orrbine, P. C. Rowe, L. Hyde, F. Chan, W. Johnson, P. N. McLaine, and The Synsorb Pk Study Investigators.** 1998. Sensitivities and specificities of Premier *E. coli* O157 and Premier EHEC enzyme immunoassays for diagnosis of infection with verotoxin (Shiga-like toxin)-producing *Escherichia coli. J. Clin. Microbiol.* **36:**1608–1611.
24. **Molbak, K., P. S. Mead, and P. M. Griffin.** 2002. Antimicrobial therapy in patients with *Escherichia coli* O157:H7 infection. *JAMA* **288:**1014–1016.
25. **Safdar, N., A. Said, R. E. Gangnon, and D. G. Maki.** 2002. Risk of hemolytic uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7. A meta-analysis. *JAMA* **288:**996–1001.
26. **Savola, K. L., E. J. Baron, L. S. Tompkins, and D. J. Passaro.** 2001. Fecal leukocyte stain has diagnostic value for outpatients but not inpatients. *J. Clin. Microbiol.* **39:**266–269.
27. **Schiemann, D. A.** 1979. Synthesis of a selective agar medium for *Yersinia enterocolitica. Can. J. Microbiol.* **25:**1298.
28. **Schiemann, D. A.** 1982. Development of a two-step enrichment procedure for recovery of *Yersinia enterocolitica* from food. *Appl. Environ. Microbiol.* **43:**14.
29. **Sirisanthana, T., and A. E. Brown.** 2002. Anthrax of the gastrointestinal tract. *Emerg. Infect. Dis.* **8:**649–651.
30. **Slutsker, L., A. A. Ries, K. D. Greene, J. G. Wells, L. Hutwagner, and P. M. Griffin.** 1997. *Escherichia coli* O157:H7 diarrhea in the United States: clinical and epidemiologic features. *Ann. Intern. Med.* **126:**505–513.
31. **Taylor, W. I.** 1965. Isolation of shigellae. I. Xylose lysine agars: new media for isolation of enteric pathogens. *Am. J. Clin. Pathol.* **44:**471–475.
32. **van Dijk, S., M. J. Bruins, and G. J. H. M. Ruijs.** 2009. Evaluation and implementation of a chromogenic agar medium for *Salmonella* detection in stool in routine laboratory diagnostics. *J. Clin. Microbiol.* **47:**456–458.

SUPPLEMENTAL READING

Ewing, W. H. (ed.). 1986. *Edwards and Ewing's Identification of Enterobacteriaceae,* 4th ed. Elsevier Science Publishing Co., New York, NY.

Gilligan, P. H., J. M. Janda, M. A. Karmali, and J. M. Miller. 1992. *Cumitech 12A, Laboratory Diagnosis of Bacterial Diarrhea.* Coordinating ed., F. S. Nolte. American Society for Microbiology, Washington, DC.

Jeppesen, C. 1995. Media for *Aeromonas* spp., *Plesiomonas shigelloides* and *Pseudomonas* spp. from food and environment. *Int. J. Food Microbiol.* **26:**25–41.

Karch, H., and M. Bielaszewska. 2001. Sorbitol-fermenting Shiga toxin-producing *Escherichia coli* O157:H(−) strains: epidemiology, phenotypic and molecular characteristics, and microbiological diagnosis. *J. Clin. Microbiol.* **39:**2043–2049.

McFaddin, J. F. 1985. *Media for Isolation, Cultivation, Identification, and Maintenance of Bacteria,* vol. I. Williams & Wilkins, Baltimore, MD.

Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* **5:**607–625.

APPENDIX 3.8.1–1

Observe standard precautions.

Detection of Somatic O Antigen Serogroups of Bacteria

I. PRINCIPLE

Gram-negative rods possess somatic antigens in their cell walls, called O antigens, that are useful in the identification of specific serovars or serotypes. These antigens are polysaccharide side chains of endotoxin in the outer membrane of the cell wall. Antibody to these antigens will agglutinate a suspension of the organism.

II. SPECIMEN

Prepare milky suspension of pure culture from fresh plate in normal sterile saline. If culture is not pure, erroneous results can occur.

III. MATERIALS

A. Antisera

1. *Salmonella* polyvalent, A, B, C1, C2, D, E, etc., and Vi; *Shigella* groups A, B, C, and D; *E. coli* O157; *V. cholerae* O1, or any other serotype
2. Store antisera at 4°C.

B. Petri dishes and either glass slides or disposable cards with black background (preferred)

IV. QUALITY CONTROL

Test each lot of reagent with a known positively and negatively reacting organism prior to putting into use it and every 6 months thereafter. Organisms can be stored in formalin at 4°C for QC use.

V. PROCEDURE

Mix drop of antiserum with drop of suspension of organism. Rotate slide for 1 min and observe for agglutination.

VI. INTERPRETATION

Positive agglutination is an indication that the strain contains O antigen.

VII. REPORTING RESULTS

Report presumptive identification as "[organism name]" followed by serotype alpha and numeric designation.

VIII. LIMITATIONS

A. Some organisms with either capsules or flagella can give a false-negative result. Boil the suspension for 5 min or test *Salmonella* using Vi antigen for the capsule of this genus.

B. Serological identification should be confirmed with biochemical testing, since serotypes can be shared or cross-react with other species.

Supplemental Reading

Ewing, W. H. 1986. *Edwards and Ewing's Identification of Enterobacteriaceae,* 4th ed. Elsevier Science Publishing Co., New York, NY.

APPENDIX 3.8.1–2

Detection of *Escherichia coli* O157 by Latex Agglutination

I. PRINCIPLE

The *Escherichia coli* serotype most frequently isolated in cases of HC and HUS is O157:H7. Detection of this serotype from a strain of *E. coli* is presumptive for the agent of HUS and HC. Cytotoxin testing confirms the identification as a strain that produces verocytotoxin. Colonies are mixed with latex particles coated with antibody against the cell wall O antigen of *E. coli* O157. *E. coli* O157 will bind with the antibody, causing the latex particles to visibly agglutinate. Bacteria which are not O157 will not bind to the antiserum and will not result in agglutination (1–3).

II. SPECIMEN

Indole-positive, oxidase-negative, MUG-negative, non-sorbitol-fermenting colonies from CT-SMAC medium. Test multiple colonies if a negative result is obtained.

III. MATERIALS

A. *E. coli* O157 kit stored at 2 to 8°C. Some suppliers are listed below.
   1. Oxoid Incorporated, Ogdenburg, NY
   2. Pro-Lab Diagnostics, Austin, TX
   3. Remel Microbiology Products, Lenexa, KS
   4. Meridian Diagnostics, Cincinnati, OH

B. Kits usually contain the following.
   1. One dropper vial of *E. coli* O157 latex reagent containing latex particles coated with purified rabbit immunoglobulin G (IgG) which reacts with *E. coli* somatic antigen O157
   2. One dropper vial of negative control latex containing latex particles coated with purified rabbit IgG which does not react with *E. coli* serotype O157
   3. One dropper vial of positive control suspension containing *E. coli* serotype O157:H7 antigen
   4. A negative control *E. coli* suspension (a culture of *E. coli* ATCC 25922 can be used in place of the negative control)
   5. Test cards
   6. Mixing sticks

IV. QUALITY CONTROL

Check each lot of reagents prior to use and every 6 months thereafter with the suspension of *E. coli* O157:H7 (positive control) and a suspension of *E. coli* (ATCC 25922) (negative control).

V. PROCEDURE

Follow manufacturer's instructions and observe for agglutination. An example is below.

A. Bring reagents to room temperature before testing.
B. Resuspend colony in 0.5 ml of normal sterile saline in a culture tube to McFarland 3 to 5.
C. Place 1 drop of *E. coli* O157 latex reagent onto a test circle.
D. Using a sterile Pasteur pipette, add 1 drop of the colony suspension to the test circle. Alternatively, add a drop of saline to the card and emulsify the colony directly in the saline until a smooth suspension is obtained.
E. Mix reagent with the bacterial suspension in the test circle.
F. Rock card gently and examine for agglutination over a 1- to 2-min period.
G. Specimens showing positive agglutination within 2 min must be examined further.
H. Test positive specimens again by repeating the procedure using negative control latex reagent.

VI. INTERPRETATION

A. Positive agglutination indicates the presence of *E. coli* serogroup O157 in the recommended time. Grade the agglutination as follows.
   1. 4+: large clumps against a very clear background
   2. 3+: large and small clumps against a clear background
   3. 2+: small but definite clumps against a slightly clouded background
   4. 1+: fine granulation against a milky background
   5. Negative: a homogeneous suspension of particles with no visible clumping.

B. A lack of agglutination indicates the absence of *E. coli* serogroup O157.

APPENDIX 3.8.1–2 *(continued)*

C. Results cannot be interpreted if there is agglutination of both the test and control latex.

VII. REPORTING RESULTS

A. Report positive agglutination with O157 latex test reagent, no agglutination with negative control reagent, and identification as *E. coli* as "*E. coli* serotype O157."

B. Testing for the H antigen or VTEC may be needed. See procedure 3.8.1 for further testing.

VIII. LIMITATIONS

A. Some strains of *E. coli* are difficult to emulsify in saline and may give a stringy-type reaction with both the test and the control reagents. If this stringiness is found, suspend the colony in 0.5 ml of saline. Allow the lumps to settle, and retest the smooth supernatant.

B. Neither growth of colorless colonies on CT-SMAC nor a positive reaction in the *E. coli* O157 latex test will directly confirm the isolate as a toxin-producing strain.

C. The latex test does not confirm the isolate as having the H7 flagellar type or that the strain is a toxin producer. Further studies are needed to determine these characteristics.

D. Other serotypes which produce verocytotoxin are not detected in this assay.

E. Some strains of *Escherichia hermannii* may cross-react with *E. coli* O157 antiserum and the latex test due to a shared antigen. *E. hermannii* may be differentiated from *E. coli* by the former's ability to ferment cellobiose and yellow pigmentation, which may be delayed.

References

1. **Mackenzie, A. M. R., P. Lebel, E. Orrbine, P. C. Rowe, L. Hyde, F. Chan, W. Johnson, P. N. McLaine, and The Synsorb Pk Study Investigators.** 1998. Sensitivities and specificities of Premier *E. coli* O157 and Premier EHEC enzyme immunoassays for diagnosis of infection with verotoxin (Shiga-like toxin)-producing *Escherichia coli*. *J. Clin. Microbiol.* **36:**1608–1611.
2. **Sowers, E. G., J. G. Wells, and N. A. Strockbine.** 1996. Evaluation of commercial latex reagents for identification of O157 and H7 antigens of *Escherichia coli*. *J. Clin. Microbiol.* **34:**1286–1289.
3. **Thompson, J. S., D. S. Hodge, and A. A. Borczyk.** 1990. Rapid biochemical test to identify verocytototoxin-positive strains of *Escherichia coli* serotype O157. *J. Clin. Microbiol.* **28:**2165–2168.

3.8.2 Fecal Culture for *Campylobacter* and Related Organisms

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Using recently developed genetic techniques, the genus *Campylobacter* was divided into three genera based on 16S rRNA homology tests (22). The groups *Campylobacter*, *Helicobacter*, and *Arcobacter* are microaerobic to anaerobic organisms. These curved, helical, gram-negative rods are among the most challenging bacteria for the clinical microbiologist to cultivate and identify. In addition, the disease spectrum with these genera ranges from "well established" (as in the association of *Campylobacter jejuni* and diarrhea) to "speculative" (associated with clinical strains such as *Arcobacter* species that have unproven etiology) to "emerging" (such as *C. upsaliensis* and *Helicobacter rappini* ["*Flexispira rappini*"]).

Table 3.8.2–1 lists the taxonomic positions, known sources, and common disease associations for campylobacters, helicobacters, arcobacters, and other, unrelated genera that are sometimes confused with campylobacters (16). In Table 3.8.2–2, clinical diseases and their possible causative agents are listed. Because of diverse growth requirements, such as temperature, atmosphere of incubation, etc., most clinical laboratory methods are designed to recover only the most common pathogenic strains of these genera. While this procedure focuses on fecal cultures, these basic methods can be used to identify campylobacters from most cultures. For *Helicobacter pylori*, refer to procedure 3.8.4.

It is estimated that *Campylobacter* causes approximately 2.4 million cases of disease each year in the United States. *C. jejuni* is the most commonly identified bacterial pathogen isolated from fecal cultures, with greater than 90% of campylobacter infections caused by *C. jejuni* (7). *C. jejuni* gastroenteritis is a self-limiting disease, but severe infections occur in the young and immunocompromised. The procedure that follows is designed to detect and identify *C. jejuni*. Although other species have been associated with gastroenteritis, they are more difficult to isolate and identify to the species level. Consequently, culture of these other species cannot be justified in most clinical laboratories.

Because *Campylobacter* spp. and *Escherichia coli* O157:H7 are the major contributors to blood in the stool, it is important to detect the presence of the infecting organism, since the use of antimicrobial treatment for *E. coli* O157 infection is thought to increase the risk of hemolytic-uremic syndrome. The drug of choice for treatment of campylobacters was ciprofloxacin, because it is effective for both *Campylobacter* and other enteric pathogens. The use of quinolones in the poultry industry has increased resistance of campylobacters to quinolones, with rates of 10% (1) to as high as 40% (15). Erythromycin is returning as the drug of choice for treatment, although occasional resistance is also seen with this agent. Newer macrolides are also effective but are more expensive.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. See procedure 3.8.1 for fecal and rectal swab collection.

2. *Campylobacter* can be isolated from routine anaerobic cultures of blood or wound specimens. If isolate does not grow on subculture, incubate in a microaerobic atmosphere at 35°C for possible campylobacter or helicobacter isolation.

Table 3.8.2–1 Taxonomic position, known sources, and common disease associations of *Campylobacter*, *Arcobacter*, *Helicobacter*, and related species[a]

Taxon[b]	Known source(s)	Disease association(s)	
		Human	Veterinary
rRNA homology group I			
***Campylobacter fetus* subsp. *fetus* ('*Campylobacter fetus* subsp. *intestinalis*')**	Cattle, sheep	Septicemia, gastroenteritis, abortion, meningitis	Bovine and ovine spontaneous abortion
***Campylobacter fetus* subsp. *venerealis* ('*Campylobacter fetus* subsp. *fetus*')**	Cattle	Septicemia	Bovine infectious infertility
***Campylobacter hyointestinalis* subsp. *hyointestinalis*[c]**	Pigs, cattle, hamsters, deer	Gastroenteritis	Porcine and bovine enteritis
Campylobacter hyointestinalis subsp. *lawsonii*[b]	Pigs	None at present	Unknown
Campylobacter concisus	Humans	Periodontal disease, gastroenteritis	None at present
Campylobacter mucosalis ("*Campylobacter sputorum* subsp. *mucosalis*")	Pigs	None at present	Porcine necrotic enteritis and ileitis
***Campylobacter sputorum* bv. Sputorum (incorporating bv. Bubulus[d])**	Humans, cattle, pigs	Abscesses, gastroenteritis	None at present
Campylobacter sputorum bv. Faecalis ('*Campylobacter fecalis*')	Sheep, bulls	None at present	None at present
***Campylobacter curvus* ("*Wolinella curva*")**	Humans	Periodontal disease, gastroenteritis	None at present
***Campylobacter rectus* ("*Wolinella recta*")**	Humans	Periodontal disease	None at present
***Campylobacter showae*[b] ('*Wolinella curva* subsp. *intermedius*')**	Humans	Periodontal disease	None at present
***Campylobacter gracilis*[d] ("*Bacteroides gracilis*")**	Humans	Periodontal disease, empyema, abscesses	None at present
***Campylobacter upsaliensis* ('CNW *Campylobacter*')**	Dogs, cats	Gastroenteritis, septicemia, abscesses	Canine and feline gastroenteritis
Campylobacter helveticus[c]	Cats, dogs	None at present	Feline and canine gastroenteritis
Campylobacter hyoilei[c,h]	Pigs	None at present	Porcine proliferative enteritis
Campylobacter coli	Pigs, poultry, bulls, sheep, birds	Gastroenteritis, septicemia	Gastroenteritis
Campylobacter jejuni* subsp. *jejuni	Poultry, pigs, bulls, dogs, cats, water, birds, mink, rabbits, insects	Gastroenteritis, septicemia, meningitis, abortion, proctitis, Guillain-Barré syndrome	Gastroenteritis, avian hepatitis
***Campylobacter jejuni* subsp. *doylei* ('NNC group')**	Humans	Gastroenteritis, gastritis, septicemia	None at present
***Campylobacter lari* ("*Campylobacter laridis.*" 'NARTC')**	Birds (including poultry), river water and seawater, dogs, cats, monkeys, horses, fur seals	Gastroenteritis, septicemia	Avian gastroenteritis
***Bacteroides* [*Campylobacter*] *ureolyticus*[e]**	Humans	Nongonococcal urethritis, necrotic tissue lesions, infected amniotic fluid, wound infections	None at present
"rRNA homology group Ia"			
Campylobacter-like, unnamed free-living species[e]	Anaerobic sludge	None at present	None at present

Table 3.8.2–1 *(continued)*

Taxon[b]	Known source(s)	Disease association(s)	
		Human	Veterinary
rRNA homology group II			
Arcobacter nitrofigilis ("*Campylobacter nitrofigilis*")	Plant roots and associated sediment	None at present	None at present
***Arcobacter butzleri* ("*Campylobacter butzleri*")**	Pigs, bulls, monkeys, humans, poultry, drinking water, river water, sewage, horses	Gastroenteritis, septicemia	Porcine, bovine, and primate gastroenteritis; porcine abortion
Arcobacter skirrowii	Sheep, bulls, pigs	None at present	Ovine and bovine gastroenteritis, porcine and bovine abortion
***Arcobacter cryaerophilus* ("*Campylobacter cryaerophila*")**	Pigs, bulls, sheep, horses, sewage	Gastroenteritis, septicemia	Porcine, bovine, ovine, and equine abortion
rRNA homology group III			
Wolinella succinogenes ("*Vibrio succinogenes*")	Bulls	None at present	None at present
***Helicobacter pylori* ("*Campylobacter pylori*," "*Campylobacter pyloridis*")**	Humans, nonhuman primates	Gastritis, peptic ulcer disease, gastric lymphoma, gastric adenocarcinoma	Gastritis in rhesus monkeys
Helicobacter acinonychis[c]	Cheetahs	None at present	Gastritis in cheetahs
Helicobacter nemestrinae[c]	Nonhuman primates	None at present	Gastritis in macaque monkeys
Gastrospirillum lemur[c]	Lemurs (presumed)	Unknown	Unknown
***Helicobacter heilmannii* ("*Gastrospirillum hominis* 1")**[f]	Humans	Gastritis	None at present
***Helicobacter heilmannii* ("*Gastrospirillum hominis* 2")**[f]	Humans	Gastritis	None at present
Gastrospirillum suis[c]	Pigs	None at present	Porcine gastritis
Helicobacter bizzozeronii[g] ("*Helicobacter bizazzoro*")	Dogs	None at present	Canine gastritis
Helicobacter felis[c]	Cats, dogs	None at present	Feline and canine gastritis
Helicobacter pullorum[i]	Poultry	Gastroenteritis	Avian hepatitis
***Helicobacter* CLO-3 ("CLO-3")**	Humans	Proctitis	None at present
***Helicobacter fennelliae* ("*Campylobacter fennelliae*")**	Humans	Gastroenteritis, septicemia, proctocolitis	None at present
***Helicobacter* sp. strain Mainz**[c]	Humans	Septic arthritis	None at present
***Helicobacter cinaedi* ("*Campylobacter cinaedi*")**	Humans, hamsters	Gastroenteritis, septicemia, proctocolitis	Hamster enteritis
'*Helicobacter westmeadii*'	Unknown	Bacteremia	Unknown
Helicobacter bilis[c]	Mice	None at present	Murine chronic hepatitis and hepatocellular tumors
***"Flexispira rappini"*[c] ("*Helicobacter rappini*")**	Humans, sheep, mice	Gastroenteritis, bacteremia	Ovine abortion
Helicobacter canis	Dogs	Gastroenteritis	Canine gastroenteritis
Helicobacter muridarum[c] ("*Helicobacter muridae*")	Rodents	None at present	Murine gastritis
Helicobacter hepaticus[c]	Mice	None at present	Murine necrotizing hepatitis
Helicobacter pametensis[c]	Birds, pigs	None at present	None at present
Helicobacter sp. strain Bird-B[c]	Birds	None at present	None at present
Helicobacter sp. strain Bird-C[c]	Birds	None at present	None at present
Helicobacter mustelae[e] ("*Campylobacter pylori* subsp. *mustelae*")	Ferrets, mink	None at present	Gastritis in ferrets

(continued)

Table 3.8.2–1 Taxonomic position, known sources, and common disease associations of *Campylobacter*, *Arcobacter*, *Helicobacter*, and related species[a] *(continued)*

Taxon[b]	Known source(s)	Disease association(s)	
		Human	Veterinary
Other campylobacter-like organisms phylogenetically distinct from rRNA superfamily VI			
Anaerobiospirillum succiniciproducens	Dogs	Gastroenteritis, septicemia	None at present
Anaerobiospirillum sp. strain Malnick et al. 1983	Dogs, cats	Gastroenteritis	None at present
Lawsonia intracellularis ("ileal symbiont intracellularis," "*Campylobacter intracellulare*")	Pigs, hamsters, ferrets	None at present	Proliferative enteritis in pigs and hamsters

[a] Table adapted from reference 16.

[b] All organisms listed belong to rRNA superfamily VI. Organisms in boldface type are associated wih human sources. Validly published or most commonly used nomenclature of the taxa described are given priority, with superseded (in quotation marks) or less common nomenclature given in parentheses. Names that have not been validly published are given in single quotation marks. Abbreviations: CNW, catalase negative—weak; NNC, nitrate-negative *Campylobacter;* NARTC, NA-resistant thermophilic *Campylobacter;* CLO, *Campylobacter*-like organism; IDO, intracellular *Desulfovibrio* organism.

[c] Likely phylogenetic position of taxon in rRNA superfamily VI based on 16S rRNA sequence comparisons or DNA-DNA hybridization data.

[d] Biovar descriptions conform with recent suggestions by On (16).

[e] Original phylogenetic position in rRNA superfamily VI emended with reference to available 16S rRNA sequence comparisons. Brackets indicate that the taxon is generically misnamed.

[f] The proposed name of '*H. heilmannii*' did not distinguish between the two phylogenetically distinct taxa referred to as '*G. hominis* 1' and '*G. hominis* 2.'

[g] Taxonomic position based upon marked morphological similarity to '*Gastrospirillum*' spp.

[h] Most current taxonomy indicates *C. hyoilei* to be indistinguishable from *C. coli* (23).

[i] *Helicobacter canadensis* is a newly described agent of human gastroenteritis and is closely related to *H. pullorum* (6).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

B. Specimen transport

1. Process within 1 to 2 h or place in transport medium.

◩ **NOTE:** *C. jejuni* is stable in feces for 3 days at 4°C and 2 days at 25°C and is unstable at freezing temperatures. It has a similar stability at the respective temperatures in modified Cary-Blair medium, which will inhibit the growth of normal fecal microbiota (11).

2. Transport in modified Cary-Blair medium (formulation modified with reduced agar content of 1.6 g/liter rather than 5 g/liter).

a. Purchase from commercial source *or*

b. Prepare as follows.

(1) Dispense the following ingredients into 991 ml of H_2O.

sodium thioglycolate ..... 1.5 g
disodium phosphate ..... 1.1 g
sodium chloride ..... 5.0 g
phenol red ..... 0.003 g
agar ..... 1.6 g

(2) Heat to dissolve and cool to 50°C. Then add 9 ml of 1% $CaCl_2$.

(3) Adjust pH to 8.4; dispense in vials and steam for 15 min.

(4) Cool and tighten caps.

3. *Avoid phosphate-buffered glycerol transport media.*

4. If there is a delay in transport, refrigerate transport vial at 4°C.

C. Refer to procedure 3.8.1 for other collection, labeling, transport, QC, and rejection criteria.

Table 3.8.2–2 Human disease associations of *Campylobacter* species by clinical syndrome

Organism(s)	Human disease association						
	GI[a]	Bacteremia	Meningitis	Abortion	Periodontitis	Wounds/abscesses	Other
C. jejuni	×	×	×	×		×	Guillain-Barré syndrome
C. jejuni subsp. *doylei*	×						
C. coli	×						
C. fetus (subsp. *fetus* and *venerealis*)	×	×	×	×		×	
C. upsaliensis	×	×				×	
C. hyointestinalis	×						
C. showae					×		
C. concisus	×				×		
C. curvus					×		
C. mucosalis	×						
C. rectus					×	×	
C. sputorum						×	
C. gracilis (*Sutterella wadsworthensis*)					×	×	
C. lari		×					Urinary tract infections
C. sputorum						×	
C. ureolytism					×		
Arcobacter butzleri	×	×					
A. cryaerophilus	×	×					
H. fennelliae	×	×					Proctocolitis
H. cinaedi	×	×				×	Cellulitis, proctocolitis
H. pullorum	×						
H. canadensis	×						
H. westmeadii		×					
H. pylori							Duodenal and gastric ulcer, mucosa-associated lymphoid tissue, gastric carcinoma
"*Flexispira rappini*"	×	×					

[a] GI, gastrointestinal.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

D. Other rejection criteria

1. Reject fecal specimen if it has been frozen.

2. Reject fecal specimen in transport media other than *modified* Cary-Blair medium.

III. MATERIALS

A. Media

◩ **NOTE:** The use of more than one type of selective medium increases the yield from stools by as much as 15% (4). Cephalothin, colistin, and polymyxin B in some selective media are inhibitory to *C. fetus*, *C. jejuni* subsp. *doylei*, and *C. upsaliensis* and may inhibit some strains of *C. jejuni* and *C. coli*. Cefoperazone is now considered to be the preferred antimicrobial agent, as it inhibits *Pseudomonas* species and other gram-negative normal microbiota but does not inhibit growth of campylobacters

1. Blood-free charcoal contains Columbia agar, charcoal, pyruvate, and hemin and cefoperazone, vancomycin, and amphotericin B (or cycloheximide) (4).

III. MATERIALS *(continued)*

2. Blood-free charcoal-cefoperazone-desoxycholate agar (CCDA) contains desoxycholate, charcoal base (CM 739; Oxoid), and cefoperazone (9).
3. Campy-CVA contains brucella agar with 5% sheep blood, cefoperazone, vancomycin, and amphotericin B.

B. Stain reagents

1. Gram stain (procedure 3.2.1) with carbol fuchsin or 0.1% basic fuchsin for colony smears
2. Acridine orange (procedure 3.2.2) for problem cultures
3. Wet mount (procedure 3.2.3) for direct specimen

C. Other supplies

1. A microaerobic environment

   ◩ **NOTE:** Most campylobacters require a microaerobic environment of approximately 5% O_2 and 85% N_2 for optimal growth. Increased H_2 appears to be a growth requirement for the more fastidious campylobacters (*C. sputorum, C. concisus, C. curvus, C. mucosalis,* and some strains of *C. hyointestinalis*). These strains are not of significance in fecal cultures, but they may be isolated from blood and may require anaerobic incubation for growth.

   a. Use an anaerobic jar, without a catalyst, and a gas mixture containing 10% CO_2–5% H_2–balance N_2. Evacuate and fill jar two times to achieve a final atmosphere of 5% O_2, 7% CO_2, and 4% H_2.

   b. Alternatively, use a leakproof Saranex zipper bag (Associated Bag Company, [800] 926-6100) and fill once with 5% O_2, 10% CO_2, balance N_2 (Campy Gas Cylinder, catalog no. S0730; PML Microbiologicals, Inc., Wilsonville, OR, [800] 547-0659, http://www.pmlmicro.com)

   c. See Table 3.8.2–3 for commercial gas systems.

2. Incubator at 42 and 35°C
3. Identification—media and biochemical tests

   a. Heart infusion agar with 5% rabbit blood (BBL catalog no. 297472; BD Diagnostic Systems), BAP, or brucella agar with 5% sheep blood for disk tests and temperature growth tests

   b. Heart infusion broth (HIB) or BHI for inoculum preparation (BD Diagnostic Systems)

   c. Oxidase (procedure 3.17.39)

   d. Catalase (3% H_2O_2) (procedure 3.17.10)

   e. Hippurate (procedure 3.17.21 and reference 13)

   f. Indoxyl acetate disks (procedure 3.17.24 and reference 19)

   g. Rapid urea hydrolysis (procedure 3.17.48 and references 12 and 17)

   h. Disks—store at −20°C (BD Diagnostic Systems; Remel, Inc.).
      (1) Nalidixic acid (NA) disks, 30 µg
      (2) Cephalothin or cefazolin (CF [abbreviation stands for either drug]) disks, 30 µg
      (3) Ciprofloxacin, 5 µg, and erythromycin, 15 µg, disks (optional)

   i. Nitrate test (procedure 3.17.35)

   j. Triple sugar iron (TSI) (procedure 3.17.22)

      ◩ **NOTE:** For use in the identification of campylobacters, TSI must be freshly prepared and used within 1 week (*CDC Campylobacter Reference Lab Procedure Manual*). Incubate test at 35°C for 72 h before calling result negative.

Table 3.8.2–3 Commercial systems for generating microaerobic environments and the approximate atmospheric content

System	Final atmosphere
BBL Campy Pak	6.9% O_2, 5.5–7.0% CO_2, 0.2% H_2
BBL Campy Pouch	7.5–9.0% O_2, 11.5–14% CO_2, 0.5–1.5% H_2
BBL Bio Bag Cfj	10.5–15% O_2, 7–15% CO_2, 6.5–12.5% H_2
Oxoid CampyGen[a]	6% O_2, 15% CO_2
Mitsubishi Pack-Campylo[a]	6% O_2, 14% CO_2

[a] This system produces negligible H_2 and may not grow H_2-requiring species.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Verify that media meet expiration date and QC parameters per Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22-A3 (3). See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

B. QC test *each lot* of campylobacter medium for growth requirements and inhibition of fecal microbiota, per CLSI document M22-A3 (3). See Table 3.8.1–3 for QC organisms and testing details.

C. QC each lot of transport media with *C. jejuni* ATCC 33291 by following the method outlined in section 14, Table 14.2–2, for QC listed under "Transport."

D. Test each new lot of CF and NA disks before use in the manner described in item V.D.6 below.

1. *E. coli* ATCC 25922; zone size range for NA = 22 to 28 mm; that for CF = 15 to 21 mm

2. *C. jejuni* ATCC 33291; >20-mm zone for NA; no zone for CF

◩ **NOTE:** QC erythromycin and ciprofloxacin disks as part of the QC for susceptibility testing, using the usual QC organisms listed in section 5.

E. Test each lot of gas system to ensure that it supports the growth of *C. jejuni.*

F. For testing the ability of an organism to grow in a microaerobic atmosphere at various temperatures, perform QC on each incubator or temperature condition, when put into use for this test, with the following to determine if incubator temperatures and atmospheric conditions are in control.

1. *Arcobacter cryaerophilus* ATCC 43158 (aerobic growth; no microaerobic growth at 42°C)

2. *C. jejuni* ATCC 33291 (no microaerobic growth at 25°C)

3. *C. fetus* subsp. *fetus* ATCC 27374 (microaerobic growth at 25, 35 to 37, and 42°C)

V. PROCEDURE

Observe standard precautions.

A. Microscopic (rarely performed but can be helpful in isolated cases)

1. Perform Gram stain in selected cases during the acute phase of diarrhea for visualization of campylobacters (sensitivity, 66 to 94%) (18, 20).

2. Visualize motile campylobacters in wet mounts of very fresh stools (<30 min old) as darting across the field in a zigzag fashion (procedure 3.2.3).

3. Use acridine orange stain for problem cultures (procedure 3.2.2).

B. Direct antigen detection

ProSpecT *Campylobacter* microplate assay for stool specimens (Remel, Inc.) is an EIA for detection of *Campylobacter* specific antigen found in *C. jejuni* and *C. coli.* There is no differentiation between the two species. The test cross-reacts with *C. upsaliensis* but not other species of *Campylobacter* (8). Performance analysis demonstrated the assay to be 96 and 89% sensitive and 99% specific in two independent studies (8, 21), with a cost in excess of that of culture. See Appendix 3.8.2–1 for details.

C. Culture

◩ **NOTE:** Use of enrichment broth cultures (Preston enrichment, Campy-THIO, Campylobacter enrichment) has been reported to increase isolation of campylobacters when specimens are obtained after the acute stage of diarrhea and the broth is incubated overnight at 42°C and subcultured to selective media. However, the value of these enrichments has not been studied adequately, although the use of two primary media or the addition of the filtration technique also increases recovery.

1. Feces—routine plating

a. Place 1 to 3 drops of stool or broth from transport vial on one or two of the following selective agars: blood-free charcoal, CCDA, or Campy-

V. PROCEDURE *(continued)*

CVA. Alternatively inoculate plates using a swab dipped into the transport vial, and completely roll the swab on the medium plate in an area the size of a nickel.

b. Streak in four quadrants.

c. Place the plate *immediately* in a microaerobic environment.

d. Incubate at 42°C for 72 h to screen for thermotolerant *Campylobacter* and *Helicobacter* species.

◩ **NOTE:** If detection of species other than *C. jejuni* and *C. coli* is desired, incubate a second plate at 35 to 37°C. Since most species that do not grow at 42°C will grow at 40°C, use of a 40°C incubator will allow one temperature to be used for all species.

2. Feces—filtration technique

◩ **NOTE:** This technique has been successful for isolation of campylobacters that are susceptible to antimicrobial agents in media. Passage of motile campylobacters through the pores of the filter allows for selective separation of campylobacters from the nonmotile stool constituents. This method will yield a large variety of *Campylobacter* species, not all of which have been clearly linked with disease.

a. Place a sterile cellulose acetate membrane filter, with a 47-mm diameter and a 0.65-µm pore size (Sartorius Corp., Edgewood, NY, [800] 635-2906, http://www.sartorius.com), directly on the surface of a BAP.

b. Add 6 to 8 drops of the stool directly on the filter.

c. Incubate for 45 min to 1 h in ambient air at 35 to 37°C.

d. Remove filter with sterile forceps, taking care not to allow any residual stool on the filter to drip on the plate. *Do not streak for isolation.*

e. Immediately place the plate in a microaerobic environment at 35 to 37°C for a *minimum of 72 h or up to 6 days* for recovery of fastidious and slow-growing organisms (5).

D. Identification methods

1. Examine direct plates for growth of campylobacters.

a. On freshly prepared media, colonies are gray, flat, irregular, and spreading; sometimes they may appear mucoid.

b. Some strains will appear as a thin film on the medium and require careful observation to detect growth (best done by scraping a loop over the surface of the medium). They can also appear as colonies that tail along the line of streaking.

c. On less fresh medium, colonies, 1 to 2 mm in diameter, appear as gray, round, convex, and glistening, with little or no spreading.

d. Colonies can be yellowish to gray or pinkish and are nonhemolytic.

e. *Helicobacter cinaedi* and *Helicobacter fennelliae* growth on fresh media in a moist environment may spread as a uniform film over the entire surface of the plate. *H. fennelliae* has the distinct odor of hypochlorite. They require 5 to 10% hydrogen for growth and generally do not grow at 42°C.

f. Check aerobic BAP (procedure 3.8.1) for oxidase-positive colonies at 24 h, which are likely *Pseudomonas* or *Aeromonas.* Then reincubate if negative and recheck at 96 h. If positive at 4 to 5 days and not at 48 h, *Arcobacter* could be present.

2. Perform Gram stain on suspicious colonies using basic fuchsin or carbol fuchsin counterstain promptly after removal from the microaerobic environment.

a. Look for small gram-negative rods shaped like spirals, gull wings, archery bows, or commas, consistent with *Campylobacter* species. Coccoidal forms may be seen in the Gram stain, especially in older cultures or cultures exposed to ambient air for prolonged periods.

V. PROCEDURE *(continued)*

b. *H. cinaedi* and *H. fennelliae* Gram stain as long, wavy, gram-negative rods.

c. "*Flexispira rappini*," also called *Helicobacter* sp. strain *flexispira*, closely associated with helicobacters, is a long, fusiform, gram-negative rod, slightly curved.

(1) Perform urea test if Gram stain suggests fusiform rods.

(2) *Helicobacter* sp. strain *flexispira* as a cause of gastroenteritis is generally catalase negative.

(3) *Helicobacter* sp. strain *flexispira* grows at 42°C and is urea positive.

3. Perform direct wet mount examination for motility (*see* procedure 3.17.31, with the following caveats).

a. Emulsify a loopful of 24- to 48-h bacterial growth in broth (not saline or distilled water).

b. Observe microscopically for darting motility typical of spiral rods or vibrio-like organisms.

4. Perform the following rapid assays from colonies isolated at 42°C and use Fig. 3.8.2–1 to identify to the species level.

a. Oxidase (procedure 3.17.39)

b. Catalase (procedure 3.17.10)

c. Hippurate hydrolysis (13); use a 48-h culture if growth is poor at 24 h (procedure 3.17.21)

5. Incubate a plate aerobically at 35°C; *C. jejuni* should *not* grow under these conditions.

6. Perform disk susceptibility tests (14).

a. Prepare a bacterial suspension of a 24- to 48-h culture in HIB or BHI to a turbidity equal to a McFarland no. 1 standard.

b. Dip cotton- or polyester-tipped swab in the suspension, drain against side of tube, and inoculate entire surface of BAP.

c. Place NA and CF disks in separate quadrants of the plate surface.

d. It is helpful for both identification and treatment to add a 5-μg ciprofloxacin and 15-μg erythromycin disk to the plate.

e. Incubate the plate overnight at 40 to 42°C (use 35 to 37°C if the isolate does not grow at 40 to 42°C) in a microaerobic atmosphere.

f. Read and record presence (susceptible) or absence (resistant) of zone of inhibition. If background growth is poorly defined, reincubate the plate and read in 48 h.

g. If species can be determined from Fig. 3.8.2–1, omit further testing.

7. If isolate is hippurate negative, perform rapid indoxyl acetate hydrolysis test (procedure 3.17.24 and reference 19), and identify using Fig. 3.8.2–1.

8. For all other identifications, inoculate the following biochemical tests and use Table 3.8.2–4 to identify species.

a. Nitrate (procedure 3.17.35)

b. Urease (procedure 3.17.48)

c. TSI (procedure 3.17.22)

d. Prepare inoculum from a 24- to 48-h culture on heart infusion agar with 5% rabbit blood or BAP at 35 to 37°C in a microaerobic atmosphere. Alternatively, use growth from a 72-h culture, if the organism grows slowly. If primary isolation was at 42°C, the biochemical tests may be performed at 42°C.

e. Test for the ability of an organism to grow in a microaerobic atmosphere at various temperatures (2).

(1) Prepare a bacterial suspension of a 24- to 48-h culture in HIB or BHI to a turbidity equal to a McFarland no. 1 standard.

(2) Label four BAPs: microaerobic, 25°C; microaerobic, 35°C; microaerobic, 42°C; and aerobic, 35°C.

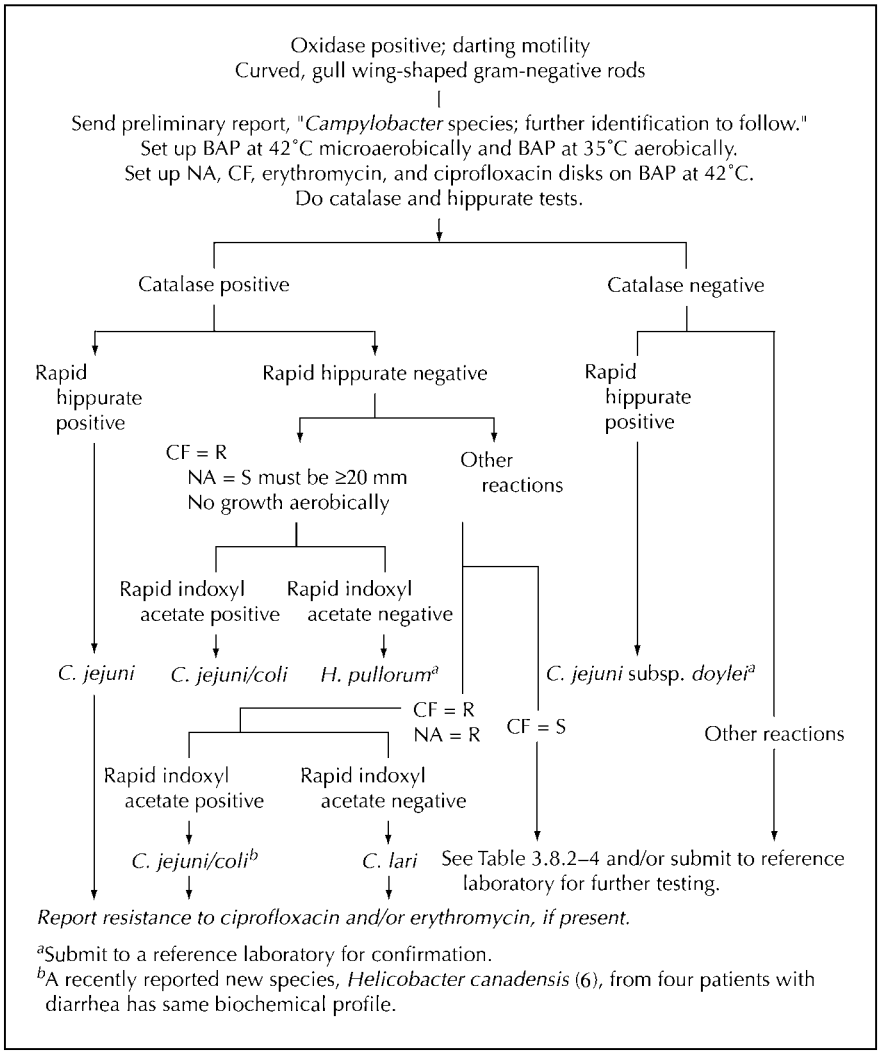

Oxidase positive; darting motility
Curved, gull wing-shaped gram-negative rods
Send preliminary report, "*Campylobacter* species; further identification to follow."
Set up BAP at 42°C microaerobically and BAP at 35°C aerobically.
Set up NA, CF, erythromycin, and ciprofloxacin disks on BAP at 42°C.
Do catalase and hippurate tests.
Catalase positive
Catalase negative
Rapid hippurate positive
Rapid hippurate negative
Rapid hippurate positive
CF = R
NA = S must be ≥20 mm
No growth aerobically
Other reactions
Rapid indoxyl acetate positive
Rapid indoxyl acetate negative
C. jejuni
C. jejuni/coli
H. pullorum[a]
C. jejuni subsp. doylei[a]
CF = R
NA = R
CF = S
Other reactions
Rapid indoxyl acetate positive
Rapid indoxyl acetate negative
C. jejuni/coli[b]
C. lari
See Table 3.8.2–4 and/or submit to reference laboratory for further testing.
Report resistance to ciprofloxacin and/or erythromycin, if present.
[a]Submit to a reference laboratory for confirmation.
[b]A recently reported new species, Helicobacter canadensis (6), from four patients with diarrhea has same biochemical profile.

Figure 3.8.2–1 *Campylobacter* identification flowchart for minimum identification of *C. jejuni* from stool specimens. Abbreviations: R, no zone; S, zone.

V. PROCEDURE *(continued)*

(3) Mark lines across the bottom of each plate approximately 3/4 in. apart to indicate where inoculum will be placed. Three cultures can be streaked on each agar plate. Label lines with culture numbers.

(4) Moisten a cotton swab in the bacterial suspension and make a single streak across each plate, rotating the swab one-third of a turn before inoculating the next plate.

(5) Incubate each plate under its stated temperature and atmospheric conditions for 3 days.
   (a) Aerobic at 35°C (to screen for *Arcobacter* species)
   (b) Microaerobic at 35°C (to screen for nonthermotolerant *Campylobacter* and *Helicobacter* species)
   (c) Microaerobic at 40 to 42°C (to screen for thermotolerant *Campylobacter* and *Helicobacter* species)
   (d) Microaerobic at 25°C (to screen for *C. fetus*).

Table 3.8.2–4 Phenotypic reactions of clinically important *Campylobacter* and *Helicobacter* species[a]

Identification test	*C. jejuni*	*C. jejuni* subsp. *doylei*	*C. coli*/ *C. jejuni*, hippurate negative	*C. lari*[b]	*C. fetus* subsp. *fetus*	*C. upsaliensis*	*A. cryaerophilus*/ *A. butzleri*[c]	*C. hyointestinalis*	*H. cinaedi*/ *CLO1B*[d]	*H. fennelliae*[d]	*H. pullorum*
Oxidase	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	V	+	+	+	0/W	V	+	+	+	+
Aerobic growth, 35–37°C	0	0	0	0	V[e]	0	+	0	0	0	0
Microaerobic growth, 25°C	0	0	0	0	V	0	+	V	0	0	0
Microaerobic growth, 35–37°C	+	+	+	+	+	+	+	+	+	+	+
Microaerobic growth, 40–42°C	+	V	+	+	V	+	V	+	V	V	+
Hippurate hydrolysis	+	+	0	0	0	0	0	0	0	0	0
Indoxyl acetate	+	+	+	0	0	+	+	0	0	+	0
NA resistant	0[f]	0	0[f]	+	+	0[f]	V	+	0	0	0
CF resistant	+	V	+	+	V	0	V	0	0	V	+
Nitrate reduction	+	0	+	+	+	+	V	+	+	0	+
H_2S in TSI agar	0	0	V[g]	V	0	0	0	+	0	0	0
Urea hydrolysis[h]	0	0	0	0	0	0	0	0	0	0	0

[a] +, positive reaction; 0, negative reaction; w, weakly positive; V, variable reaction, NA, not available. See procedure 3.8.4 for *H. pylori* identification.

[b] Urease-positive thermophilic campylobacters or *C. lari*-like strains may be found (12).

[c] Growth at 42°C; catalase negativity suggests *A. butzleri*.

[d] *H. cinaedi*/*CLO1B* can be separated by DNA homology tests. *H. cinaedi*/*CLO1B*, *H. fennelliae*, and *H. pylori* can be definitively identified by cellular fatty acid analysis (10).

[e] Rare *C. fetus* subsp. *fetus* strains are aerobic.

[f] These species are historically sensitive to NA; however, resistant strains are seen in as high as 35% of isolates due to acquired fluoroquinolone resistance, which may make this assay less useful in identification.

[g] H_2S in TSI suggests *C. coli*.

[h] There are isolated reports of *Helicobacter* species that are urease producing other than *H. pylori* (24).

V. PROCEDURE *(continued)*

(6) Interpretation: record the amount of 72-h growth as follows.
   (a) No growth
   (b) Trace
   (c) 1+ (light)
   (d) 2+ (moderate)
   (e) 3+ (heavy)

9. Other tests

a. Cellular fatty acid analysis (MIDI Laboratories, Newark, DE, [302] 737-4297, http://www.midi-inc.com/) (10)

◩ **NOTE:** This method cannot differentiate *C. jejuni* and *C. coli*, the two most common species.

b. Species-specific PCR or 16S rDNA sequencing (CDC Campylobacter Laboratory, Atlanta, GA; MIDI Laboratories)

c. *C. jejuni*-specific DNA probe (16) (BioProbe; Enzo Diagnostics, Farmingdale, NY, [631] 694-7070, http://www.enzobio.com).

d. AccuProbe *Campylobacter* culture identification test (Gen-Probe, Inc., http://www.gen-probe.com/) for DNA identification of *C. jejuni*/*coli*/*lari* colonies does not differentiate among these three species.

◩ **NOTE:** The AccuProbe system uses a single-stranded DNA probe with a chemiluminescent label that recognizes RNA sequences that are unique to *C. jejuni*, *C. coli*, and *C. lari*. Following the release of the target RNA, the DNA probe forms a stable DNA:RNA hybrid. A selection reagent inactivates unhybridized probe and the labeled DNA:RNA hybrids are detected in a luminometer.

(1) Label a sufficient number of probe reagent tubes to test the number of culture isolates and a positive (*C. jejuni* ATCC 33560) and negative (*C. fetus* ATCC 27374) control.
(2) Pipette 50 µl of lysis reagent into all tubes.
(3) Transfer a 1-µl loopful of cells or several small colonies (<48 h old) to the tubes and twirl the loop to remove the cells. Test a positively and negatively reacting organism with each run.
(4) Pipette 50 µl of hybridization buffer into all tubes and mix by vortexing.
(5) Incubate tubes for 15 min at 60°C in a water bath or dry-heat block.
(6) Remove and cool the tubes. Pipette 300 µl of selection reagent to each tube and mix by vortexing.
(7) Incubate tubes for 5 min at 60°C in a water bath or dry-heat block.
(8) Cool the tubes to room temperature and read results in the luminometer within 30 min. Cutoff values for positive and negative signals are established by the manufacturer for the type of luminometer used.

◩ **NOTE:** The AccuProbe assay was compared to standard culture and biochemical identification using 301 isolates of thermophilic *Campylobacter* species (*C. jejuni*, *C. coli*, and *C. lari*), 41 isolates of other *Campylobacter* spp., and 300 other bacterial cultures from 53 genera at three clinical sites. The sensitivities and specificities were 100 and 99.1%, 100 and 100%, and 100 and 100%, respectively. One false positive was detected with *C. hyointestinalis* (product insert).

e. Oxoid Dryspot *Campylobacter* test (latex agglutination), product code DR0150

◩ **NOTE:** The Oxoid Dryspot *Campylobacter* test is a latex agglutination test for the identification of enteropathogenic campylobacters from solid culture media. *C. jejuni*, *C. lari*, *C. coli*, *C. fetus*, and *C. upsaliensis* will give positive results. Isolates of other *Campylobacter* spp. such as *C. fetus* subsp. *fetus* will give variable results.

V. PROCEDURE *(continued)*

(1) Test 48-h colonies with morphology suggestive of *Campylobacter* spp. from *Campylobacter* selective media.
(2) Remove the reagents from the refrigerator and allow to reach room temperature.
(3) Label a sufficient number of reagent tubes to test the number of culture isolates (and a positive and negative control, which should be tested with each new lot and every 6 months thereafter).
(4) Add 1 drop of extraction reagent 1 into the extraction tube.
(5) Remove sufficient suspect growth to fill the internal diameter of the sterile loop.
(6) Thoroughly suspend the cells in the drop of reagent 1. Allow the loop to stand in reagent 1 for 3 min. Do not discard the loop.
(7) Add 2 drops of extraction reagent 2 to the extract. Mix using the retained loop.
(8) Using a paddle pipette, place 1 drop (50 μl) of the neutralized extract onto the test circle and 1 drop onto the control circle.
(9) Using the flat end of the paddle pastette, mix the extract into the dry control reagent spot until completely resuspended. Spread to cover the reaction area. Use the same pastette to repeat this procedure for the test reagent.
(10) Pick up and rock the card for 3 min. Look for agglutination under normal lighting conditions. Do not use a magnifying glass.
(11) Interpretation
A result is positive if agglutination of the latex particles occurs within 3 min; this indicates the presence of *Campylobacter* spp. A negative result is obtained if no agglutination occurs and a smooth blue suspension remains in the test area after 3 min of mixing. This indicates that the isolate is not *C. jejuni, C. coli, C. upsaliensis,* or *C. lari.* Reactions occurring after 3 min should be ignored. The test is uninterpretable if the control reagent shows agglutination. This indicates that the culture causes autoagglutination.
(12) The performance of the Oxoid Dryspot *Campylobacter* test was evaluated at one clinical center in Finland. The sensitivity and specificity in the study were 100 and 100%, respectively. In addition, a total of 93 stock cultures and clinical isolates were tested at a clinical center in the United Kingdom. Strains were cultured on Columbia blood agar and/or modified CCDA and CAT (charcoal-based medium containing cefoperazone, amphotericin, and teicoplanin) agars at 37 or 42°C, under microaerobic conditions for 48 to 72 h. The assay detected all *C. jejuni, C. coli, C. lari, C. upsaliensis, C. hyointestinalis,* and *C. mucosalis* isolates tested, six of eight *C. fetus* subsp. *fetus* isolates, and one of two *H. pylori* isolates. Other species of *Campylobacter, Arcobacter,* and *Helicobacter* as well as 23 other genera of organisms failed to react in the kit.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Direct smears

1. If Gram stain of fecal specimens demonstrates the presence of gull wing-shaped, curved, gram-negative rods, report as "Presumptive *Campylobacter* species."

2. If wet mount of stool demonstrates darting motility, report as "Presumptive *Campylobacter* species."

B. Culture

1. All *Campylobacter* and *Helicobacter* strains are oxidase positive, vibrio-like, or spiral gram-negative rods that have darting motility.

2. Report "Presumptive *Campylobacter* species" if the following criteria are met.

a. Growth has typical colonial morphology on selective media in a microaerobic environment incubated at 42°C.

b. Curved, gram-negative rods are demonstrated on Gram stain from colony.

c. Organism exhibits darting motility.

d. Organism is oxidase positive and catalase positive.

3. Use the biochemical tests listed in Table 3.8.2–4 and the flowchart in Fig. 3.8.2–1 to differentiate *Campylobacter* and *Helicobacter* species of human importance. Report *C. jejuni, C. jejuni* subsp. *doylei, C. jejuni/coli, C. lari,* or *Helicobacter pullorum* based on the flowchart in Fig. 3.8.2–1.

4. If isolate is not identified by Fig. 3.8.2–1, report species as denoted by biochemical characterization in Table 3.8.2–4.

5. If unable to identify isolates using the above schemes or to confirm identification, submit to a reference laboratory. Report "Campylobacter-like organism; submitted to reference laboratory for definitive identification."

◩ **NOTE:** For definitive identification, cellular fatty acid analysis and/or molecular analysis may be required.

C. Susceptibility testing

1. Standardized antimicrobial susceptibility testing methods for testing the susceptibility of *Campylobacter* isolates, with the exception of the agar dilution method, are not available.

2. However, the lack of a zone around either erythromycin or ciprofloxacin disks indicates that the isolate is likely resistant to the antimicrobial agent.

◩ **NOTE:** In over 200 disk diffusion tests with erythromycin and ciprofloxacin, there was consistently a bimodal distribution of results, with most results either at ≥25 mm or showing no zone (6 mm). More recently, zone sizes have been found to be smaller, but the bimodal distribution is still valid. No *Campylobacter* isolates have been found that are susceptible to NA (large zone) but resistant to ciprofloxacin (no zone) (R. Nadarajah, personal communication).

3. If there is no zone around the ciprofloxacin or erythromycin disk, enter the following: "By a nonstandard susceptibility test method, isolate is resistant to [drug]."

4. Do not report any isolate as susceptible, since there are no standardized interpretive criteria for *Campylobacter.*

D. Notification

1. Notify caregiver of positive findings.

2. Report *Campylobacter* in fecal cultures to the local health department, per local reportable-disease regulations.

VII. INTERPRETATION

A. Treatment with hydration and electrolyte balance is the cornerstone of therapy for *C. jejuni.* In cases with high fever, bloody stools, and symptoms lasting greater than 1 week, antimicrobial treatment is indicated and can reduce the symptoms and severity as well as reduce the likelihood of complications, such as Guillain-Barré syndrome. Because of these complications, resistance to quinolones needs to be communicated to the physician.

B. *C. jejuni* is a major cause of bloody diarrhea that can have serious sequelae. Isolation of other species in fecal cultures, especially *C. upsaliensis, Arcobacter* spp., and *H. pullorum,* is generally associated with milder diseases.

C. Because campylobacters and helicobacters are biochemically inert bacteria, few phenotyic tests are available in the clinical laboratory to group them by species. Most clinical laboratories are capable of characterizing the common species that are of medical importance.

VIII. LIMITATIONS

A. Methods presented here are designed to recover and identify only the most common pathogenic strains of *Campylobacter,* with isolation and definitive identification of *C. jejuni,* the most common species. The optimal methods to recover campylobacters from clinical specimens have yet to be determined. While confidence can be placed in routine methods for detection of the most common agents in diarrhea *(C. jejuni* and *C. coli),* recovery procedures for other agents are not clearly defined.

B. For recovery of campylobacters from nonfecal specimens, it is essential that there be communication between the clinician and the laboratory. It is impractical and unreasonable for a laboratory to routinely plate specimens under multiple atmospheric conditions and at different temperatures. An understanding of the critical growth requirements is necessary to establish optimal conditions for recovery of these organisms in clinical specimens.

C. If *C. jejuni* is resistant to fluoroquinolones, it will be resistant to NA, and the disk will not be useful for identification.

D. Other strains of *Helicobacter* besides *H. pylori* produce urease, including "*Flexispira rappini*"; these isolates are best identified with molecular assays (24).

REFERENCES

1. **Allos, B. M.** 2001. *Campylobacter jejuni* infections: update on emerging issues and trends. *Clin. Infect. Dis.* **32:**1201–1206.
2. **Barrett, T. J., C. M. Patton, and G. K. Morris.** 1988. Differentiation of *Campylobacter* species using phenotypic characterization. *Lab. Med.* **19:**96–102.
3. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved Standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Endtz, H. P., G. J. Ruijs, A. H. Zwinderman, T. van der Reijden, M. Biever, and R. P. Mouton.** 1991. Comparison of six media, including semisolid agar, for the isolation of various *Campylobacter* species from stool specimens. *J. Clin. Microbiol.* **29:**1007–1010.
5. **Engberg, J., S. L. On, C. S. Harrington, and P. Gerner-Smidt.** 2000. Prevalence of *Campylobacter, Arcobacter, Helicobacter,* and *Sutterella* spp. in human fecal samples as estimated by a reevaluation of isolation methods for campylobacters. *J. Clin. Microbiol.* **38:**286–291.
6. **Fox, J. G., C. C. Chien, F. E. Dewhirst, B. J. Paster, Z. Shen, P. L. Melito, D. L. Woodward, and F. G. Rogers.** 2000. *Helicobacter canadensis* sp. nov. isolated from humans with diarrhea as an example of an emerging pathogen. *J. Clin. Microbiol.* **38:**2546–2549.
7. **Friedman, C. R., J. Neimann, H. C. Wegener, and R. V. Tauxe.** 2000. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations, p. 121–138. *In* I. Nachamkin and M. J. Blaser (ed.), *Campylobacter,* 2nd ed. ASM Press, Washington, DC.
8. **Hindiyeh, M., S. Jense, S. Hohmann, H. Benett, C. Edwards, W. Aldeen, A. Croft, J. Daly, S. Mottice, and K. C. Carroll.** 2000. Rapid detection of *Campylobacter jejuni* in stool specimens by enzyme immunoassay and surveillance for *Campylobacter upsaliensis* in the greater Salt Lake City area. *J. Clin. Microbiol.* **38:**3076–3079.
9. **Hutchinson, D. N., and F. J. Bolton.** 1984. Improved blood free selective medium for the isolation of *Campylobacter jejuni* from faecal specimens. *J. Clin. Pathol.* **37:**956–957.

REFERENCES *(continued)*

10. **Lambert, M. A., C. M. Patton, T. J. Barrett, and C. W. Moss.** 1987. Differentiation of *Campylobacter* and *Campylobacter*-like organisms by cellular fatty acid composition. *J. Clin. Microbiol.* **25:**706–713.
11. **Luechtefeld, N. W., W. L. Wang, M. J. Blaser, and L. B. Reller.** 1981. Evaluation of transport and storage techniques for isolation of *Campylobacter fetus* subsp. *jejuni* from turkey cecal specimens. *J. Clin. Microbiol.* **12:**438–443.
12. **Megraud, F., D. Chevrier, N. Desplaces, A. Sedallian, and J. L. Guesdon.** 1988. Urease-positive thermophilic *Campylobacter* (*Campylobacter laridis* variant) isolated from an appendix and from human feces. *J. Clin. Microbiol.* **26:**1050–1051.
13. **Morris, G. K., M. R. el Sherbeeny, C. M. Patton, H. Kodaka, G. L. Lombard, P. Edmonds, D. G. Hollis, and D. J. Brenner.** 1985. Comparison of four hippurate hydrolysis methods for identification of thermophilic *Campylobacter* spp. *J. Clin. Microbiol.* **22:**714–718.
14. **Morris, G. K., and C. M. Patton.** 1985. *Campylobacter*, p. 302–308. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), *Manual of Clinical Microbiology*, 4th ed. American Society for Microbiology, Washington, DC.
15. **Nachamkin, I., H. Ung, and M. Li.** 2002. Increasing fluoroquinolone resistance in *Campylobacter jejuni*, Pennsylvania, USA, 1982–2001. *Emerg. Infect. Dis.* **8:**1501–1503.
16. **On, S. L.** 1996. Identification methods for campylobacters, helicobacters, and related organisms. *Clin. Microbiol. Rev.* **9:**405–422.
17. **Owen, R. J., S. R. Martin, and P. Borman.** 1985. Rapid urea hydrolysis by gastric campylobacters. *Lancet* **1:**111.
18. **Park, C. H., D. L. Hixon, A. S. Polhemus, C. B. Ferguson, S. L. Hall, C. C. Risheim, and C. B. Cook.** 1983. A rapid diagnosis of *Campylobacter* enteritis by direct smear examination. *Am. J. Clin. Pathol.* **80:**388–390.
19. **Popovic-Uroic, T., C. M. Patton, M. A. Nicholson, and J. A. Kiehlbauch.** 1990. Evaluation of the indoxyl acetate hydrolysis test for rapid differentiation of *Campylobacter*, *Helicobacter*, and *Wolinella* species. *J. Clin. Microbiol.* **28:**2335–2339.
20. **Sazie, E. S. M., and A. E. Titus.** 1982. Rapid diagnosis of *Campylobacter* enteritis. *Ann. Intern. Med.* **96:**62–63.
21. **Tolcin, R., M. M. LaSalvia, B. A. Kirkley, E. A. Vetter, F. R. Cockerill III, and G. W. Procop.** 2000. Evaluation of the Alexon-Trend ProSpectT *Campylobacter* microplate assay. *J. Clin. Microbiol.* **38:**3853–3855.
22. **Vandamme, P., E. Falsen, R. Rossau, B. Hoste, P. Segers, R. Tytgat, and J. De Ley.** 1991. Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. *Int. J. Syst. Bacteriol.* **41:**88–103.
23. **Vandamme, P., L. J. Van Doorn, S. T. al Rashid, W. G. Quint, J. van der Plas, V. L. Chan, and S. L. On.** 1997. *Campylobacter hyoilei* Alderton et al. 1995 and *Campylobacter coli* Veron and Chatelain 1973 are subjective synonyms. *Int. J. Syst. Bacteriol.* **47:**1055–1060.
24. **Weir, S., B. Cuccherini, A. M. Whitney, M. L. Ray, J. P. MacGregor, A. Steigerwalt, M. I. Daneshvar, R. Weyant, B. Wray, J. Steele, W. Strober, and V. J. Gill.** 1999. Recurrent bacteremia caused by a "*Flexispira*"-like organism in a patient with X-linked (Bruton's) agammaglobulinemia. *J. Clin. Microbiol.* **37:**2439–2445.

SUPPLEMENTAL READING

Nachamkin, I. 2003. *Campylobacter* and *Arcobacter*, p. 902–914. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, DC.

Nachamkin, I., and M. J. Blaser (ed.). 2000. *Campylobacter*, 2nd ed. ASM Press, Washington, DC.

Penner, J. L. 1988. The genus *Campylobacter:* a decade of progress. *Clin. Microbiol. Rev.* **1:**157–172.

Versalovic, J., and J. G. Fox. 2003. *Helicobacter*, p. 915–928. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, DC.

APPENDIX 3.8.2–1

Observe standard precautions.

Direct Detection of *Campylobacter* by EIA Method (ProSpecT)

I. PRINCIPLE

Diagnosis of campylobacteriosis by isolation of the organism can take 2 days to a week. Direct detection of the *Campylobacter* surface antigen (SA) from fecal specimens in an EIA can be done the same day the specimen is received. In this test two molecules with molecular masses of 15 and 66 kDa are detected. Cross-reactivity studies indicate that the antigens are shared by *C. jejuni* and *C. coli.*

The test can be performed directly on extracts of fecal specimens or fecal enriched broth cultures. Diluted specimens are added to breakaway microplate wells on which rabbit polyclonal anti-*Campylobacter* SA antibody is bound. If antigen is present, it is captured by the bound antibody. When an enzyme conjugate (polyclonal rabbit anti-

APPENDIX 3.8.2–1 *(continued)*

Campylobacter SA labeled with horseradish peroxidase enzyme) is added, captured *Campylobacter*-specific antigen binds the enzyme conjugate to the well. After the substrate for the enzyme is added, it is converted to a colored reaction product. Color development can be detected visually or spectrophotometrically. In a negative reaction, there is no *Campylobacter* SA or an insufficient amount of antigen present to bind the enzyme conjugate to the well and no colored reaction product develops.

II. SPECIMEN COLLECTION AND HANDLING

A. Collection of direct stool (feces)

1. Unpreserved stool specimens, specimens in Cary-Blair medium, or specimens diluted in the ProSpecT bacterial specimen diluent can be stored at 2 to 8°C for up to 72 h, although this storage can be extended to up to 7 days if in Cary-Blair medium.
2. If testing is to be delayed, store frozen at −20°C or lower immediately after collection.
3. Optimal specimen results will be obtained if the specimen is tested immediately upon receipt in the laboratory.

B. Specimen preparation for assay

1. Direct method

   Dilute unpreserved specimens approximately 1:3 by mixing 1 part specimen with 2 parts bacterial specimen diluent as follows.

   a. Add 0.6 ml of bacterial specimen diluent to a clean plastic or glass disposable tube.
   b. Thoroughly mix stool.
   c. Using a transfer pipette, remove approximately 0.3 ml of specimen (third mark from the tip of the pipette). Expel sample into bacterial specimen diluent and mix by drawing up and down once. Leave transfer pipettes in the tubes.
   d. For specimens that will not pipette, use an applicator stick to add 0.3 g (~6 mm in diameter) of specimen. Vigorously stir specimen into bacterial specimen diluent. Add a transfer pipette to the tube and mix by drawing up and down once. Leave transfer pipettes in the tubes.

2. For specimens in Cary-Blair transport, mix well and transfer to the microplate well.
3. Broth method

   a. Inoculate 150 μl or 3 drops of fresh stool or stool in Cary-Blair transport medium into 5 ml of GN broth (Table 3.8.1–1).
   b. Incubate at 35 ± 2°C under ambient atmospheric conditions for 18 to 24 h.
   c. Add 0.6 ml of bacterial specimen diluent to a clean 12- by 75-mm tube.
   d. Transfer 0.3 ml of broth culture into 0.6 ml of bacterial specimen diluent using a transfer pipette. Leave the transfer pipette in the tube.

III. MATERIALS

A. Reagents

1. The ProSpecT *Campylobacter* microplate assay (48- or 96-test microplate assay) catalog no. is 760-96 (Remel, Inc.).

   a. 100 ml of bacterial specimen diluent, containing buffered solution with rabbit serum with 0.02% thimerosal
   b. Microwell eight-well strips coated with rabbit polyclonal anti-*Campylobacter* SA antibody
   c. Positive control containing *C. jejuni* culture supernatant suspended in negative control with fetal bovine serum and 0.02% thimerosal
   d. Low positive control containing *C. jejuni* culture supernatant suspended in negative control with fetal bovine serum and 0.02% thimerosal
   e. Negative control containing human fecal material with rabbit serum and 0.02% thimerosal
   f. Enzyme conjugate: horseradish peroxidase-labeled rabbit polyclonal anti-*Campylobacter* SA and 0.01% thimerosal
   g. Wash buffer: 10× concentrated buffered solution with 0.1% thimerosal
   h. Color substrate in buffer
   i. Stop solution containing 0.5 N hydrochloric acid (corrosive)

2. Store reagents at 2 to 8°C until use.

APPENDIX 3.8.2–1 *(continued)*

3. Preparation of wash buffer
   a. Dilute 10× wash buffer concentrate to 1× by adding 1 part concentrate to 9 parts distilled or deionized water.
   b. Label diluted wash buffer with 1-month expiration date, and store at 2 to 8°C.

B. Materials
1. Disposable applicator sticks
2. Plastic or glass disposable tubes for specimen preparation, ~1 to 2-ml capacity
3. 1- to 10-ml pipettes
4. Wash bottle or dispenser for wash buffer or microwell plate washer
5. Deionized or distilled water
6. Plate reader capable of reading at 450 nm or 450 and 630 nm (optional)
7. Vortex mixer with microplate adapter or shaker (optional)

IV. QUALITY CONTROL

A. Test the positive and negative controls each time the test is performed.

B. Optionally, the low positive control may be run when an indeterminate or very low positive result is repeated. The low positive control may be included in the repeat run as an indication of the performance of the assay near the cutoff level.

C. The optical density (OD) of the negative control should be <0.100 at 450 nm or <0.070 at 450 and 630 nm. or colorless when read visually. If yellow color equal to 1+ or greater on the procedure card is present in the negative control, the test should be repeated with careful attention to the wash procedure.

D. The OD of the positive control should be >0.500 at 450 nm or 450 and 630 nm. Visually the intensity of color in the positive control should be equal to or greater than the 2+ reaction on the procedure card.

V. PROCEDURE

A. Allow all reagents and specimens to reach room temperature (20 to 25°C) before use.

B. Open the foil pouch, remove the required number of microplate strip wells, and place into a microplate strip holder. Use one well for the negative control and one well for the positive control. If using fewer than eight wells, break off the required number of wells from the strips and return the unused microwells to the foil pouch. *Reseal pouch tightly to exclude moisture and return to the refrigerator.*

C. Add 4 drops of negative control to the first well. Add 4 drops of positive control to a second well. (Optionally, add 4 drops of low positive control to a third well.)

D. Using a transfer pipette, add 4 drops of diluted specimen or enriched broth culture, or 4 drops of specimen in transport medium per well. *Note:* Place the opening of the transfer pipette just inside the well to avoid splashing into adjacent wells.

E. Cover the microplate and incubate at room temperature (20 to 25°C) for 60 min. Begin timing after the addition of the last specimen.

F. Shake out or aspirate the contents of the wells. Wash by completely filling each well with diluted wash buffer (350 to 400 µl/well) a total of three times. After the last wash, remove all fluid from the wells.

G. Add 4 drops (200 µl) of enzyme conjugate to each well.

H. Cover the microplate and incubate at room temperature for 30 min.

I. Decant or aspirate. Wash each well five times.

J. Add 4 drops (200 µl) of color substrate to each well.

K. Cover the microplate and incubate at room temperature for 10 min.

L. Add 1 drop (50 µl) of stop solution to each well. Gently tap or vortex the wells until the yellow color is uniform. Read reactions within 10 min after adding stop solution.

M. Read visually or spectrophotometrically at 450 nm (single wavelength) and/or 450 and 630 to 650 (dual wavelength).

VI. INTERPRETATION

A. Interpretation of visual results: refer to the procedure card for color interpretations.
1. Negative: a colorless reaction is a negative result and indicates that no *Campylobacter* SA or an undetectable level of *Camplyobacter* SA is present in the sample tested.
2. Indeterminate: if faint yellow color that is less than the 1+ reaction develops, the test is indeterminate. Tests with indeterminate results should be repeated.

APPENDIX 3.8.2–1 *(continued)*

3. Positive: if yellow color of at least 1+ intensity develops in the test wells, the sample contains *Campylobacter* SA and the test is positive.

B. Read spectrophotometric results at the single (450 nm) and/or dual (450 and 630 to 650 nm) wavelength. Tests with indeterminate results should be repeated.

Single wavelength

	Transport media	Fresh stool
Negative:	OD $<$ 0.100	OD $<$ 0.130
Indeterminate:	OD $=$ 0.100 to 0.130	OD $=$ 0.130 to 0.170
Positive:	OD $>$ 0.130	OD $>$ 0.170

Dual wavelength

	Transport media	Fresh stool
Negative:	OD $<$ 0.070	OD $<$ 0.100
Indeterminate:	OD $=$ 0.070 to 0.100	OD $=$ 0.100 to 0.140
Positive:	OD $>$ 0.100	OD $>$ 0.140

VII. LIMITATIONS

A. The ProSpecT *Campylobacter* microplate assay was evaluated in three studies and was found to have sensitivities of 80, 89, and 96% (1, 2, 3). Specificities were 99%.

B. The overall performance of the ProSpecT *Campylobacter* microplate assay for broth-enriched culture was a sensitivity of 90% and specificity of 100%, as indicated in the package insert.

C. The test detects the presence of *Campylobacter* SA in diarrheal stools and cultures. Correlation between the amount of antigen in a sample and clinical presentation has not been established.

D. A negative test result does not exclude the possibility of the presence of *Campylobacter* and may occur when the antigen level in the sample is below the detection level of the test.

E. The ProSpecT *Campylobacter* microplate assay does not differentiate *C. jejuni* and *C. coli*, and there are other serotypes and subspecies that may or may not be detected.

F. It is not known whether *C. upsaliensis*, *C. hyointestinalis*, or *C. helveticus* cross-reacts.

References

1. **Endtz, H. P., C. W. Ang, N. van den Braak, A. Luijendijk, B. C. Jacobs, P. de Man, J. M. van Duin, A. van Belkum, and H. A. Verbrugh.** 2000. Evaluation of a new commercial immunoassay for rapid detection of *Campylobacter jejuni* in stool samples. *Eur. J. Clin. Microbiol. Infect. Dis.* **19:**794–797.
2. **Hindiyeh, M., S. Jense, S. Hohmann, H. Benett, C. Edwards, W. Aldeen, A. Croft, J. Daly, S. Mottice, and K. C. Carroll.** 2000. Rapid detection of *Campylobacter jejuni* in stool specimens by an enzyme immunoassay and surveillance for *Campylobacter upsaliensis* in the greater Salt Lake City area. *J. Clin. Microbiol.* **38:**3076–3079.
3. **Tolcin, R., M. M. LaSalvia, B. A. Kirkley, E. A. Vetter, F. R. Cockerill III, and G. W. Procop.** 2000. Evaluation of the Alexon-Trend ProSpectT *Campylobacter* microplate assay. *J. Clin. Microbiol.* **38:**3853–3855.

3.8.3 *Clostridium difficile* Toxin Detection

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Clostridium difficile is an opportunistic pathogen present in healthy adults and children and is responsible for 300,000 to 3,000,000 cases of diarrhea and colitis in hospitalized patients in the United States every year (12). When the intestinal microbiota is altered, toxigenic *C. difficile* flourishes and toxin production increases. *C. difficile* toxin is responsible for 25% of antimicrobial agent-associated diarrhea cases and almost all cases of pseudomembranous colitis (11). Patients with the latter present with pseudomembranous nodules or plaques in the distal and sigmoid colon and rectum, which lead to colonic dilation (toxic megacolon) or even perforation. If unrecognized or untreated, it can be fatal. The definitive diagnosis of pseudomembranous colitis is made by the endoscopic detection of pseudomembranes or microabscesses.

For hospitalized patients, *C. difficile*-associated diarrhea (CDAD) correlates with antimicrobial therapy within the last 30 days and either three unformed stools over a 24-h period or abdominal pain (6). Although clindamycin and cephalosporin usage is highly associated with the disease, any antimicrobial agent can precipitate toxin production, as can various anticancer chemotherapeutic agents and diseases, such as AIDS. Generally, the implicated antimicrobial agent is discontinued, and treatment with oral vancomycin or metronidazole may be needed. The latter agent is preferred to prevent the emergence of vancomycin-resistant enterococci.

Nosocomial spread of *C. difficile* can be a problem, particularly in hospitals with neutropenic patients or long-term- care patients. The Society for Healthcare Epidemiology of America (SHEA) and America College of Gastroenterology have issued separate guidelines for prevention and control of *C. difficile* disease (3, 4). *C. difficile* produces two toxins, enterotoxin (toxin A) and cytotoxin (toxin B). Toxin A causes extensive tissue damage (lesions) to the mucosal wall of the intestine. Toxin B is a highly potent cytotoxin, with as little as 1 pg of the toxin causing rounding of tissue culture cells (9). The toxins are very unstable, even at refrigerator temperatures and in the frozen state. Several immunologic tests are available to detect the toxin, using antibody to the toxin. Culture for the organism is very sensitive but not specific, since it detects colonization with the organism, even when no toxin is produced (5). In addition, it takes several days for a positive result. Hence, it is used only to collect isolates for an epidemiologic investigation (*see* procedure 3.8.1 for culture methods). A latex agglutination test detects glutamate dehydrogenase, a nontoxin protein associated with *C. difficile* and other anaerobes that may correlate with CDAD. This test will yield false-positive results with non-toxin-producing isolates of *C. difficile, Clostridium botulinum, Clostridium sporogenes, Peptostreptococcus anaerobius,* and *Bacteroides asaccharolyticus* (10).

The reference method for laboratory diagnosis of CDAD has been detection of toxin B by cytotoxin (tissue culture) assay. Yet the sensitivity of this "gold standard" method has recently been reevaluated, and in some studies, it is reported to be about 85 to 90% (15) compared to cytotoxin assay of broth cultures from the organism isolated from anaerobic fecal cultures on prereduced selective medium (procedure 3.8.1). EIAs, for either toxin A or toxins A and B are less sensitive than tissue culture cytotoxicity (generally 70 to 82% sensitive [13, 15]) but may be more practical, because of their rapid turnaround time and the instability of the toxins, especially if a tissue culture assay must be sent to a reference laboratory. Many commercial tests detect toxin A, which is present in greater concentration. Some also detect toxin B, and a third type detects toxin A and glutamate dehydrogenase (Triage; Biosite Diagnostics, San Diego, CA). Tests that detect only toxin A may miss some isolates that produce toxin B only (2, 11). However, since toxin B is present in lower quantities, tests that detect both toxin A and toxin B may still not be able to detect low levels of toxin B in stool specimens and may not have much advantage over those tests that detect only toxin A. The Triage is less likely to miss a positive and has a reported sensitivity of 97.5% (package insert) to 89% compared to cytotoxin assay (15). However, the specificity of the test is 89%. Thus, those results that are toxin A negative and glutamate dehydrogenase positive should be confirmed by a toxin B assay or reported with a qualification that they may not be positive for the toxin (8). Since procedural methods for the immunologic tests are available in the package inserts and the options, sensitivities, and specificities are discussed elsewhere (1, 15), this procedure presents only the cytotoxin assay. However, the speci-

men collection, reporting, and some limitations apply to all methods.

Cytotoxin B tissue culture assay is based on the principle that a mixture of a bacterial-cell-free stool filtrate containing *C. difficile* toxin will show cytopathic effect (CPE) (rounding of the cells) in a susceptible cell line. The filtrate of *C. difficile* toxin mixed with *C. difficile* antitoxin will not produce a CPE. The neutralization of the toxin confirms the presence of *C. difficile* cytotoxin in the patient's stool sample.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Collect stool specimens in leakproof container, and transport and store without preservative at 2 to 8°C until processing (within 24 h of collection).

B. Also acceptable are lumen contents and surgical or autopsy samples of the large bowel.

C. Rejection criteria

◩ **NOTE:** The SHEA recommends that testing not be performed on asymptomatic patients, even for test of cure (4, 5, 6).

1. A rectal swab will not provide enough specimen for the test and therefore is unacceptable.

2. Reject stools that are not liquid or soft.

3. Request repeat collection if there is not enough specimen for test; 10 to 20 ml of watery, diarrheal stool is preferred, or a minimum of 3 ml or 3 g is required.

4. Request recollection if test will not be performed within 24 h of receipt.

5. Meconium may interfere with the assay.

6. Discourage testing specimens from infants less than 1 year of age (reference 6 and SHEA guidelines). Include comment on report indicating lack of specificity of test for this population.

◩ **NOTE:** Infants have been shown to be asymptomatic carriers, with colonization rates as high as 50% (7, 16, 17).

7. Limit testing of stools from cystic fibrosis patients (4), because these patients have been shown to have colonization rates as high as 32% (14).

8. Patients with positive tests should *not* have repeat testing for cure, unless they again become symptomatic after completion of therapy (6).

◩ **NOTE:** In one study, limiting the number of stool samples processed for *C. difficile* toxin to one per 72 h would have reduced the number of duplicates by 40%, without adversely affecting the identification of toxin-positive patients (M. LaRocco, personal communication).

III. MATERIALS

A. Minimum essential medium (MEM) with 2% fetal bovine serum (FBS), gentamicin (50 μg/ml), vancomycin (500 μg/ml), and nystatin (10,000 U/ml) (*see* procedure 10.3 for details of preparation of tissue cultures and media)

B. 96-well flat-bottomed microtiter plate with monolayer of diploid fibroblasts (e.g., human fetal foreskin, MRC-5, Chinese hamster ovary K1 cells) and 0.1 ml of MEM per well

1. Preparation of cell monolayer

a. Make a suspension of cells at a concentration of 4×10^4 cells per ml in growth medium (90% MEM plus 10% FBS).

b. Inoculate 100 μl of suspension into each well.

c. Incubate at 35°C aerobically with 5% CO_2 for several days until a monolayer forms.

d. Aspirate growth medium and replace with 100 μl of MEM with 2% FBS.

e. To avoid drying of outer wells, add 200 μl of MEM with 2% FBS to perimeter wells and do not use them for the assay if they do not look healthy.

2. Store at 35°C aerobically with 5% CO_2.

III. MATERIALS *(continued)*

3. Use trays up to 3 weeks after preparation.

◩ **NOTE:** The Bartels (Issaquan, WA) cytotoxicity assay for *C. difficile* toxin utilizes a microtiter tray containing tissue culture cells, thus allowing laboratories not equipped for standard tissue culture to use the assay.

C. *C. difficile* culture filtrate, lyophilized (Techlab Inc., Blacksburg, VA)
- **1.** Contains toxins A and B
- **2.** Add 1 ml of sterile distilled water to vial.
- **3.** Aliquot small volumes (50 to 100 µl) of the reconstituted filtrate in an airtight container at −20°C.

D. *C. difficile* antitoxin, lyophilized (Techlab Inc.)
- **1.** Add 3 ml of sterile distilled water for stock.
- **2.** Aliquot small volumes (50 to 100 µl) of the reconstituted antitoxin in an airtight container at −20°C.
- **3.** Place a 6-month expiration date on reconstituted antitoxin.
- **4.** For working dilution, dilute stock 1:25 with 98% MEM.
  - **a.** Add 0.1 ml of stock to 2.4 ml of MEM.
  - **b.** Prepare fresh every 2 weeks.
  - **c.** Store at 2 to 8°C.
- **5.** Determine the titer of each new lot of both toxin and antitoxin before use.
  - **a.** Make 10-fold dilutions from 10^{-1} to 10^{-5} in 98% MEM.
  - **b.** Remove MEM from right wells of double rows of microtiter plate.
  - **c.** Inoculate 0.1 ml of working dilution of antitoxin into each right well.
  - **d.** Inoculate 0.1 ml of culture filtrate into the right and left wells.
  - **e.** Incubate in 3 to 5% CO_2 at 33 to 37°C for 48 h.
  - **f.** The titer is the highest dilution showing 4+ toxin CPE and no CPE in the tube with antitoxin. The working dilution is 10 times this dilution. (If the highest dilution showing 4+ CPE is 10^{-5} the working dilution is 10^{-4}.)

E. Materials required but not provided
- **1.** Pipettors that dispense 100 µ1
- **2.** Powderless disposable latex or polypropylene gloves
- **3.** 1-, 5-, and 10-ml sterile pipettes
- **4.** *Refrigerated* centrifuge, capable of centrifugation at ≥12,000 × *g*
- **5.** 15-ml round-bottomed polypropylene or other appropriate centrifuge tubes for specimen dilution and centrifugation
- **6.** Syringe, 3 ml
- **7.** Repeating pipetting device
- **8.** Millipore 0.45-µm-pore-size filters
- **9.** Incubator with 5% CO_2
- **10.** Inverted microscope

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Each time the test is run, even if on the same tray, set up the following controls after aspirating the maintenance medium (*see* Fig. 3.8.3–1).
- **1.** 100 µl of toxin plus 100 µl of MEM
- **2.** 100 µl of toxin plus 100 µl of antitoxin

B. In addition, set up the following controls once per plate after aspirating the maintenance medium.
- **1.** 100 µl of antitoxin plus 100 µl of MEM
- **2.** 200 µl of MEM

C. Expected results
- **1.** Well with toxin shows typical CPE within 12 to 18 h. The cells round up and appear evenly throughout the cell sheet.
- **2.** Well with toxin plus antitoxin shows no CPE.
- **3.** Wells with MEM and wells with antitoxin alone show no CPE.

	Day 1				Day 2						
A/1	2	3	4	5	6	7	8	9	10	11	12
B	Toxin	Toxin + A	Patient D 1:20	Patient D 1:20 + A	Toxin	Toxin + A					
C	MEM	A	Patient D 1:100	Patient D 1:100 + A	Patient F 1:20	Patient F 1:20 + A					
D	Patient A 1:20	Patient A 1:20 + A	Patient E 1:20	Patient E 1:20 + A	Patient F 1:100	Patient F 1:100 + A					
E	Patient A 1:100	Patient A 1:100 + A	Patient E 1:100	Patient E 1:100 + A	Patient G 1:20	Patient G 1:20 + A					
F	Patient B 1:20	Patient B 1:20 + A			Patient G 1:100	Patient G 1:100 + A					
G	Patient B 1:100	Patient B 1:100 + A									
H											

Figure 3.8.3–1 Example of worksheet diagram to accompany a 96-well microtiter tray previously inoculated with a monolayer of fibroblasts and subsequently inoculated with patient specimen, controls, toxin, and antitoxin. Day 1 is the first date the tray is used. Day 2 illustrates the controls omitted on subsequent days; i.e., MEM and antitoxin control wells are not needed. Patient stool specimens in dilutions of 1:20 and 1:100 are indicated as patient A, B, C, D, and E, inoculated on day 1, and patients F and G, inoculated on day 2. Note that outer wells are not used. A, antitoxin.

IV. QUALITY CONTROL *(continued)*

D. Troubleshooting

Problem	Corrective action
Positive control shows no CPE.	1. Positive control has deteriorated. Dilute new aliquot. 2. Cells insensitive—rerun test with another cell lot.
Toxin-antitoxin well shows CPE.	1. Antitoxin deteriorated. Dilute and use frozen aliquot of the same lot. 2. Dilute and use different lot of antitoxin. 3. Cell sheet contaminated. Try new lot of cells or reagents.

V. PROCEDURE

Observe standard precautions.

A. Preparation of stool sample

1. Add 3 ml of 98% MEM to a 15-ml centrifuge tube.
2. Add enough stool sample to bring the level of medium up to the 5-ml mark (2:5 dilution).
3. Vortex at the highest speed.
4. Centrifuge at 12,000 × *g* for 15 min in refrigerated centrifuge.
5. Filter the supernatant into a tube using a 0.45-μm-pore-size filter attached to a 3-ml syringe.
6. Add 100 μl of supernatant to 300 μl of 98% MEM in a 12- by 75-mm tube (final dilution, 1:10).
7. Add 100 μl of the 1:10 dilution to 400 μl of 98% MEM in a 23- by 75-mm tube (final dilution, 1:50)

V. PROCEDURE *(continued)*

B. Inoculation (*see* Fig. 3.8.3–1)
- **1.** Remove medium from right wells of double rows.
- **2.** Add 100 µl of working dilution antitoxin to each right well immediately after medium is removed but before adding patient's specimen.
- **3.** Add 100 µl of the 1:50 dilution of patient's specimen to the left and right wells of the double rows (1:100 dilution).
- **4.** Add 100 µl of the 1:10 dilution of patient's specimen to the left and right wells of the double rows (1:20 dilution).
- **5.** The same pipette tip can be used for pipetting both dilutions of the same specimen if the higher dilution is inoculated first.
- **6.** Save dilutions and stool at 4°C for further testing.
- **7.** Inoculate toxin and toxin-antitoxin controls.

C. Incubate the tray for a minimum of 12 to 18 h at 33 to 35°C with 3 to 5% CO_2.

D. Read for CPE at 24 and 48 h.

POSTANALYTICAL CONSIDERATIONS

VI. INTERPRETATION

A. If stool filtrate and stool filtrate plus antitoxin are both negative, this is interpreted as negative for *C. difficile* toxin.

B. If stool filtrate is positive and stool filtrate plus antitoxin is negative at the same dilution, this is interpreted as positive for *C. difficile* toxin.

C. If stool filtrate and stool filtrate plus antitoxin are both positive, this is an equivocal result.
- **1.** Wells with 1:20 dilution stool extract plus antitoxin can show breakthrough CPE in a strongly positive specimen. In this case a definite reduction (50%) in CPE of the 1:100 dilution stool extract plus antitoxin compared to the 1:20 dilution plus antitoxin well suggests presence of *C. difficile* toxin.
  - **a.** If the well with the 1:100 dilution plus antitoxin also shows breakthrough CPE, set up further dilutions of 1:200, 1:400, etc., with and without the antitoxin.
  - **b.** Use the 1:20 dilution extract to make further dilutions.
  - **c.** Determine the highest dilution of stool extract plus antitoxin that shows no CPE. If the stool extract at that dilution shows CPE, the specimen is positive for *C. difficile* toxin.
- **2.** If CPE shows in stool extract-plus-antitoxin well of the highest dilution of stool extract that shows CPE, the CPE is not due to *C. difficile* toxin. Request another sample, since the results cannot be interpreted.
- **3.** If the cell sheet is deteriorating nonspecifically, request another sample, since these results cannot be interpreted.

VII. REPORTING RESULTS

A. Report positive results as "*C. difficile* toxin positive."
- **1.** Notify the caregiver who ordered the test.
- **2.** Report positives as soon as neutralized CPE is observed.

B. Report preliminary negative results as "No *C. difficile* toxin detected at 24 h; final reading at 48 h."

C. Report final negative results as "No *C. difficile* toxin detected."

VII. REPORTING RESULTS *(continued)*

D. For equivocal results (cells show rounding that is not neutralized by antitoxin), questionable weak results, or specimens that are toxic to the cell sheet (cell sheet falls off sides of well), report "Specimen result not interpretable. Suggest repeat sample."

1. Notify caregiver to submit another specimen if diarrhea persists.

2. Communicate this result as soon as possible to avoid delays in obtaining a repeat specimen. It is likely that if the patient has *C. difficile* toxin, the repeat specimen will be a strong positive.

VIII. LIMITATIONS

A. The etiology of diarrhea caused by microorganisms other than *C. difficile* will not be established with this assay.

B. Levels of toxin can be low. A negative result alone may not rule out the possibility of CDAD.

C. Always evaluate assay result along with clinical signs and patient history.

D. Certain isolates of *Clostridium sordellii* produce the same type of rounding on tissue culture cells as toxigenic *C. difficile*, due to the similarities of the toxins. *C. sordellii* has not been detected in patients with antimicrobial agent-associated diarrhea and colitis. It is also unlikely that *C. sordellii* will be present in human fecal specimens.

E. False-positive results are associated with misinterpretation of CPE and its neutralization.

F. Lack of homogeneity in a stool sample may lead to incorrect results. Thorough mixing of stool specimens is essential to avoid this problem.

G. Stool specimens that appear to have large amounts of fat, mucus, or blood present are more likely to give toxic results.

H. Healthy newborns are frequently toxin positive.

REFERENCES

1. **Allen, S. A., C. L. Emery, and J. A. Siders.** 2002. Anaerobic bacteriology, p. 69–76. *In* A. L. Truant (ed.), *Manual of Commercial Methods in Microbiology.* ASM Press, Washington, DC.
2. **Depitre, C., M. Delmee, V. Avesani, R. L'Haridon, A. Roels, M. Popoff, and G. Corthier.** 1993. Serogroup F strains of *Clostridium difficile* produce toxin B but not toxin A. *J. Med. Microbiol.* **38:**434–441.
3. **Fekety, R.** 1997. Guidelines for the diagnosis and management of *Clostridium difficile*-associated diarrhea and colitis. American College of Gastroenterology, Practice Parameters Committee. *Am. J. Gastroenterol.* **92:**739–750.
4. **Gerding, D. N., S. Johnson, L. R. Peterson, M. E. Mulligan, and J. Silva, Jr.** 1995. *Clostridium difficile*-associated diarrhea and colitis. *Infect. Control Hosp. Epidemiol.* **16:**459–477.
5. **Gerding, D. N., M. M. Olson, L. R. Peterson, D. G. Teasley, R. L. Gebhard, M. L. Schwartz, and J. T. Lee, Jr.** 1986. *Clostridium difficile*-associated diarrhea and colitis in adults. A prospective case-controlled epidemiologic study. *Arch. Intern. Med.* **146:**95–100.
6. **Johnson, S., and D. N. Gerding.** 1998. *Clostridium difficile*-associated diarrhea: a review. *Clin. Infect. Dis.* **26:**1027–1034.
7. **Kader, H. A., D. A. Piccoli, A. F. Jawad, K. L. McGowan, and E. S. Maller.** 1998. Single toxin detection is inadequate to diagnose *Clostridium difficile* diarrhea in pediatric patients. *Gastroenterology* **115:**1329–1334.
8. **Landry, M. L., J. Topal, D. Ferguson, D. Giudetti, and Y. Tang.** 2001. Evaluation of Biosite Triage *Clostridium difficile* panel for rapid detection of *Clostridium difficile* in stool samples. *J. Clin. Microbiol.* **39:**1855–1858.
9. **Lyerly, D., H. Krivan, and T. Wilkins.** 1988. *Clostridium difficile:* its diseases and toxins. *Clin. Microbiol. Rev.* **1:**1–18.
10. **Lyerly, D. M., D. W. Ball, J. Toth, and T. D. Wilkins.** 1988. Characterization of cross-reactive proteins detected by Culturette brand rapid latex test for *Clostridium difficile. J. Clin. Microbiol.* **26:**397–400.
11. **Moncrief, J. S., L. Zheng, L. M. Neville, and D. M. Lyerly.** 2000. Genetic characterization of toxin A-negative, toxin B-positive *Clostridium difficile* isolates by PCR. *J. Clin. Microbiol.* **38:**3072–3075.
12. **Mylonakis, E., E. T. Ryan, and S. B. Calderwood.** 2001. Clostridium difficile-associated diarrhea: a review. *Arch. Intern. Med.* **161:**525–533.

REFERENCES *(continued)*

13. **O'Connor, D., P. Hynes, M. Cormican, E. Collins, G. Corbett-Feeney, and M. Cassidy.** 2001. Evaluation of methods for detection of toxins in specimens of feces submitted for diagnosis of *Clostridium difficile*-associated diarrhea. *J. Clin. Microbiol.* **39:**2846–2849.
14. **Peach, S. L., S. P. Borriello, H. Gaya, F. E. Barclay, and A. R. Welch.** 1986. Asymptomatic carriage of *Clostridium difficile* in patients with cystic fibrosis. *J. Clin. Pathol.* **39:**1013–1018.
15. **Turgeon, D. K., T. J. Novicki, J. Quick, L. Carlson, P. Miller, B. Ulness, A. Cent, R. Ashley, A. Larson, M. Coyle, A. P. Limaye, B. T. Cookson, and T. R. Fritsche.** 2003. Six rapid tests for direct detection of *Clostridium difficile* and its toxins in fecal samples compared with the fibroblast cytotoxicity assay. *J. Clin. Microbiol.* **41:**667–670.
16. **Viscidi, R., S. Willey, and J. G. Bartlett.** 1981. Isolation rates and toxigenic potential of *Clostridium difficile* isolates from various populations. *Gastroenterology* **81:**5–9.
17. **Wilson, K. H.** 1993. The microbiology of *Clostridium difficile*. *Clin. Infect. Dis.* **16**(suppl. 4)**:**S214–S218.

3.8.4 *Helicobacter pylori* Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

In 1984, a campylobacter-like organism, *Helicobacter pylori*, was reported to be present in high numbers in the deep mucus layer of the gastric surface epithelium in patients with chronic active gastritis (11). Since its discovery, *H. pylori* has been estimated to be present in 50% of the population (10, 11), and it is known to be associated with an increased risk for the development of peptic ulcer disease, gastric adenocarcinoma, and gastric non-Hodgkin's B-cell lymphomas (mucosa-associated lymphoid tissue) (2). While *H. pylori* is the primary agent of disease, there are increasing reports of evidence of other spiral organisms (e.g., *Helicobacter heilmannii*) present as part of the human microbiota (1, 2, 14), which are often uncultivable but may be responsible for gastritis.

There are a number of tests available to diagnose *H. pylori* infection which can be categorized into invasive (requiring endoscopy and biopsy) and noninvasive. The most common noninvasive test is serology to determine the presence of antibodies to the organism (*see* procedure 11.9). Serologic testing, if positive, for a symptomatic patient who has not been previously treated supports the diagnosis of *H. pylori*. Caution should be exercised in interpretation in areas of high prevalence of *H. pylori*, as antibodies generally remain for life. These tests have been reviewed by Laheij et al. (6) and are not discussed further here. Another noninvasive test is the urea breath test (3), which is an accurate means of identifying the presence of *H. pylori* infection before and after antimicrobial therapy. The test is labor-intensive and requires expensive instrumentation for analysis (scintillation counter or mass spectroscope), yet for the patient it is simple, rapid, and relatively inexpensive. A stool EIA antigen test (Appendix 3.8.4–1) is also a sensitive noninvasive test that has been shown to be a good method for initial diagnosis and for monitoring eradication after antimicrobial therapy (13). However, the cost of this assay is high.

There are three tests available to diagnose the disease invasively from gastric biopsy specimens. Because of their ease of use, rapidity, and cost-effectiveness, rapid urease biopsy tests are the most common test. Several commercial tests are approved for performance by nonlaboratory scientists ("waived" tests) in the gastroenterologists' offices, with sensitivities of 89 to 98% and specificities of 93 to 98% (3, 5). Other tests include histologic examination and culture. Culture is demanding, requires special conditions of transport and incubation, and does not provide a result in a timely manner, making it useful only in selected cases.

H. pylori organisms appear in gull wing formation on Gram stain, although after culture they may form a U shape. With age or after prolonged exposure to ambient air, the organisms may appear as coccoidal forms. Unlike most other campylobacters, *H. pylori* organisms possess abundant amounts of urease. They produce superoxide dismutase and catalase in much greater amounts than other campylobacters. The presence of gull wing or U-shaped gram-negative organisms from a gastric specimen that are microaerophilic and urease, catalase, and oxidase positive makes the diagnosis of *H. pylori.*

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. Obtain biopsy specimens.

a. Pass an endoscope orally.

b. Obtain specimens through a channel in the endoscope by using one of the following procedures.

(1) Using biopsy forceps, obtain samples from the stomach or duodenum.

(2) Using a sheathed brush, brush suspicious areas several times to obtain adequate cellular material.

c. If a gastric ulcer is seen, obtain biopsy samples from the base, the surrounding gastric mucosa, and each of the four quadrants of the margin.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

2. Transport
- **a.** Urease test
  - (1) Prewarm agar.
  - (2) Inoculate several biopsy samples by pushing into the urea agar, or inoculate into broth or other urea test (7).
  - (3) Seal tube and incubate at 25°C or, preferably, at 35°C until submitted to laboratory (8) or agar turns pink.
- **b.** Submit biopsy samples within 3 h in 1 ml of modified Cary-Blair transport medium or place in saline on ice.

  ◩ **NOTE:** Prompt submission of biopsy specimens to the laboratory for homogenization and urease testing has a higher yield than direct inoculation.
- **c.** Label all tubes and specimens with date and time of collection.

3. Optional: collect a stool specimen for direct antigen test. Store at 4°C for up to 72 h or at −20°C until tested. Do not freeze-thaw more than two times.

B. Rejection criteria

1. Do not accept specimens in formalin for culture.

2. For specimens not cultured within 3 h, document in the final report that specimen quality may have been compromised.

3. Gastric washes are not acceptable specimens for culture.

III. MATERIALS

A. Urease tests—choose from the following or use other commercially available sensitive test for urease detection (9).
- **1.** Pyloritek urease test (Bard, Inc., Billerica, MA)
- **2.** Rapid selective urea agar (Hardy Diagnostics; Remel, Inc.) (procedure 3.17.48)
- **3.** CLO test (Ballard Medical Products, Draper, UT)
- **4.** HPFAST (GI Supply, Camp Hill, PA)
- **5.** 0.5 ml of Christensen's urea solution without agar (procedure 3.17.48)

B. Optional: Premier Platinum HpSA direct antigen test (Meridian Diagnostics, Cincinnati, OH). See Appendix 3.8.4–1.

C. Media for culture—for best results, choose one selective and one nonselective medium.
- **1.** Nonselective media: BAP, BHI with 7% horse blood, brucella agar, or CHOC
- **2.** Selective media: modifed Thayer-Martin medium, Campy-CVA, or Columbia agar with 10% egg yolk emulsion, 1% Vitox, and 40 mg of 2,3,5-triphenyltetrazolium chloride per liter with cefsulodin, trimethoprim, vancomycin, and amphotericin B (CEYE)

  ◩ **NOTE:** CHOC and CEYE were reported in one study to provide the best combination (12).

D. Gram stain reagents

E. Other supplies
- **1.** Microaerobic environment (*see* Table 3.8.2–3 in procedure 3.8.2)
- **2.** Catalase (procedure 3.17.10) and oxidase (procedure 3.17.39) reagents

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Verify that media meet expiration date and QC parameters per Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22-A3 (4). See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

B. Check plate media for ability to support growth of *H. pylori* ATCC 43504.

IV. QUALITY CONTROL *(continued)*

C. Urea direct tests

1. Examine each lot visually to determine that no reaction has occurred and that the test is yellow or straw colored.

2. Prior to each use, make sure there are no signs of contamination or deterioration, (shrinking, cracking, or discoloration) and that the tube is yellow. Discard any defective tubes and obtain a new one.

3. Check each lot prior to putting it into use with a positively and negatively reacting organism.

Test organism	Result
Proteus mirabilis ATCC 12453	Positive; color change from the original yellow to pink-red, slower reaction time than *H. pylori*
Escherichia coli ATCC 25922	Negative; no color change observed

4. For proficiency, QC, and validation of direct urease test, obtain urease tablets (Kimberly Clark, Draper, UT). As a positive patient control, insert the tablet into a negative test, as you would a biopsy sample. After 5 min, inspect for a positive color change. If the test remains negative, take corrective action.

V. PROCEDURE

Observe standard precautions.

A. Inoculation

1. Gently homogenize the tissue (refer to procedure 3.13.1 for details on grinding).

◩ **NOTE:** This method of processing results in heavier yields of organisms compared to either mincing the specimen or direct plating of the specimen.

2. Place several pieces of biopsy material into one of the urea test media and incubate at 35°C under aerobic conditions.

3. Inoculate plate media and immediately incubate under strict microaerophilic conditions at 35°C. When using an airtight jar, add a moistened gauze pad in the bottom. Use of the Anoxomat system (MART Microbiology) may improve the yield from *H. pylori* cultures, since the proper atmospheric conditions are immediately established (http://www.anoxomat.com).

4. Inoculate additional CHOC plate at 35°C under aerobic conditions in 5% CO_2.

B. Direct tests

1. Examine the urea test for a pink to magenta color change at 30 min, 4 h, and 24 h, depending on the manufacturer's instructions for incubation and reading.

2. Gram stain and examine for gull wing-shaped, gram-negative rods.

C. Culture methods (*also see Campylobacter* procedure [3.8.2])

1. Incubate plates at 35 to 37°C for 3 to 5 days. Some strains require as long as 7 days for growth on primary isolation.

2. Examination of culture media

a. Colonies of *H. pylori* appear as small, gray, and translucent. Some strains demonstrate weak beta-hemolysis with growth on the microaerobic plates but *not* on the aerobically incubated plate.

b. Gram stain suspicious colonies. *H. pylori* organisms from solid media appear as slightly curved gram-negative rods.

c. Perform oxidase, catalase, and urea tests from colonies. *H. pylori* is positive in all three tests.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report direct results.
- **1.** Report results of urea test as "positive" if any pink to magenta color is observed or "negative" if there is no color change in 24 h.
- **2.** Report results of stool direct antigen test as "positive" or "negative for *Helicobacter pylori* antigen" per criteria in package insert.
- **3.** If the direct urease is positive (pink) and "gull wings" are seen on Gram stain, a preliminary culture report can be sent as "Probable *Helicobacter pylori* present."

B. Report culture as "*Helicobacter pylori*" if the following are true.
- **1.** The colony Gram stain demonstrates curved, gram-negative rods.
- **2.** Oxidase and catalase reactions are positive.
- **3.** Urease reaction is positive (usually within several minutes).

C. Refer to procedure 3.3 for general criteria for reporting.

VII. INTERPRETATION

A. The presence of a urease-positive curved rod in a gastric specimen is indicative of the presence of *H. pylori,* although there are reports of other urea-positive curved rods in gastric specimens (14).

B. The presence of *H. pylori* indicates the need for aggressive treatment of the infection to eradicate the organism.

C. There are a number of current protocols for therapy of *H. pylori.* In general, they consist of agents to decrease acidity to allow healing and multiple antimicrobial agents to eradicate the organism. Common regimens include amoxicillin, clarithromycin, and omeprazole (triple therapy) and bismuth, tetracycline, metronidazole and a proton pump inhibitor (quadruple therapy) (10). In addition, pharmaceutical companies have packaged antihelicobacter therapy in convenient dosing formats that include "PrevPak" and "Helidac." In >90% of treated patients, there is healing of the gastric inflammation, prevention and healing of peptic ulcers, and loss of the symptoms of gastritis (1, 5).

D. Perform antimicrobial susceptibility testing in cases of treatment failures.

VIII. LIMITATIONS

A. No one test has 100% sensitivity in detecting *H. pylori,* and several tests, including serologic assays and tests described in this procedure, may be necessary.

B. False-negative urease tests may occur when very low numbers of *H. pylori* organisms are present in the stomach or when the bacterium has a patchy distribution. Testing multiple biopsy samples will increase the likelihood of a positive test result.

C. The urease test and culture are less sensitive if the patient has recently taken antimicrobial agents or bismuth. Suppression of *H. pylori* by these agents makes the organism difficult to detect by any means. Regrowth of *H. pylori* may be patchy in the first 3 weeks after treatment.

D. After treatment, the stool antigen test and the urea breath test are the most accurate to monitor therapy and cure.

E. Since there are now a number of urease-positive spiral organisms, the preliminary testing described in this procedure cannot be considered definitive without further molecular testing (1, 14).

REFERENCES

1. **Blaser, M. J.** 1998. Helicobacters are indigenous to the human stomach: duodenal ulceration is due to changes in gastric microecology in the modern era. *Gut* **43:**721–727.
2. **Blaser, M. J.** 1999. Hypothesis: the changing relationships of *Helicobacter pylori* and humans: implications for health and disease. *J. Infect. Dis.* **179:**1523–1530.
3. **Chey, W. D.** 2000. Accurate diagnosis of *Helicobacter pylori.* 14C-urea breath test. *Gastroenterol. Clin. N. Am.* **29:**895–902.
4. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Dunn, B. E., H. Cohen, and M. J. Blaser.** 1997. *Helicobacter pylori. Clin. Microbiol. Rev.* **10:**720–741.
6. **Laheij, R. J. F., H. Straatman, J. B. M. J. Jansen, and A. L. M. Verbeek.** 1998. Evaluation of commercially available *Helicobacter pylori* serology kits: a review. *J. Clin. Microbiol.* **36:**2803–2809.
7. **Laine, L., D. Chung, C. Stein, I. El-Beblawi, V. Sharma, and P. Chandra-Soma.** 1996. The influence of size or number of biopsies on rapid urease test results: a prospective evaluation. *Gastroinest. Endosc.* **43:**49–53.
8. **Laine, L., R. Estrada, D. N. Lewin, and H. Cohen.** 1996. The influence of warming on rapid urease test results—a prospective evaluation. *Gastrointest. Endosc.* **44:**429–432.
9. **Laine, L., D. Lewin, W. Naritoku, R. Estrada, and H. Cohen.** 1996. Prospective comparison of commercially available rapid urease tests for the diagnosis of *Helicobacter pylori. Gastrointest. Endosc.* **44:**523–526.
10. **Leung, W. K., and D. Y. Graham.** 2000. Clarithromycin for *Helicobacter pylori* infection. *Exp. Opin. Pharmacother.* **1:**507–514.
11. **Marshall, B. J., and J. R. Warren.** 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **i:**1311–1315.
12. **Piccolomini, R., G. Di Bonaventura, D. Festi, G. Catamo, F. Laterza, and M. Neri.** 1997. Optimal combination of media for primary isolation of *Helicobacter pylori* from gastric biopsy specimens. *J. Clin. Microbiol.* **35:**1541–1544.
13. **Vaira, D., P. Malfertheiner, F. Mégraud, A. T. R. Axon, M. Deltenre, A. M. Hirschl, G. Gasbarrini, C. O'Morain, J. M. Pajares Garcia, M. Quina, G. N. J. Tytgat, and the HpSA European Study Group.** 1999. Diagnosis of *Helicobacter pylori* infection with a new non-invasive antigen-based assay. *Lancet* **354:**30–33.
14. **Weir, S., B. Cuccherini, A. M. Whitney, M. L. Ray, J. P. MacGregor, A. Steigerwalt, M. I. Daneshvar, R. Weyant, B. Wray, J. Steele, W. Strober, and V. J. Gill.** 1999. Recurrent bacteremia caused by a "*Flexispira*"-like organism in a patient with X-linked (Bruton's) agammaglobulinemia. *J. Clin. Microbiol.* **37:**2439–2445.

APPENDIX 3.8.4–1

Observe standard precautions.

Helicobacter pylori Antigen Assay (HpSA)

I. PRINCIPLE

A microplate EIA is commercially available for the qualitative detection of *Helicobacter pylori* antigens in human stool (HpSA) by a noninvasive method (1). The test can identify current infections and can be used to confirm the eradication of the microorganism after the end of the therapy. Unlike serologic tests (procedure 11.9), which are unable to differentiate between current and past infections, the HpSA test detects current infection only. The HpSA EIA is a standard microtiter well assay using polyclonal antibodies.

II. SPECIMENS

Collect a stool specimen in an empty container.

A. Store for up to 3 days at 2 to 8°C before testing.

B. If testing cannot be performed within this time frame, store specimens frozen upon receipt at −20 to −80°C.

III. MATERIALS

Premier Platinum HpSA, catalog no. 601348—48 tests (Meridian Diagnostics, Cincinnati, OH).

The kit contains antibody-coated microwells, a positive control and negative control, sample diluent, wash buffer, enzyme conjugate, substrate, stop solution, transfer pipettes, strip holder, strip sealer, and wooden stick applicators. Store kit at 2 to 8°C.

IV. QUALITY CONTROL

Test the positive and negative controls upon receipt of new lots and each time patient testing is performed.

V. PROCEDURE

A. Emulsify 5- to 6-mm-diameter portion of stool into 200 μl of sample diluent in a test tube.

APPENDIX 3.8.4–1 *(continued)*

B. Transfer 50 μl of diluted stools and controls in the appropriate wells in the microtiter plate.
C. Add 1 drop of enzyme conjugate to each well, seal, and incubate at room temperature for 1 h.
D. Wash five times with the wash buffer.
E. Add 2 drops of substrate to all wells and incubate at room temperature for 10 min.
F. Add 1 drop of stop solution and read at dual wavelength (450 and 630 nm).

VI. INTERPRETATION

Cutoff values (absorbance at 450 nm) for HpSA test are ≥0.160 for positive results, 0.159 to 0.140 for indeterminate results, and <0.140 for negative results.

VII. LIMITATIONS

A. At present no single test can be relied upon to detect definitely colonization by *H. pylori*, and a combination of two is recommended if this is feasible.
B. In a large prospective evaluation comparing the HpSA test to invasive tests, the sensitivity and the specificity of the HpSA were 94 and 92%, respectively, compared to biopsy-based tests (3).
C. In an evaluation of eradication (4) on day 35 after antimicrobial therapy, the urea breath test had a sensitivity of 94% and a specificity of 100%. The stool antigen test had a sensitivity of 94% and a specificity of 97%. On day 7 after treatment, the stool antigen test was predictive of eradication (positive predictive value, 100%; negative predictive value, 91%).
D. The test has been shown to be accurate for pediatrics patients, for whom serology tests are less sensitive (2).

References

1. **Evangelista, A. T., A. L. Truant, and P. Bourbeau.** 2002. Rapid systems and instruments for the identification of bacteria, p. 38–39. *In* A. L. Truant (ed.), *Manual of Commercial Methods in Microbiology.* ASM Press, Washington, DC.
2. **Oderda, G., A. Rapa, B. Ronchi, P. Lerro, M. Pastore, A. Staiano, G. L. dè Angelis, and P. Strisciuglio.** 2000. Detection of *Helicobacter pylori* in stool specimens by non-invasive antigen enzyme immunoassay in children: multicentre Italian study. *BMJ* **320:**347–348.
3. **Vaira, D., P. Malfertheiner, F. Mègraud, A. Axon, M. Deltenre, A. M. Hirschl, G. Gasbarrini, C. O'Morain, J. M. Pajares, M. Quina, G. N. J. Tytgat, and the HpSA European Study Group.** 1999. Diagnosis of *Helicobacter pylori* infection with a new non-invasive antigen-based assay. *Lancet* **354:**30–33.
4. **Vaira, D., N. Vakil, M. Menegatti, B. van Hoff, C. Ricci, L. Gatta, G. Gasbarrini, and M. Quina.** 2002. The stool antigen test for detection of *Helicobacter pylori* after eradication therapy. *Ann. Intern. Med.* **136:**280–287.

3.8.5 Screen for Vancomycin-Resistant Enterococci in Fecal Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Since the first report of enterococci resistant to high concentrations of glycopeptide antimicrobial agents in 1988, a sudden and widespread increase in the incidence of infection and colonization with vancomycin-resistant enterococci (VRE) has occurred over the last two decades (7). Specific classes of antimicrobial agents appear to be responsible for the spread, including extended-spectrum cephalosporins and drugs with activity against anaerobic bacteria. The use of vancomycin to treat *Clostridium difficile* colitis has also been implicated (7). Transmission of VRE can be through patient-to-patient contact, health care workers, and contaminated environmental surfaces and patient care equipment. Control methods include routine screening for vancomycin resistance among clinical isolates, active surveillance for VRE in intensive care units, contact isolation to minimize person-to-person transmission, rigorous decontamination of patient contact areas, and restriction of the use of vancomycin and other antimicrobial agents (1, 2). Patients who are colonized with VRE should be identified so that contact isolation can be instituted. Screening for VRE may facilitate removal of patients from isolation if three consecutive fecal cultures are negative for VRE. This procedure describes a method to screen for fecal carriage of VRE.

Various types of vancomycin resistance have been genotypically characterized, including *vanA, vanB, vanC, vanD,* and *vanE*. *vanA* and *vanD* are most often seen in *Enterococcus faecium*. *vanB* and *vanE* are mostly seen in *Enterococcus faecalis*. The mechanism of resistance of *vanA* and *vanB* is a plasmid-mediated transposon capable of altering the dipeptide terminus of peptidoglycan from a D-alanyl-D-alanine to the depsipeptide D-alanyl-D-lactate, which then binds vancomycin with a very low affinity. The MICs of vancomycin for enterococci with the *vanA* or *vanB* genotype are typically greater than 32 μg/ml. It is these genotypes that have been associated with spread of resistance within hospitals and nursing homes. Phenotypically, the definition of VRE includes those *E. faecalis, E. faecium,* and *Enterococcus raffinosus* organisms that are resistant to vancomycin with MICs usually ≥32 μg/ml (3). It does not include motile enterococci, *Enterococcus gallinarum*, and *Enterococcus casseliflavus,* which have intrinsic low-level vancomycin resistance mediated by *vanC.* These species do not account for the spread of vancomycin resistance and are generally susceptible to other agents, such as ampicillin and aminoglycosides. Although most commercially available identification systems adequately differentiate *E. faecalis* from other species of enterococci, additional tests, including motility, are required to distinguish the motile enterococci, *E. gallinarum,* and *E. casseliflavus* from *E. faecium* and *E. raffinosus* (2). Such identification to the species level is necessary to limit the reporting of VRE to only those strains that are of epidemiologic concern.

Screening for fecal carriage of VRE is accomplished by fecal or rectal swab culture on a selective and differential medium. Most laboratories rely on bile-esculin-azide (BEA) agar and broth with vancomycin, but chromogenic agar is also available and demonstrates improved detection and workload by decreased subculture and biochemical testing of colonies (http://www.chromagar.com; available from bioMérieux) (9, 10). Esculin is included to detect esculin-hydrolyzing enterococci, ferric citrate to provide ferric ions, bile salts to inhibit gram-positive bacteria other than enterococci, and sodium azide to inhibit gram-negative bacteria. Esculin forms glucose and esculetin when hydrolyzed by enterococci. Esculetin reacts with the ferric ions to produce dark brown to black color development in the medium, allowing recognition of the enterococci (5). The vancomycin concentration ranges from 6 to 10 μg/ml depending on the manufacturer; sensitivity and specificity will vary with the concentration used (8). CHROMagar VRE contains inhibitory and chromogenic substrates that allow presumptive identification of *E. faecium* as mauve colonies and *E. faecalis* as blue colonies. Also see procedures 5.6 and 13.17.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. Collect fecal specimen and submit immediately or place in VRE broth.

◩ **NOTE:** The yield from feces has been shown to be greater than that from perirectal swabs that are not soiled with feces (6). Rectal swabs should be obtained by inserting the swab 1 to 2 in. into the rectal canal and collecting feces. Rectal swabs for VRE screening that are submitted without visible fecal soiling should be rejected, and new swabs should be collected.

2. Swabs of wounds or urine may also be submitted.

B. Generally submit three specimens from different days to rule out the presence of VRE in a patient who was previously positive.

C. Rejection criterion

Reject specimens if VRE were isolated from the patient in other cultures collected in the previous 2 weeks.

III. MATERIALS

A. Media

1. Agar

a. BEA agar with 6 μg of vancomycin per ml (Remel, Inc.) *or*

b. BEA agar with 10 μg of vancomycin per ml (Hardy Diagnostics) *or*

c. Vancomycin-supplemented Enterococcosel agar (BD Diagnostic Systems) with either 6 or 8 μg of vancomycin per ml

◩ **NOTE:** With 10 μg of vancomycin per ml, BEA agar with vancomycin is less likely to support the growth of *E. casseliflavus* and *E. gallinarum*, owing to their low-level resistance. True VRE may be inhibited as well.

d. CHROMagar VRE

2. VRE broth containing BEA broth with vancomycin

◩ **NOTE:** Use of both broths in conjunction with agar culture has been shown to increase the detection of VRE by 50% but also increases the yield of motile enterococci (6).

3. BAP

4. Store media at 2 to 8°C. Allow media to come to room temperature before use.

B. Gram stain reagents

C. Other reagents

1. Pyrrolidonyl-β-naphthylamide (PYR) (procedure 3.17.41)

2. Catalase (procedure 3.17.10)

3. 0.5 ml of BHI broth for motility (procedure 3.17.31)

4. Methyl glucopyranoside MGP broth (procedure 3.17.30) (optional)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that media meet expiration date and QC parameters per Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22-A3 (4). See procedures 14.2 and 3.3.1 for further QC procedures, especially for in-house-prepared media.

B. BEA broth or agar with vancomycin should appear clear and light amber.

C. Subculture the following QC strains to VRE broth and agar containing vancomycin prior to use, and incubate at 35°C aerobically for 18 to 24 h. Verify that they produce the desired reactions listed. Repeat QC of agar containing vancomycin at weekly intervals with both enterococcal species (3).

IV. QUALITY CONTROL
(continued)

Test organism	Result
Enterococcus faecalis ATCC 51299	Growth with blackening in medium surrounding colony or blackening and turbidity of broth
Enterococcus faecalis ATCC 29212	Inhibition (clear broth or no growth on agar)
Escherichia coli ATCC 25922	Inhibition (clear broth or no growth on agar)

D. Subculture of the following strains should be done with each new lot and shipment of CHROMagar VRE: *Enterococcus faecalis* ATCC 51299 (growth of bluish green colonies), *Enterococcus faecium* ATCC 700221 (growth of mauve-violet colonies), and *Enterococcus gallinarum* ATCC 49573 (growth is inhibited).

V. PROCEDURE

Observe standard precautions.

A. Fecal VRE culture on BEA

1. Inoculation

a. Process specimen not in VRE broth as soon as received.

◩ **NOTE:** Use of biosafety cabinet will avoid contamination of the culture or specimen as well as protect laboratory processing personnel.

b. Inoculate first quadrant of vancomycin-containing BEA agar heavily with stool or swab; with a loop, streak in three other quadrants, as illustrated in Fig. 3.3.1–1.

c. Inoculate VRE broth with a small amount of fecal specimen.

2. Incubate plate and broth at 35°C aerobically for up to 72 h.

3. Check agar plates daily.

a. If brown-black color is diffused in medium, then consider it to be bile-esculin positive. Using a stereoscope, check for different morphotypes. With a plate marker, number the colonies, and Gram stain each.

b. Subculture only gram-positive cocci to BAP.

c. Incubate subculture at 35°C for 24 h.

4. Subculture turbid broth, if black, to vancomycin-containing BEA agar. Incubate for 24 h and subculture black colonies to BAP if they are gram-positive cocci on Gram stain.

5. Perform catalase and PYR tests on suspected colonies from BAP. Because of exposure to vancomycin, the PYR test might be negative; it should turn positive on a second subculture.

6. Lightly inoculate each different morphology of catalase-negative, PYR-positive, gram-positive cocci to 0.5 ml of BHI and incubate at 30°C for 2 h. Observe microscopically at ×400 for motile cocci.

a. Nonmotile organisms are considered to be VRE.

b. Optionally, inoculate MGP broth. VRE do *not* produce a yellow color.

c. A rapid D-xylose tablet test also differentiates *E. gallinarum/E. casseliflavus* from *E. faecium/E. faecalis* (11).

d. Further species identification and antimicrobial susceptibility testing (AST) are necessary only if patient has not had a prior positive result (*see* Table 3.18.1–3).

e. Notify caregiver of positive culture findings.

f. Hold positive culture plates for at least 7 days.

B. Fecal VRE culture on CHROMagar VRE

1. Process the specimen as soon as received.

◩ **NOTE:** Use of a biosafety cabinet will avoid contamination of the culture specimen as well as protect laboratory processing personnel.

V. PROCEDURE *(continued)*

2. Inoculate the first quadrant of the agar heavily with stool or swab; with a loop, streak in three other quadrants, as illustrated in Fig. 3.3.1–1.
3. Incubate the chromogenic media for 18 to 24 h at 35°C before initially checking the plate for growth of mauve or blue colonies. If there is no growth on the chromogenic agar at 24 h, it should be reincubated for another day before reporting as negative for VRE after 48 h.
4. Pick mauve colonies (i.e., presumptive *E. faecium*) and perform a Gram stain to confirm as gram-positive cocci. Perform a group D typing and PYR to confirm as *Enterococcus* spp.
5. Pick blue-green colonies (i.e., presumptive *E. faecalis*) and perform a Gram stain to confirm as gram-positive cocci. Perform a group D typing and PYR to confirm as *Enterococcus* spp.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. If no VRE are present, report "No vancomycin-resistant *Enterococcus* organisms isolated."
B. If culture is positive for VRE, report "Vancomycin-resistant *Enterococcus* present."
C. Document notification to caregiver of positive findings.

VII. INTERPRETATION

A. The presence of VRE in fecal specimens is an indication of carriage or colonization with the organism but does not indicate infection, since enterococci are a part of the normal intestinal microbiota.
B. It is recommended that VRE be confirmed by a CLSI MIC method (3) if the patient has not previously been positive for VRE.

VIII. LIMITATIONS

A. Organisms other than enterococci *(Pediococcus, Leuconostoc,* and *Weissella)* may grow on these media and produce a positive reaction for esculin. These genera are PYR negative.
B. False-negative results can be caused by low numbers of organisms or prior antimicrobial treatment.

REFERENCES

1. **Centers for Disease Control and Prevention.** 1994. Preventing the spread of vancomycin resistance—report from the Hospital Infection Control Practices Advisory Committee. *Fed. Regist.* **59:**25758–25763.
2. **Centers for Disease Control and Prevention.** 1995. Recommendations for preventing the spread of vancomycin resistance: recommendations of the Hospital Infection Control Practices Advisory Committee (HICPAC). *MMWR Morb. Mortal. Wkly. Rep.* **44**(RR-12)**:**1–13.
3. **Clinical and Laboratory Standards Institute.** 2006. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically,* 7th ed. Approved standard M7-A7. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Difco Laboratories.** 1984. *Difco Manual,* 10th ed., p. 129–131. Difco Laboratories, Detroit, MI.
6. **Edberg, C. E., C. J. Hardalo, C. Kontnick, and S. Campbell.** 1994. Rapid detection of vancomycin-resistant enterococci. *J. Clin. Microbiol.* **32:**2182–2184.
7. **Gold, H. S.** 2001. Vancomycin-resistant enterococci: mechanisms and clinical observations. *Clin. Infect. Dis.* **33:**210–219.

REFERENCES *(continued)*

8. **Ieven, M., E. Vercauteren, P. Descheemaeker, F. van Laer, and H. Goossens.** 1999. Comparison of direct plating and broth enrichment culture for the detection of intestinal colonization by glycopeptide-resistant enterococci among hospitalized patients. *J. Clin. Microbiol.* **37:**1436–1440.
9. **Landman, D., J. M. Quale, E. Oydna, B. Willey, V. Ditore, M. Zaman, K. Patel, G. Saurina, and W. Huang.** 1996. Comparison of five selective media for identifying fecal carriage of vancomycin-resistant enterococci. *J. Clin. Microbiol.* **34:**751–752.
10. **Ledeboer, N. A., K. Das, M. Eveland, C. Roger-Dalbert, S. Mailler, S. Chatellier, and W. M. Dunne.** 2007. Evaluation of a novel chromogenic agar medium for isolation and differentiation of vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis. J. Clin. Microbiol.* **44:**4561–4563.
11. **Willey, B. M., R. N. Jones, A. McGeer, W. Witte, G. French, R. B. Roberts, S. G. Jenkins, H. Nadler, and D. E. Low.** 1999. Practical approach to the identification of clinically relevant *Enterococcus* species. *Diagn. Microbiol. Infect. Dis.* **34:**165–171.

SUPPLEMENTAL READING

Boyce, J. M. 1997. Vancomycin-resistant enterococcus: detection, epidemiology and control measures. *Infect. Dis. Clin. N. Am.* **11:**367–368.

3.9.1 Guidelines for Performance of Genital Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Specimens from genital sites are sent to the clinical microbiology laboratory for detection of microorganisms from females presenting with clinical syndromes such as cervicitis, vulvovaginitis, urethritis, bacterial vaginosis (BV), salpingitis (pelvic inflammatory disease [PID]), endometritis, or genital ulcers and from males exhibiting urethritis, epididymitis, prostatitis, or genital ulcers (Tables 3.9.1–1 to 3.9.1–3) (3). Specimens are also submitted from pregnant females to diagnose the presence of organisms that may cause disease in the neonate. Less commonly, specimens are sent from children and postmenopausal women (16, 17, 22, 25, 26).

The syndromes that are associated with female and male genital tract infections are listed in Tables 3.9.1–1 to 3.9.1–3, with the major pathogens for each syndrome indicated. For surgically collected specimens and those from normally sterile sites, a routine wound and abscess culture will detect most bacterial pathogens, as long as selective media for *Neisseria gonorrhoeae* are included. Anaerobic cultures are often indicated for these specimens (14), and mycoplasma cultures may be appropriate in selected cases (procedure 3.15).

The human vagina is lined with 25 layers of epithelium cells. Many indigenous microorganisms colonize these surfaces. Accurate diagnosis of genital infections from the male and female genitalia depends on the separation of microbial pathogens from the normal genital microbiota. The microorganisms colonizing the female genital tract have been studied extensively and include lactobacilli, *Corynebacterium* spp., *Gardnerella vaginalis,* coagulase-negative staphylococci, *Staphylococcus aureus, Streptococcus agalactiae, Enterococcus* spp., *Escherichia coli,* anaerobes, and yeasts (19). For prepubescent females, diphtheroids and coagulase-negative staphylococci predominate; lactobacilli predominate in the adult female. Postmenopausal women are generally colonized with fewer lactobacilli but have a greater number of *Enterobacteriaceae* than premenopausal women. They also lack the presence of yeasts and mycoplasmas. Many adult female genital tract infections arise from endogenous microorganisms, the pathogenicity of which has been activated by host factors and other microorganisms. Various viruses, including herpes simplex virus (HSV), human papillomavirus (HPV), and human immunodeficiency virus, may also influence the receptivity of the host surface to microorganisms. The male urethra normally contains relatively few skin microbiota, consisting of coagulase-negative staphylococci, micrococci, *Corynebacterium* spp., and viridans group streptococci.

Because the agents of disease have diverse culture and detection requirements and selective media are often needed, *a "routine" genital culture, with the intent to "detect what is there," rarely has an indication.* Occasionally such specimens are appropriate for prepubescent or postmenopausal women. Otherwise, laboratories should provide a special requisition, such as in Appendix 3.9.1–1, to be used to request the appropriate tests in the outpatient setting. For certain pathogens, such as *N. gonorrhoeae, Haemophilus ducreyi,* or *S. agalactiae* (group B streptococcus [GBS]), cultures are ordered specifically for those pathogens, with proper collection and selection media. Because of the special nature of cultures for these pathogens, procedures to detect them are listed separately in this handbook following this procedure.

To diagnose vulvovaginitis and BV, routine bacterial cultures are *not* helpful (1, 27). In Table 3.9.1–4, the recommended tests are indicated to make these diagnoses. Vaginitis is caused by BV, *Trichomonas vaginalis,* or an overgrowth of yeasts, typically *Candida albicans.* Many laboratories still rely on the Gram stain using the Nugent scoring system to diagnose BV (21) (Appendix 3.2.1–3) and to determine if there is an overgrowth of yeasts. Rapid tests that detect a high level of sialidase produced by organisms associated with BV are also available and have a sensitivity comparable to that of microscopic examination. A yeast culture may also be done if there is a history of recurrent vaginitis or treatment failure. Wet-mount examination for detection of *T. vaginalis* has a low sensitivity, particularly when specimens are delayed in transit. It should be noted that performing only a wet mount without *Trichomonas* culture may miss 50% of cases (3). Non-culture-based methods are increasingly being used to improve detection of *T. vaginalis,* since culture requires the maintenance of specialized media and may also have decreased sensitivity when specimens are delayed in transit (Appendix 9.9.4). *Trichomonas* rapid antigen and nucleic acid amplification tests (NAT) are available and have greater sensitivity for detecting infection (5, 18). A sensitive DNA probe assay is also available that combines the

Table 3.9.1–1 Female genital infections that are sexually transmitted

Clinical syndrome	Location of infection	Clinical symptoms/signs	Primary pathogen(s)	Specimen(s) collected
Bartholinitis	Bartholin gland	Pain, induration, and redness of gland area	*Chlamydia trachomatis* *Neisseria gonorrhoeae* *Staphylococcus aureus* Enteric bacteria (e.g., *Escherichia coli*, *Proteus mirabilis*, and other enteric GNB[a]) plus anaerobes *Ureaplasma urealyticum*	Aspirate of gland Swab of abscess pus
Cervicitis	Endocervical canal	Mucopurulent discharge, pain with movement of the cervix (dyspareunia). HSV primarily causes blisters and painful ulcers of the external cervical os.	*Chlamydia trachomatis* *Neisseria gonorrhoeae* HSV If above tests are negative, then BV and *Trichomonas* should be ruled out (7).	Endocervical swab Ulcer swab for viral culture
Genital ulcer with inguinal lymphadenopathy	Skin and soft tissues of the genital area and inguinal lymph nodes	Genital ulcer(s) in syphilis, LGV,[b] and granuloma inguinale (donovanosis) are usually painless. Chancroid ulcer is typically painful, with undermined edges. Inguinal lymphadenopathy may occur on one or both sides.	*Calymmatobacterium granulomatis* (donovanosis) *Chlamydia trachomatis*—LGV serovars *Haemophilus ducreyi* (chancroid) *Treponema pallidum* (syphilis)	Ulcer scrapings Inguinal lymph node aspirate Dark-field exam Syphilis serology LGV serology
Endometritis[c]	Endometrial lining of the uterus	Fever, leukocytosis, pelvic pain, cramps, abnormal bloody discharge or spotting	*Actinomyces* spp. *Chlamydia trachomatis* Enteric bacteria (e.g., *Escherichia coli*, *Proteus mirabilis*, and other enteric GNB) plus anaerobes *Neisseria gonorrhoeae* *Streptococcus pyogenes*	Transvaginal aspirate or biopsy sample of endometrium
Salpingitis (PID)	Ascending infection of the fallopian tubes and peritoneal cavity, with the formation of abscesses	Fever, leukocytosis, lower abdominal and pelvic pain associated with discharge	*Chlamydia trachomatis* *Neisseria gonorrhoeae* Enteric bacteria (e.g., *Escherichia coli*, *Proteus mirabilis*, and other enteric GNB) plus anaerobes *Staphylococcus aureus* *Streptococcus pyogenes*	Culdocentesis Laparoscopy sample of fallopian tube and pelvic abscesses
Skenitis	Skene's gland	Pain, induration, and redness in the gland area	*Neisseria gonorrhoeae* *Staphylococcus aureus*	Aspirate of gland Swab of abscess pus
Urethritis, urethral syndrome	Urethra	Dysuria with initiation of urination, urethral discharge, and pain. Cystitis should be ruled out by doing a urine culture.	*Chlamydia trachomatis* *Neisseria gonorrhoeae* *Mycoplasma genitalium* *Ureaplasma urealyticum*	Urethral swab Urine culture
Vaginosis	Vagina	Vaginal discharge that may be fishy or foul smelling, pruritus, terminal dysuria, painful intercourse	*Candida* spp. *Trichomonas vaginalis* BV *(G. vaginalis* plus anaerobes: *Atopobium vaginae*, *Mobiluncus*, and *Prevotella)*	Vaginal swab

(continued)

Table 3.9.1–1 Female genital infections that are sexually transmitted *(continued)*

Clinical syndrome	Location of infection	Clinical symptoms/signs	Primary pathogen(s)	Specimen(s) collected
Vulvovaginitis	Vagina and vulva	Pruritus, redness of the skin, white curd-like discharge, macerate skin, and terminal dysuria	*Candida* spp. *Staphylococcus aureus*[d] Methicillin-resistant *S. aureus*	Vaginal swab Vulval swab
Vulvovaginal, perineal ulcers	Vagina, vulva, perineum	Painful blisters on an erythematous base, ulcers	HSV	Ulcer swab for viral culture

[a] GNB, gram-negative bacillus.

[b] LGV, lymphogranuloma venereum.

[c] Associated with use of intrauterine device.

[d] In wounds, TSS, and tampon-associated ulcerations.

Table 3.9.1–2 Female genital infection clinical syndromes associated with intrapartum, postpartum, and postabortal infections

Clinical syndrome	Location of infection	Clinical symptoms and signs	Primary pathogen(s)	Specimen(s) collected
Intra-amniotic infection syndrome	Uterus and its contents during pregnancy, including the amniotic membrane and fluid	Fever, leukocytosis, abdominal pain, uterine tenderness, premature rupture of membranes, bacteremia. May cause premature labor and delivery.	Enteric bacteria (e.g., *Escherichia coli*, *Proteus mirabilis*, and other enteric GNB[a]) plus anaerobes *Listeria monocytogenes* *Neisseria gonorrhoeae* *Streptococcus agalactiae* *Streptococcus pyogenes*	Amniotic fluid Amniotic tissue Blood cultures
Postpartum endometritis, endomyometritis, endoparametritis	Endometrium and uterine muscle	Fever, leukocytosis, abdominal pain, uterine tenderness, foul-smelling lochia/discharge, bacteremia	*Chlamydia trachomatis* Enteric bacteria (e.g., *Escherichia coli*, *Proteus mirabilis*, and other enteric GNB) plus anaerobes *Gardnerella vaginalis* *Streptococcus agalactiae* *Streptococcus pyogenes*	Transvaginal aspirate of endometrium Endometrial tissue Blood cultures
Puerperal sepsis/septic abortion	Endometrium and uterine muscle and pelvis	Fever, leukocytosis, abdominal pain, uterine tenderness, foul-smelling lochia/discharge, hypotension, bacteremia	*Clostridium perfringens* and other anaerobes Enteric bacteria (e.g., *Escherichia coli*, *Proteus mirabilis*, and other enteric GNB) *Streptococcus agalactiae* *Streptococcus pyogenes*	Transvaginal aspirate of endometrium Endometrial tissue Blood cultures
Septic pelvic thrombophlebitis	Deep pelvic veins	Refractory postpartum fever and leukocytosis with no other obvious focus of infection. CT[b] scan shows uterine vein thrombosis.	Enteric bacteria (e.g., *Escherichia coli*, *Proteus mirabilis*, and other enteric GNB) plus anaerobes *Streptococcus agalactiae* *Streptococcus pyogenes*	Blood cultures

[a] GNB, gram-negative bacillus.

[b] CT, computed tomography.

detection of yeasts, *T. vaginalis*, and *G. vaginalis* as a marker for BV (4).

For primary syphilis, a dark-field exam is useful but is rarely performed (Appendix 3.2.3–1). *Calymmatobacterium granulomatis* is detected by demonstration of Donovan bodies (intracytoplasmic cysts of enlarged mononuclear cells containing 10 or more deeply staining pleomorphic rods). These bodies are usually stained either by the Giemsa method or by silver stains of formalin-fixed biopsy material. (See procedure 9.8.5 for details of Giemsa stain preparation.) *Chlamydia* and herpes viral cultures are also important diagnostic

Table 3.9.1–3 Male genital infections

Clinical syndrome	Location of infection	Clinical symptoms and signs	Primary pathogen(s)	Specimen(s) collected
Balanoposthitis	Head of penis and foreskin	Pain, swelling, exudates and pus under foreskin	Enteric bacteria (e.g., *Escherichia coli* and other *Enterobacteriaceae*) plus anaerobes *Streptococcus pyogenes* *Staphylococcus aureus*	Swab from under foreskin
Epididymitis	Epididymus	Pain, swelling, tenderness of scrotum and epididymus	*Chlamydia trachomatis* Enteric bacteria (e.g., *Escherichia coli* and other *Enterobacteriaceae, Enterococcus* spp., *Pseudomonas* spp.) *Mycobacterium tuberculosis* *Neisseria gonorrhoeae*	Urethral swab Urine culture Epididymus tissue
Orchitis	Testes	Pain, swelling, tenderness of scrotum	*Chlamydia trachomatis* Enteric bacteria (e.g., *Escherichia coli* and other *Enterobacteriaceae, Enterococcus* spp., *Pseudomonas* spp.) *Mycobacterium tuberculosis* *Neisseria gonorrhoeae*	Urethral swab Urine culture Testicular tissue
Prostatic abscess	Prostate gland	Dysuria, frequency, decreased urinary stream or obstruction, passing air on urination if vesiculoenteric fistula	Enteric bacteria (e.g., *Escherichia coli* and other *Enterobacteriaceae, Enterococcus* spp., *Pseudomonas* spp.) *Staphylococcus aureus*	Swab abscess Abscess fluid/pus
Prostatitis	Prostate gland	Dysuria, frequency, decreased urinary stream or obstruction	Enteric bacteria (e.g., *Escherichia coli* and other *Enterobacteriaceae, Enterococcus* spp., *Pseudomonas* spp.) *Neisseria gonorrhoeae*	Urine culture[a] Urethral swab Prostatic fluid
Urethritis	Urethra	Dysuria, mucopurulent discharge, urethral Gram stain shows >4 PMNs/per oil immersion field	*Chlamydia trachomatis* *Neisseria gonorrhoeae* HSV *Mycoplasma genitalium* *Ureaplasma urealyticum* *Trichomonas vaginalis*	Urethral swab DFA[b]/viral culture Wet mount of urethral secretions

[a] See Table 3.9.1–6 and Appendix 3.9.1–2.

[b] DFA, direct fluorescent antibody.

tools in the evaluation of sexually transmitted diseases.

For the recognition of toxic shock syndrome (TSS), isolation of *S. aureus* is difficult and not sufficient for the diagnosis. Further characterization of the strain is necessary to confirm the diagnosis. Two commercial kits are available for testing, which is usually done in reference laboratories (Toxic Shock ELISA [Toxin Technologies, Inc., Madison, WI] and TST-RPLA reverse passive latex agglutination [Oxoid, Columbia, MD]). Alternatively, testing acute- and convalescent-phase sera for antibodies to the exotoxin (TSST-1) in a reference laboratory can be helpful. Most patients with TSS lack antibodies at the onset of infection but then produce them in response to the infection. Greater than 90% of women have antibodies to the exotoxin.

Table 3.9.1–4 Diagnostic characteristics for vaginitis and vaginosis[a]

Disease or condition	pH	Observation on wet mount	Amine odor[b]	Other test(s) for diagnosis[c]
None (normal)	4–4.5	Long rod form bacteria	Absent	
Atrophic	>6		Absent	
Candidiasis	4–4.5	Pseudohyphae or budding yeasts	Absent	Yeast culture
BV	>4.5	Clue cells	Present	Gram stain (Nugent's criteria) OSOM BV Blue test (Genzyme)
Trichomoniasis	5–6	Flagellated parasites[d]	Usually present	*Trichomonas* culture Rapid antigen test (OSOM Trich [Genzyme]) Nucleic acid amplification test (APTIMA TV [Gen-Probe])

[a] Data for table extrapolated from Sobel (27).

[b] A drop of 10% KOH is added to vaginal fluid and sniffed for release of fishy or amine odor.

[c] These tests are *recommended* for increased sensitivity and might be offered as a panel by laboratory.

[d] Caution: occasional contaminants from the environment, such as *Colpoda*, while ameboid in motion, may be seen.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

◩ **NOTE:** Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and other caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection. In many cases the procedures to collect the specimens listed below should be performed by qualified physicians with appropriate training.

A. Specimen collection (*see* Tables 3.9.1–4 and 3.9.1–5 and Appendix 3.9.1–1 for options for outpatient testing)

1. Female specimens

a. Amniotic fluid

(1) Aspirate fluid by catheter at cesarean section or at amniocentesis.

(2) Order body fluid culture (procedure 3.5).

b. Bartholin cyst

(1) Decontaminate the skin with povidone-iodine, 3% chloroxylenol, and 3% cocamidopropyl PG-dimonium chloride phosphate (Techni-Care, Care-Tech, St. Louis, MO) or other surgical disinfectant, and aspirate material from the duct(s).

◩ **NOTE:** Bartholin glands are small mucus-secreting glands located beneath the posterior portion of the labia majora.

(2) Order aerobic and anaerobic wound/abscess cultures (procedure 3.13.1).

c. Cervical

(1) Clear away vaginal mucus and exudate with large swab. Moisten speculum with warm water, not lubricants, which can be antibacterial. Using a small swab (not cotton or wood shaft) inserted through a speculum, sample endocervical canal. Avoid the vaginal walls during collection.

(2) See Table 3.9.1–4 for ordering options.

d. Culdocentesis

(1) After cleaning the vaginal wall with surgical disinfectant, such as povidone-iodine or 3% chloroxylenol and 3% cocamidopropyl PG-dimonium chloride phosphate, perform transvaginal puncture of the cul-de-sac to aspirate fluid.

◩ **NOTE:** The cul-de-sac is the pouch between the anterior wall of the rectum and the posterior wall of the uterus. Collection is often done to diagnose PID without more invasive laparoscopy; however, results may not correlate with more invasive testing.

Table 3.9.1–5 Diagnostic tests for combined detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis*[a]

Disease or condition	Collection	Order	Comments
Cervicitis[c]	Swab using the specific manufacturer's collection kit.	NAT[b]	Preferred approach due to high sensitivity and combined detection of *N. gonorrhoeae* and *C. trachomatis* (*see* section 12).
	Swab using Gen-Probe PACE 2 collection kit.	Hybridization probe	Provides combined detection of *N. gonorrhoeae* and *C. trachomatis* but is less sensitive than NAT (*see* section 12)
	Take a swab for GC culture plus a swab for *C. trachomatis* testing by another method.	*N. gonorrhoeae* culture plus *C. trachomatis* testing by another method	*N. gonorrhoeae* culture done for recurrent infections or a history of treatment failure
Urethritis, or epididymitis, or prostatitis	Swab using the specific manufacturer's collection kit *or* submit first-void urine in a clean container.	NAT	Preferred approach due to high sensitivity and combined detection of *N. gonorrhoeae* and *C. trachomatis* (*see* section 12).
	Swab using Gen-Probe PACE 2 collection kit.	Hybridization probe	Provides combined detection of *N. gonorrhoeae* and *C. trachomatis* but is less sensitive than NAT (*see* section 12).
	Take a swab for *N. gonorrhoeae* culture plus a swab for *C. trachomatis* testing by another method.	*N. gonorrhoeae* culture plus *C. trachomatis* testing by another method	*N. gonorrhoeae* culture done for recurrent infections or a history of treatment failure

[a] Because of the high rate of dual infection with both *C. trachomatis* and *N. gonorrhoeae*, testing for both pathogens should be routinely performed for patients suspected of having either urethritis and/or cervicitis.

[b] Self-collected vaginal swab FDA cleared by some manufacturers for NAT.

[c] If tests for *C. trachomatis* and/or *N. gonorrhoeae* and HSV are negative, BV and *Trichomonas* should be ruled out. *Trichomonas* culture or a rapid antigen test or NAT is recommended (7).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

(2) Order aerobic and anaerobic wound/abscess cultures.

e. Endometrium

(1) Insert endometrial suction curette or catheter-protected Dacron swab through the cervical os and transfer beyond the cervical opening into the uterine cavity. Collect sample from within the cavity.

(2) Order aerobic and anaerobic wound/abscess cultures (14).

f. Fallopian tubes and pelvic cavity

(1) Collection: obtain aspirates and biopsy samples during laparoscopy. Also sample the pelvic peritoneum. Biopsies often yield better diagnostic specimens.

(2) Order aerobic and anaerobic wound/abscess cultures (14).

g. Skene's glands

(1) Decontaminate the skin with surgical disinfectant, and aspirate material from the gland(s).

◩ **NOTE:** Skene's glands are paraurethral glands located at both sides of the outer end of the urethra.

(2) Order aerobic and *N. gonorrhoeae* culture.

h. Vagina

(1) Collect fluid from the vagina with sterile pipette or Dacron swab. Successful self-collection of vaginal swabs can be done (23).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

(2) See Appendix 3.9.1–1 and Table 3.9.1–4 for ordering options.

◩ **NOTE:** Some manufacturers have been given FDA approval to submit a self-collected vaginal swab for NAT. Check the manufacturer's package insert for the types of specimens approved for testing with their product kits. A vaginal swab may also be submitted for microscopic examination to detect leukocytosis. A finding of a WBC count of >10 cells in vaginal fluid in the absence of *Trichomonas* infection might indicate endocervical inflammation due specifically to *Chlamydia trachomatis* or *Neisseria gonorrhoeae* infection (13, 29).

i. Vulva

(1) Collect only if pain, erythema, or edema is present.

(2) Clean the surface of the lesion with 0.85% NaCl and collect by one of the methods below.

(a) Sample exudate or area of erythema with swab for yeast culture.

(b) If there is a vesicle present, collect for HSV culture.

i. Unroof vesicle.

ii. Collect fluid with a sterile swab *or*

iii. Aspirate vesicular fluid with a 26- to 27-gauge needle and syringe.

iv. Then scrape the base of the vesicle with a sterile scalpel blade, and collect specimen with a Dacron swab by vigorously rubbing the base of the vesicle.

(c) If there is a crust on the lesion, gently remove it.

i. Moisten swab with saline and collect specimen by vigorously rubbing the base of the lesion for *H. ducreyi* culture.

ii. Alternatively, gently abrade the lesion with a sterile scalpel or needle until serous fluid emerges. (Try to avoid bleeding.) Irrigate with saline.

(i) For *H. ducreyi* culture, rub the base vigorously with a sterile swab or aspirate fluid with flamed smoothed Pasteur pipette or needle and syringe.

(ii) For *Treponema pallidum*, wipe away fluid, blood, and debris with sterile gauze. Apply gentle pressure to the base of the lesion until clear fluid is expressed. Touch a slide to the fluid, and cover the fluid on the slide with a coverslip. If no exudate is present, add a drop of saline to the lesion or insert a needle and syringe at the lesion base, aspirate, and then draw a drop of saline into the needle. Express the material onto a slide (*see* Appendix 3.2.3–1).

(3) Order *T. pallidum* dark-field microscopy, *H. ducreyi* culture, or HSV culture or request yeast culture for most cases showing only erythema or edema.

2. Male specimens

a. Epididymitis or testicular abscess

(1) The specimen of choice for diagnosis of an infected epididymis is a urethral swab or first-void urine submitted for NAT for simultaneous detection of *N. gonorrhoeae* and *C. trachomatis* (*see* Table 3.9.1–4 and section 12). A midstream urine specimen should also be submitted for bacterial culture (*see* section 12).

(2) Fluid/pus will only be collected if there is a testicular abscess. Fluid/pus is aspirated from the abscess after disinfecting the scrotal skin.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

(3) The following tests should be ordered on testicular fluid/pus.
   (a) Routine aerobic bacterial culture, most commonly members of the family *Enterobacteriaceae* and pseudomonads causing complex urinary tract infection in older men or those with a spinal cord injury
   (b) Although rare, *Mycobacterium tuberculosis* infection generally also involves the prostate or seminal vesicles and usually occurs in immunocompromised patients.

b. Penile lesion or vesicle
   (1) Clean the surface of the lesion with 0.85% NaCl and collect by one of the methods below.
      (a) If there is a vesicle present, collect for HSV culture.
         i. Unroof vesicle
         ii. Collect fluid with a sterile swab *or*
         iii. Aspirate vesicular fluid with a 26- to 27-gauge needle and syringe.
         iv. Then scrape the base of the vesicle with a sterile scalpel blade, and collect specimen with a Dacron swab by vigorously rubbing the base of the vesicle.
      (b) If there is a crust on the lesion, gently remove it.
         i. Moisten swab with saline and collect specimen by vigorously rubbing the base of the lesion for *H. ducreyi* culture.
         ii. Alternatively, gently abrade the lesion with a sterile scalpel or needle until serous fluid emerges. (Try to avoid bleeding.) Irrigate with saline.
            (i) For *H. ducreyi* culture, rub the base vigorously with a sterile swab or aspirate fluid with flamed smoothed Pasteur pipette or needle and syringe.
            (ii) For *T. pallidum*, wipe away fluid, blood, and debris with sterile gauze. Apply gentle pressure to the base of the lesion until clear fluid is expressed. Touch a slide to the fluid, and cover the fluid on the slide with a coverslip. If no exudate is present, add a drop of saline to the lesion or insert a needle and syringe at the lesion base, aspirate, and then draw a drop of saline into the needle. Express the material on to a slide (*see* Appendix 3.2.3–1).
   (2) Order *T. pallidum* dark-field microscopy, *H. ducreyi* culture, or HSV culture.

c. Prostate
   (1) After the patient urinates, perform a digital massage through the rectum.
   (2) Have patient pass prostatic secretions in the urethra by urinating into a cup. Alternatively, pass the urethral genital wire swab or a bacteriological loop several centimeters into the urethra.
   (3) Sequential urine cultures may be used to diagnose the location of a lower urinary tract infection in men (28). Table 3.9.1–6 outlines the various portions of the urine sample that are quantitatively cultured (see procedure in Appendix 3.9.1–2) (28). The different urine samples and the prostatic massage specimen should be clearly labeled by their clinical designations (e.g., VBS no. 1, etc.). A urethral swab may also be collected for detection of *N. gonorrhoeae* and *Chlamydia trachomatis* and other urethritis primary pathogens (Table 3.9.1–3).

Table 3.9.1–6 Specimens collected to diagnose the location of a lower urinary tract infection in men[a]

Specimen	Clinical designation	Description
Voided bladder urine no. 1	VBS no. 1	Initial 5–10 ml of urinary stream
Voided bladder urine no. 2	VBS no. 2	Midstream urine specimen
Expressed prostatic specimen	EPS	Secretions expressed from the prostate during prostatic massage
Voided bladder urine no. 3	VBS no. 3	First 5–10 ml of urinary stream collected immediately after prostatic massage

[a] Data adapted with permission from **T. Stamey.** 1980. *Pathogenesis and Treatment of Urinary Tract Infections.* Williams & Wilkins, Baltimore, MD.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

3. Male or female testing
- **a.** Rectal tests
  - (1) To perform an *N. gonorrhoeae* culture, insert swab past anal sphincter, move from side to side, allow 10 to 30 s for absorption, and withdraw. If swab is contaminated with feces, collect new sample. Order *N. gonorrhoeae* culture.
  - (2) To perform a combined NAT for detection of both *N. gonorrhoeae* and *C. trachomatis,* collect a swab according to the specific manufacturer's instructions (*see* section 12). Order NAT for *N. gonorrhoeae* and *C. trachomatis.*
- **b.** Throat tests for detection of *N. gonorrhoeae*
  - (1) To perform a throat culture, use a routine swab inserted into charcoal medium.
  - (2) To perform a throat NAT, use the swab provided in the specific manufacturer's collection kit.
  - (3) Depress tongue gently with tongue depressor.
  - (4) Extend one or two sterile swabs (one for antigen test and one for culture) between the tonsillar pillars and behind the uvula, avoiding the tongue, inner cheeks, and uvula.
  - (5) Sweep the swabs back and forth across the posterior pharynx, tonsillar areas, and any inflamed or ulcerated areas to obtain sample.
  - (6) Order *N. gonorrhoeae* culture or NAT for *N. gonorrhoeae*.
- **c.** Urethral tests
  - (1) To perform a urethral culture, use a routine swab inserted into charcoal medium.
  - (2) To perform a urethral NAT, use the swab provided in the specific manufacturer's collection kit.
  - (3) Express exudate onto swab from distal urethra.
  - (4) If there is no exudate, collect 1 h after urination. Wipe area clean, insert a urethrogenital swab 2 to 4 cm into the endourethra, gently rotate the swab, leave it in place for 1 to 2 s, and withdraw it.
  - (5) Order *N. gonorrhoeae* culture or NAT for *N. gonorrhoeae* and *Chlamydia trachomatis.*

4. Abscess material (bubo, lymph node, etc.)
- **a.** Disinfect skin with surgical disinfectant.
- **b.** Aspirate the lesion with needle and syringe (refer to Specimen Collection, Transport, and Handling in procedure 3.13.1 for other details).
- **c.** Order Gram stain, aerobic and anaerobic wound and abscess cultures, and, if indicated from a lymph node, *Chlamydia* or *H. ducreyi* culture.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

B. Specimen transport

1. Transport medium

a. For transport for specific organisms, refer to the separate procedures. Otherwise, submit swab in Amies transport tube.

b. Place immediately on ice or in the refrigerator until and during transport.

◩ **NOTE:** Previous literature indicates that *N. gonorrhoeae* does not survive well at refrigeration temperatures, but recent studies indicate otherwise (*see* procedure 3.9.3).

2. Label specimens and accompanying requisition with patient name, hospital medical record number, room number or clinic location, other patient demographics, and date, time, and site of collection.

3. Indicate the pathogens sought on requisition or computer entry. Do not order "routine culture" from sexually active patients. These are rarely indicated and are performed mostly from prepubescent or postmenopausal females.

C. Rejection criteria

1. Do not accept vaginal swabs from women in childbearing years for "routine genital culture." Using a form similar to that in Appendix 3.9.1–1, require that the disease or agent sought be ordered specifically.

2. Reject specimens not received in transport medium, since the agents of genital infections lose viability easily.

III. MATERIALS

A. Media

1. CHOC

2. Thayer-Martin (TM) or similar selective medium for *N. gonorrhoeae*

3. BAP

4. MAC or EMB

5. Columbia colistin-nalidixic acid agar (CNA)

6. Selective medium for yeasts (by separate request only).

◩ **NOTE:** For invasively collected specimens, refer to other procedures in this handbook for culture, including anaerobic cultures.

B. Identification methods

1. Gram stain (procedure 3.2.1)

2. Catalase test (procedure 3.17.10)

3. Oxidase test (procedure 3.17.39)

4. Indole test (procedure 3.17.23)

5. Aminolevulinic acid (ALA) test (procedure 3.17.3)

6. Pyrrolidonyl-β-naphthylamide (PYR) (procedure 3.17.41)

7. Sodium polyanethol sulfonate (SPS) disks (procedure 3.17.45)

8. Identification kits for gram-negative bacteria

9. Other tests as indicated in procedures 3.3.2, 3.18.1, and 3.18.2

C. Other supplies

1. Incubator at 35°C, with 5% CO_2 or ambient air

2. Self-contained CO_2-generating system for culture incubation if incubator is ambient air

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that media meet expiration date and QC parameters per Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22-A3 (9). See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

B. See procedures 3.3.1 and 3.9.3 for additional QC of CHOC and TM, respectively.

V. PROCEDURE

Observe standard precautions.

A. Inoculation

◩ **NOTE:** Use of a biosafety cabinet will avoid contamination of cultures as well as protect laboratory processing personnel.

1. If the specimen is not received on plates, inoculate plates from the swab in transport medium.

V. PROCEDURE *(continued)*

2. See Appendix 3.9.1–1 for tests for reference to specific organism procedures. For specimens from wounds, abscesses, and normally sterile sites, refer to the wound and abscess procedure (3.13.1) or body fluid culture procedure (3.5).

3. For cervical, vaginal, or other noninvasive genital source specimens submitted for unusual culture requests, inoculate the first five media listed above.

B. Direct smear

1. Prepare a Gram stain from the swab after plate inoculation.

2. Stain slide (procedure 3.2.1) and, for women in childbearing years, observe for evidence of BV from vaginal specimens (Appendix 3.2.1–3), for yeasts, and for evidence of other bacteria associated with WBCs.

C. Incubation

1. Incubate plates at 35°C in 5% CO_2, or use other CO_2-generating system to provide the proper atmosphere and moisture.

2. Observe for growth after 18 to 24 h of incubation. Hold negative plates for up to 72 h.

D. Culture examination

1. Observe plates after 24 h for growth of abnormal microbiota.

2. Correlate growth with Gram stain result to determine the extent of workup.

3. Identify the following pathogens if present, using identification kits or methods described in Table 3.3.2–5.

a. Examine non-lactose-fermenting, gram-negative rods for *Shigella* spp. or other enteric pathogens, especially *from pediatric patients* (20).

◩ **NOTE:** Do not examine cervical or vaginal specimens for other *Enterobacteriaceae*, as these microorganisms are normally found in the female genital tract.

b. *Streptococcus pyogenes* (25)

c. *S. agalactiae* (procedure 3.9.2)

d. *Listeria monocytogenes*

e. *N. gonorrhoeae* (procedure 3.9.3)

f. *Candida albicans.* Mention other yeasts. *Candida glabrata* has been implicated as a cause of vulvovaginitis (27).

4. Identify the following only if the specimen was invasively collected or there is heavy growth and they are the predominant microorganism in the culture.

a. *Haemophilus* spp. (8, 15, 30)

b. Gram-negative rods

(1) Enteric gram-negative rods (exception: *Enterobacteriaceae* are part of the normal microbiota of the vagina and should not be reported).

(2) *Pseudomonas* spp. and other non-glucose-fermenting, gram-negative rods

(3) *Pasteurella bettyae* (CDC group HB-5). See Table 3.9.1–7.

◩ **NOTE:** *P. bettyae* has been associated with genital infections, especially in neonates. It is an indole-positive gram-negative rod, but unlike *E. coli*, it is catalase negative and oxidase variable and does not grow or grows as pinpoint colonies on MAC (2, 31).

(4) *Capnocytophaga* spp.

◩ **NOTE:** This group of organisms have been associated with genital infections. They are catalase-negative, oxidase-negative glucose-fermenting gram-negative rods that do not grow on MAC. The glucose reaction may not be seen on triple sugar iron agar (TSI) or Kligler's iron agar. They are generally esculin positive (procedure 3.17.5).

(5) *Campylobacter fetus* (*see* procedure 3.8.2)

c. *S. aureus*

d. *Streptococcus pneumoniae*

Table 3.9.1–7 Biochemical reactions of *Haemophilus* and other fastidious gram-negative coccobacilli found in genital microbiota[a]

Test	*H. influenzae/ haemolyticus*[b]	*H. parahaemolyticus/ paraphrophilus/ parainfluenzae*[b]	*H. ducreyi*	*Capnocyto-phaga* DF-1 and DF-3[c]	*P. bettyae* (HB-5)	*C. fetus*	*Kingella denitrificans*[d]	*Kingella kingae*
Catalase	+	V	−	−	−	+	−	−
Oxidase	V	V	+	−	V	+	+	+
Indole	V	V	−	V	+	−	−	−
Motility	−	−	−	−	−	+	−	−
Nitrate with gas[e]	NA	NA	NA	V	+/−	+	+/V	−/V
Urease	V	V	−	−	−	−	−	−
Growth on BAP at 35°C	Satellite	Satellite	−	+	+	V	+	+; beta
ALA	−	+	−	+	NA	NA	NA	NA
TSI[f]	NA	NA	NA	Acid slant and butt	Acid butt only	−	−	Rare acid in slant and butt

[a] Strains in this table are characterized by no growth on MAC, with the exception of a rare *P. bettyae* strain. NA, not applicable; V, variable. This table is prepared from biochemical tables from Weyant et al. (31).

[b] Separate by hemolysis on horse or rabbit blood, if desired; however, *H. haemolyticus*, *H. paraphrophilus*, and *H. parahaemolyticus* generally are not found in the genital tract.

[c] *Capnocytophaga* spp. (DF-1) are rods with tapered ends and frequently have a yellow pigment; DF-3 is a coccobacillus. They both may hydrolyze esculin.

[d] Not generally a pathogen but mimics *N. gonorrhoeae;* it will grow on BAP, is nitrate positive, and is a coccobacillus.

[e] The nitrate reaction is first; if the gas reaction is known, it is listed second, preceded by a slash.

[f] May not be able to demonstrate reactions without addition of rabbit serum, if organism is fastidious.

V. PROCEDURE *(continued)*

e. *Neisseria meningitidis*

f. *G. vaginalis*

(1) For vaginal specimens, do *not* use selective medium to isolate this organism, because the importance of its isolation is determined by the quantity compared to that of lactobacilli in the culture (1, 6). *G. vaginalis* grows well on both CNA and CHOC.

(2) When present in quantities less than the other normal microbiota, it should be included as part of normal vaginal microbiota. However, for children report its presence regardless of the quantity present.

(3) If it is the predominant microorganism from the female vaginal tract and is isolated in 3 to 4 + quantities (third or fourth quadrant on the plate), report its presence.

(4) Identification

(a) Colonies appear pinpoint and transparent, with no greening of the agar

(b) Gram-variable to gram-negative small, pleomorphic coccobacilli that do not elongate into filaments or chains

(c) Catalase negative. To confirm the lack of catalase enzyme, streak colony on CHOC plate and add a dot of viridans group streptococci (*Streptococcus sanguis* ATCC 35557). A clear zone of inhibition around a dot of viridans group streptococci confirms the lack of catalase (24).

(d) SPS sensitive (1, 24) or hippurate positive (procedure 3.17.21) or beta-hemolytic on human blood agar

◩ **NOTE:** The API CORYNE strip accurately identifies this microorganism (12). It is not necessary to confirm the identification with tests other than colony morphology, catalase, and smear, if the direct Gram stain is consistent with diagnosis of BV. Aroutcheva et al. (1) report that the strains isolated from cases of BV were more likely to have a negative

V. PROCEDURE *(continued)*

hippurate or lipase test reaction than those from patients without BV. They also showed that the isolation of 3 or 4+ *G. vaginalis* from culture correlated well with BV, although 26% of the cases diagnosed by Gram stain were missed on culture using selective human blood-Tween bilayer media, which is reported to be superior to V agar (1).

E. Susceptibility testing
- **1.** Antimicrobial susceptibility testing should be performed on all aerobic bacterial isolates from pelvic or amniotic fluid or tissue specimens that are considered to be primary pathogens.
- **2.** Anaerobes isolated from pelvic or amniotic fluid or tissue specimens that are considered to be primary pathogens should have antimicrobial susceptibility testing done according to CLSI guidelines (10).
- **3.** *N. gonorrhoeae* isolated from any genital site should be referred for antimicrobial susceptibility testing (11).
- **4.** Fastidious gram-negative coccobacilli or rods that do not grow on MAC or EMB should have a beta-lactamase test.

F. Hold positive culture plates for at least 7 days should further testing be indicated.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. From surgical specimens and those from normally sterile sites, report the pathogens isolated. For cultures with mixed microbiotas, grouping of pathogens may be indicated, e.g., "Mixed enteric rods or mixed anaerobes present."

B. If no pathogens are isolated but normal microbiota is present, report as "Normal genital microbiota isolated" for vaginal and cervical specimens and as "Normal cutaneous microbiota isolated" for male urethral specimens.
- **1.** See item I of this procedure for list of organisms in the normal microbiota of male and female genitalia.
- **2.** Do not list genera and species of normal microbiota individually.
- **3.** If specific pathogens are requested, report "No [pathogen name] isolated."

C. Positive reporting
- **1.** Quantitate pathogens (Table 3.3.2–2).
- **2.** Notify physician of pathogens of serious significance in pregnant females (e.g., *L. monocytogenes*) or communicable diseases (e.g., *N. gonorrhoeae*).
- **3.** Notify the local health department of isolation of agents of reportable diseases.

VII. INTERPRETATION

A. The presence of any microorganism from a normally sterile site is generally considered significant.

B. Isolation of sexually transmitted disease pathogens (e.g., *N. gonorrhoeae* and *H. ducreyi*) is considered clinically significant from any genital site. Isolation of certain organisms such as *S. pyogenes* and *Clostridium perfringens* from soft tissue as well as endometrial tissue or transvaginal fluid samples should also be considered clinically significant.

C. The presence of other pathogens may or may not be a cause of disease and must be evaluated with consideration of relative amounts and symptoms or other conditions (e.g., pregnancy) in the patient.

D. Due to the increasing resistance of many primary pathogens to commonly used agents, antimicrobial susceptibility testing may have to be performed on clinically significant aerobic and anaerobic isolates as outlined under item V.E.

VIII. LIMITATIONS

A. Many agents of disease are difficult to culture, and the lack of isolation may not indicate that the pathogen is not the cause of disease.

B. Communication between the laboratory and the physician is necessary to provide the appropriate cultures for the disease present, since agents such as *Ureaplasma* and *H. ducreyi* may not grow on routine laboratory media.

C. Unless selective media and incubation are used, a routine genital culture will not detect carriage of GBS in all cases.

D. BV is best diagnosed by Gram stain using Nugent's criteria or a rapid test for sialidase (OSOM BV Blue; Genzyme).

E. Due to the poor sensitivity of a wet-mount examination, another method should be used for the diagnosis of *T. vaginalis* infection.

F. The presence of fastidious gram-negative rods in genital specimens may or may not indicate infection. Cases have been reported, but they are infrequent (2, 8, 15, 30).

REFERENCES

1. **Aroutcheva, A. A., J. A. Simoes, K. Behbakht, and S. Faro.** 2001. *Gardnerella vaginalis* isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems. *Clin. Infect. Dis.* **33:**1022–1027.
2. **Baddour, L. M., M. S. Gelfand, R. E. Weaver, T. C. Woods, M. Altwegg, L. W. Mayer, R. A. Kelly, and D. J. Brenner.** 1989. CDC group HB-5 as a cause of genitourinary infections in adults. *J. Clin. Microbiol.* **27:**801–805.
3. **Baron, E. J., G. H. Cassell, D. A. Eschenbach, J. R. Greenwood, S. M. Harvey, N. E. Madinger, E. M. Paterson, and K. B. Waites.** 1993. *Cumitech 17A, Laboratory Diagnosis of Female Genital Tract Infections.* Coordinating ed., E. J. Baron. American Society for Microbiology, Washington, DC.
4. **Brown, H. L., D. D. Fuller, T. E. Davis, J. R. Schwebke, and S. L. Hillier.** 2001. Evaluation of the Affirm Ambient Temperature Transport System for the detection and identification of *Trichomonas vaginalis, Gardnerella vaginalis,* and *Candida* species from vaginal fluid specimens. *J. Clin. Microbiol.* **39:**3197–3199.
5. **Campbell, L., V. Woods, T. Lloyd, S. Elsayed, and D. L. Church.** 2008. Evaluation of the OSOM *Trichomonas* rapid test versus wet preparation examination for detection of *Trichomonas vaginalis* vaginitis in specimens from women with a low prevalence of infection. *J. Clin. Microbiol.* **46:**3467–3469.
6. **Catlin, B. W.** 1992. *Gardnerella vaginalis:* characteristics, clinical considerations, and controversies. *Clin. Microbiol. Rev.* **5:**213–237.
7. **Centers for Disease Control and Prevention.** 2006. Sexually transmitted diseases treatment guidelines. *MMWR Morb. Mortal. Wkly. Rep.* **55**(RR-11)**:**1–100
8. **Chowdhury, M. N. H., and S. S. Parek.** 1983. Urethritis associated with *Haemophilus parainfluenzae:* a case report. *Sex. Transm. Dis.* **10:**45–46.
9. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
10. **Clinical and Laboratory Standards Institute.** 2007. *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria,* 7th ed. Approved standard M11-A7. Clinical and Laboratory Standards Institute, Wayne, PA.
11. **Clinical and Laboratory Standards Institute.** 2008. *Performance Standards for Antimicrobial Susceptibility Testing.* M100-S18, 18th informational supplement. Clinical and Laboratory Standards Institute, Wayne, PA.
12. **Gavin, S. E., R. B. Leonard, A. M. Briselden, and M. B. Coyle.** 1992. Evaluation of the rapid CORYNE identification system for *Corynebacterium* species and other coryneforms. *J. Clin. Microbiol.* **30:**1692–1695.
13. **Geisler, W. M., S. Yu, M. Wenglarik, and J. R. Schwebke.** 2004. Vaginal leukocyte counts in women with bacterial vaginosis and relation to vaginal and cervical infection. *Sex. Transm. Infect.* **80:**401–405.
14. **Hager, W. D., and B. Majudar.** 1979. Pelvic actinomycosis in women using intrauterine contraceptive devices. *Am. J. Gynecol.* **133:**60–63.
15. **Hall, G. D., and J. A. Washington.** 1983. *Haemophilus influenzae* in genitourinary tract infections. *Diagn. Microbiol. Infect. Dis.* **1:**65–70.
16. **Hammerschlag, M. R., S. Alpert, I. Rosnar, P. Thurston, D. Semine, D. McComb, and W. M. McCormack.** 1978. Microbiology of the vagina in children: normal and potentially pathogenic organisms. *Pediatrics* **62:**57–62.
17. **Heller, R. H., J. M. Joseph, and H. J. Davis.** 1969. Vulvovaginitis in the premenarcheal child. *J. Pediatr.* **74:**370–377.

REFERENCES *(continued)*

18. **Huppert, J. S., J. E. Mortensen, J. L. Reed, J. A. Kahn, W. C. Miller, and M. M. Hobbs.** 2007. Rapid antigen testing compares favourably with transcription-mediated amplification assay for the detection of *Trichomonas vaginalis* in young women. *Clin. Infect. Dis.* **45:**194–198.
19. **Larsen, B., and G. R. G. Monif.** 2001. Understanding the bacterial flora of the female genital tract. *Clin. Infect. Dis.* **32:**e69–e71.
20. **Murphy, T. V., and J. D. Nelson.** 1979. *Shigella* vaginitis: report of 38 patients and review of the literature. *Pediatrics* **63:**511–516.
21. **Nugent, R. P., M. A. Krohn, and S. L. Hillier.** 1991. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. *J. Clin. Microbiol.* **29:**297–301.
22. **Osborne, N. G., R. C. Wright, and L. Grubin.** 1979. Genital bacteriology: a comparative study of premenopausal women with postmenopausal women. *Am. J. Obstet. Gynecol.* **135:**195–198.
23. **Polaneczky, M., C. Quigley, L. Pollock, D. Dulko, and S. S. Whitkin.** 1998. Use of self-collected vaginal specimens for detection of *Chlamydia trachomatis* infection. *Obstet. Gynecol.* **9:**375–378.
24. **Reimer, L. G., and L. B. Reller.** 1985. Use of a sodium polyanetholesulfate disk for the identification of *Gardnerella vaginalis. J. Clin. Microbiol.* **21:**146–149.
25. **Schwartz, R. H., R. L. Wientzen, and R. G. Barsanti.** 1982. Vulvovaginitis in prepubertal girls: the importance of group A streptococcus. *South. Med. J.* **75:**446–447.
26. **Singleton, A. F.** 1980. Vaginal discharge in children and adolescents. *Clin. Pediatr.* **19:**799–805.
27. **Sobel, J. D.** 1997. Vaginitis. *N. Engl. J. Med.* **337:**1896–1903.
28. **Stamey, T.** 1980. *Pathogenesis and Treatment of Urinary Tract Infections.* Williams & Wilkins, Baltimore, MD.
29. **Steinhandler, L., J. F. Peipert, W. Heber, A. Montagno, and C. Cruikshank.** 2002. Combination of bacterial vaginosis and leukorrhea as a predictor of cervical chlamydial or gonococcal infection. *Obstet. Gynecol.* **99:**603–607.
30. **Sturm, A. W.** 1986. *Haemophilus influenzae* and *Haemophilus parainfluenzae* in nongonococcal urethritis. *J. Infect. Dis.* **153:**165–167.
31. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria,* 2nd ed. Williams & Wilkins, Baltimore, MD.

SUPPLEMENTAL READING

Casey, B. M., and S. M. Cox. 1997. Chorioamnionitis and endometritis. *Infect. Dis. Clin. N. Am.* **11:**203–222.

Hillier, S. L., and R. J. Lau. 1997. Vaginal microflora in postmenopausal women who have not received estrogen replacement therapy. *Clin. Infect. Dis.* **25**(Suppl. 2)**:**S123–S126.

Holmes, K. K., P. F. Sparling, P. A. Mardh, S. A. Lemon, W. E. Stamm, P. Piot, and J. M. Wasserheit. 1999. *Sexually Transmitted Diseases,* 3rd ed. McGraw Hill Book Co., New York, NY.

Kent, H. L. 1991. Epidemiology of vaginitis. *Am. J. Obstet. Gynecol.* **165:**1168–1176.

Landers, D. V., and R. L. Sweet. 1986. Upper genital tract infections, p. 187–207. *In* R. P. Galask and B. Larsen (ed.), *Infectious Diseases in the Female Patient.* Springer-Verlag, New York, NY.

Larsen, B. 1986. Intrauterine bacterial infections, p. 141–162. *In* R. P. Galask and B. Larsen (ed.), *Infectious Diseases in the Female Patient.* Springer-Verlag, New York, NY.

Lawson, M. A., and M. J. Blythe. 1999. Pelvic inflammatory disease in adolescents. *Pediatr. Clin. N. Am.* **46:**767–782.

APPENDIX 3.9.1–1 Sample Request Form for Submission of Specimens for Diagnosis of Genital Infections for Women in Childbearing Years and Sexually Active Adults

❑ **Bacterial vaginosis[a]:** Prepare a smear of vaginal discharge. Place slide in the slide holder and patient label on outside of holder.

Test: Gram stain (procedure 3.2.1

❑ **Candidiasis:** Prepare specimen for viewing by placing swab moistened with vaginal fluid (add small amount of saline if necessary) onto glass slide or submit swab in transport medium.

Test: wet mount for yeast (procedure 3.2.3). Yeast culture done if history of treatment failure.

❑ **Chancroid:** Submit aspirate and scraping of base of ulcer in transport medium. Contact laboratory prior to collection to arrange for appropriate transport.

Test: Haemophilus ducreyi culture (procedure 3.9.4).

❑ **Chlamydia infection[a]:** Submit ❑ cervical (females) or ❑ urethral (males) swab. ❑ First voided 20 ml of urine may be sent for nucleic acid amplification tests. Test can be done by probe technology if submitted in appropriate transport tube, but this is not accepted for abuse cases.

Test: ❑ chlamydia culture ❑ probe test *(see* procedure 10.6 and Table 12.1–2, respectively).

❑ **Trichomonas infection[a]:** Submit *vaginal* swab in InPouch after swirling swab in top portion of broth, or submit swab in charcoal transport tube.

Test: Trichomonas wet mount and culture *(see* procedures 3.2.3, 9.6.6, and 9.9.4).

❑ **Gonorrhea[a]:** Submit swab from ❑ cervix (females) or ❑ urethra (males) or ❑ rectum or ❑ throat in charcoal transport medium within 3 h of collection. ❑ First voided 20 ml of urine may be sent for nucleic acid amplification tests only. Test can be done by probe technology if submitted in appropriate transport tube, but this is not accepted for abuse cases or from throat or rectum.

Test: ❑ GC culture ❑ probe test (procedure 3.9.3 and Table 12.1–2, respectively).

❑ **Herpes infection:** Submit aspirate and scraping of base of vesicle.

Test: herpes DFA or PCR *(see* procedure 10.5). *Note:* Herpes serology can be used to document past infection.

❑ **Genital mycoplasma infection:** ❑ Vaginal or cervical (females) or ❑ urethral (males) swab for culture. ❑ Other more invasive specimens are preferred _____. This test should only be ordered for symptomatic patients and not as a screening test.

Test: Ureaplasma culture (procedure 3.15).

❑ **Prevention of neonatal group B streptococcal (GBS) disease:** Submit swab from *vaginal* and anal area in tube. Collection should be at 35 to 37 weeks gestation. Testing for GBS early in pregnancy is not recommended. Laboratory will enrich for organism in broth culture followed by plate culture.

Test: GBS culture (procedure 3.9.2).

❑ **Syphilis:** *Note:* Diagnosis is generally done by serology. Collect lesion scraping and deliver to laboratory immediately.

Test: dark-field exam.

❑ **Other:** State symptoms and organisms sought. _____

[a] Modify requisition collection and test ordering instructions for alternate methods (*see* Tables 3.9.1–5 and 3.9.1–6).

APPENDIX 3.9.1–2

Quantitative Cultures of Sequential Urine and Prostate Secretion Specimens in the Localization of Lower Urinary Tract Infection

I. PROCEDURE

A. Urine cultures and expressed prostatic secretions are collected and labeled using the correct clinical designation as outlined in Table 3.9.1–6.
   1. VBS no. 1 is the initial 5 to 10 ml of the urinary stream.
   2. VBS no. 2 is the midstream portion of the urinary stream.
   3. EPS are secretions expressed from the prostate by digital massage.
   4. VBS no. 3 is the first 5 to 10 ml of the urinary stream voided immediately after prostatic massage.

B. Each specimen is placed in a urine collection tube containing boric acid preservative and transport to the laboratory within 2 to 4 h after collection.

C. VBS no. 1, 2, and 3 and EPS are quantitatively cultured using a 0.01 loop (10 μl) and plated onto a blood agar (BA) and MAC plate.

D. Colony counts for each primary urinary tract infection pathogen are determined by the following formula: colonies on the BA plate $\times 10^2 =$ ___ CFU/ml.

E. Colony counts should be rounded and reported as a single-digit number $\times 10^e$ ___ CFU/ml.

F. Primary pathogens that should be identified include *Enterobacteriaceae; Pseudomonas aeruginosa; Enterococcus* spp.; *Staphylococcus aureus;* group A, B, C, and G streptococci; and *Candida* spp.

G. Coagulase-negative staphylococci, *Corynebacterium* spp., and other potential skin microbiota should only be worked up if isolated pure or in the same amount as the primary pathogens.

H. Antibiotic susceptibility testing should be done on all clinically significant isolates. If the same organism is isolated from more than one sample in the sequence, then the isolate from only one of the samples needs to have antibiotic susceptibility testing performed provided the morphotypes are biochemically similar in all of the samples.

II. CLINICAL SIGNIFICANCE

Diagnosis of bacterial prostatitis by the sequential localization testing requires that the colony count in the midstream urine (VBS no. 2) be increased by at least 10-fold (1 log unit) in the postprostatic massage urine (VBS no. 3) or EPS compared with the colony count in the first void urine (VBS no. 1). Chronic bacterial prostatitis patients have only low numbers of organisms in the gland itself. Quantitative culture of EPS may be particularly important for these men. EPS should also be examined microscopically to look for pus and large lipid-laden macrophages that are characteristic of the prostatic inflammatory response.

References

1. **Kreiger, J. N.** 2005. Prostatitis, epididymitis, and orchitis, p. 1381–1386. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), *Principles and Practice of Infectious Diseases*, 6th ed. Elsevier Churchill Livingstone, Philadelphia, PA.
2. **Stamey, T.** 1980. *Pathogenesis and Treatment of Urinary Tract Infections.* Williams & Wilkins, Baltimore, MD.
3. **Thin, R. N.** 1997. Diagnosis of chronic prostatitis: do urologists use the four-glass test? *Urology* **55:**403–407.

3.9.2 Group B Streptococcus Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Group B streptococcus (GBS) *(Streptococcus agalactiae)* has been recognized as the leading infectious cause of perinatal morbidity and mortality in the United States. In pregnant women, it is associated with asymptomatic bacteriuria, urinary tract infection, and amnionitis, and in women who have recently delivered, it causes endometritis and wound infection. Early-onset neonatal disease (during the first week of life) results from transmission of GBS during labor or delivery from mother to infant; late-onset disease (from 1 to 3 months after birth) is thought to be acquired in the nursery. Both are characterized by septicemia, pneumonia, or meningitis and can result in death or permanent neurological sequelae.

In 1986 Boyer and Gotoff (7), in a carefully controlled study, demonstrated that intrapartum ampicillin prophylaxis can prevent early-onset neonatal GBS disease in women with positive prenatal cultures for GBS and certain perinatal risk factors. In 1992 the American Academy of Pediatrics issued guidelines for prevention of neonatal GBS disease (1), which included prenatal cultures at 26 to 28 weeks' gestation. In 1996 and 1997, the American Academy of Pediatrics (2), the American College of Obstetricians and Gynecologists (3), and the CDC (8, 9) issued guidelines that included a combination of treating all women in risk groups and/or culturing at 35 to 37 weeks' gestation to identify carriers for whom treatment would most likely prevent disease. The rationale for this change in the timing of culturing was that at any given time 20% of women are colonized with GBS, but it is colonization at the time of delivery that is associated with disease. By the end of 1998, it was estimated that in the United States in that year alone, 3,900 cases of GBS disease in neonates and 200 deaths from early-onset GBS disease were prevented by the guidelines (22). In 2002 (10), the CDC issued new guidelines based on 6 years of experience, stating the need for doing culture and providing more exact details for the culture techniques. A preincubation step in a selective broth containing antimicrobials was included as part of the guidelines, because it is the most sensitive culture method. Newer commercial molecular and nonmolecular test systems for detection of GBS in vaginal-rectal specimens that have improved sensitivity, decreased test turnaround time, and increased test efficiency due to their ease of performance have recently been marketed (5, 11). StrepB carrot broth had sensitivity comparable to that of standard selective broth but improved test turnaround time because GBS can be directly reported based on an orange color change (11). One-day carrot broth culture with subsequent rapid PCR has recently been shown to be highly sensitive for the prompt detection of GBS (5). CHROMagar StrepB is a new chromogenic medium for screening cultures of *S. agalactiae* from vaginal-rectal swabs (http://www.chromagar.com). Detection of GBS using peptide nucleic acid in situ hybridization (AdvanDx) after broth culture has also recently been shown to be highly sensitive in enhancing the culture-based detection of *S. agalactiae* (20). Several commercial real-time PCR tests are now FDA cleared for rapid screening for prenatal GBS at 35 to 37 weeks, or during labor and delivery. Both the BD GeneOhm Strep B Assay (BD GeneOhm) and the Cepheid Xpert GBS Assay, performed on the GeneXpert System, detect GBS directly from vaginal-rectal swabs within 1 to 2 h after receipt by the laboratory. The GeneXpert has been shown to be highly sensitive compared to late antenatal screening by culture (18).

GBS disease is increasing in nonpregnant adults, especially the elderly and those with significant underlying disease (17). Diabetes mellitus, neurological impairment, and cirrhosis appear to be risk factors. Skin, soft tissue infections, pneumonia, and urosepsis are common presentations, although meningitis and endocarditis are reported. Disease is frequently nosocomial, possibly related to catheter placement (17). For diagnosis of GBS disease in nonpregnant adults and in neonates, routine culture of the symptomatic body site (e.g., blood, CSF, and amniotic and joint fluid cultures) will detect this pathogen along with the other potential pathogens which can be isolated from those cultures. This procedure addresses only the perinatal screening culturing where the physician specifically orders culture for GBS to detect colonization.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

◩ **NOTE:** Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and other caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

A. Collect specimen at 35 to 37 weeks' gestation. Using a single swab or two separate swabs, swab the distal vagina (vaginal introitus), followed by the rectum (i.e., insert swab through the anal sphincter).

◩ **NOTE:** The CDC recommends that women be empirically treated on the basis of risk factors if GBS test results are not available at the time of delivery. A vaginal-rectal swab should be immediately collected during labor and delivery and submitted to the laboratory for STAT GBS testing by a commercial real-time PCR assay to enable antimicrobial therapy to be selectively given on the basis of a positive rapid test result. Alternatively, the vaginal-rectal swab can be collected and submitted for culture in settings where rapid molecular GBS testing is not available. It is important to determine the mother's GBS colonization status at the time of delivery if prenatal testing has not previously been done, since her colonization status is clinically important in diagnosing subsequent disease in the neonate. *Rapid GBS antigen tests should not be performed during labor and delivery or in place of prenatal cultures because they are not sensitive enough to be used to direct antimicrobial treatment (10).*

B. If symptoms of sepsis or amnionitis are present, also submit amniotic fluid (procedure 3.5), blood, or urine for culture.

C. Specimen transport

1. Submit one or both swabs to the laboratory in a *single* tube of nonnutritive transport medium (Amies or Stuart's). It is not necessary to submit anal and vaginal swabs separately.

2. Label specimens and accompanying requisition with demographic information on the patient (name, identifying number, age, and diagnosis), the date and site of collection, and the week of gestation.

3. Store at 25°C or refrigerate at 4°C.

4. Order culture for GBS; if the patient is penicillin allergic, also order testing for susceptibility to clindamycin and erythromycin.

5. Submit to the laboratory within 4 days of collection.

D. Rejection criteria

◩ **NOTE:** According to an alert from the CDC on 24 March 1997, gravely erroneous information and potentially fatal misdiagnoses can result from the use of direct antigen testing to detect GBS directly in urine of pregnant women or infants.

1. If a direct antigen assay is performed on CSF, always perform a culture for confirmation of viable organisms.

2. Do not accept urine or vaginal swabs for direct antigen assay.

3. For the neonate, screening cultures (e.g., axilla, throat, etc.) and urinary antigen tests are not recommended because of their lack of sensitivity and specificity for diagnosis.

◩ **NOTE:** Collect blood, CSF, and respiratory secretions to diagnose disease in symptomatic neonates.

III. MATERIALS

A. Media

1. Selective streptococcal broth

a. LIM broth: Todd-Hewitt broth with 10 µg of colistin per ml and 15 µg of nalidixic acid per ml (available from most medium vendors)

b. Trans-Vag broth with 5% defibrinated sheep blood: Todd-Hewitt broth with 8 µg of gentamicin per ml and 15 µg of nalidixic acid per ml (Remel, Inc.)

◩ **NOTE:** Fackrell and Dick (16) reported that LIM broth supported growth of GBS better than Trans-Vag broth but was equivalent to Trans-Vag broth with blood.

c. StrepB carrot broth: modification of Granada medium that detects GBS based on a hemolytic strain's unique ability to react with substrates (e.g., starch or proteose peptone) present in the broth (Hardy Diagnostics)

2. Agar media

a. BAP

b. NNA: BAP with 30 µg of neomycin per ml and 15 µg of nalidixic acid per ml (BD Diagnostic Systems)

c. Selective streptococcal agar: BAP with neomycin and polymyxin B (Remel, Inc.; Hardy Diagnostics) or colistin and oxolinic acid (Remel, Inc.)

d. Columbia colistin-nalidixic acid agar (CNA)

e. CHROMagar StrepB: a chromogenic medium for the selective and differential screening of *S. agalactiae* (http://www.chromagar.com)

B. Identification methods

1. Gram stain (procedure 3.2.1)

2. Catalase test (procedure 3.17.10)

3. One of the following

a. *Staphylococcus aureus* ATCC 25923 for CAMP test (procedure 3.17.8)

b. Rapid hippurate (procedure 3.17.21)

c. AccuProbe GBS RNA probe assay (Gen-Probe Inc., San Diego, CA)

d. Latex or coagglutination serologic test for streptococcal grouping (*see* procedure 3.11.8 for options)

4. Other biochemical tests

a. Pyrrolidonyl-β-naphthylamide (PYR) (procedure 3.17.41)

b. Bile-esculin (procedure 3.17.5)

C. Other supplies/equipment

1. Incubator at 35°C with 5% CO_2 or other CO_2-generating system

2. Disposable cards for serologic tests

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that media meet expiration date and QC parameters per Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22-A3 (13). See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

B. Test each lot of BAP with a known GBS to verify the ability of the media to produce a positive CAMP reaction (procedure 3.17.8), or obtain documentation from medium vendor that each lot is checked for the CAMP reaction.

C. Refer to the individual biochemical test procedures for further QC requirements.

IV. QUALITY CONTROL *(continued)*

D. Perform QC with each lot of both selective streptococcal broth and agar that contain antimicrobial agents (excluding CNA). Incubate aerobically overnight at 35°C.

Test organism	Result(s)
Streptococcus agalactiae ATCC 12386	Broth: growth; beta-hemolytic colonies upon subculture to sheep blood agar. Agar: growth of beta-hemolytic colonies. StrepB Carrot broth: a positive reaction produces an orange, red, or brick red pigment. CHROMagar StrepB: a positive reaction produces mauve to pink colonies.
Proteus mirabilis ATCC 12453	Broth or agar: partial to complete inhibition. StrepB broth: no color change of carrot broth.
Streptococcus pyogenes ATCC 19615	Agar: test if agar is used to test for group A streptococcus. Growth of beta-hemolytic colonies. CHROMagar StrepB: other gram-positive bacteria may produce a blue color or be inhibited.

◩ **NOTE:** CLSI has proposed elimination of user QC of LIM broth and some selective streptococcal agars (13) purchased from commercial vendors. Consult with current regulatory agencies prior to discontinuation of user QC.

V. PROCEDURE

Observe standard precautions.

A. Inoculation
- **1.** LIM broth
  - **a.** Inoculate broth from the swab.
  - **b.** Optional, in addition to the broth inoculation: inoculate BAP, CNA, or selective streptococcal agar with swab immediately upon receipt.

    ◩ **NOTE:** Dunne and Holland-Staley (15) have shown that the addition of a selective direct plate to the broth culture increased detection by 15%, because *Enterococcus faecalis* can overgrow in the broth cultures and inhibit the growth of *S. agalactiae.*
- **2** StrepB carrot broth
  - **a.** Inoculate broth from the swab.
  - **b.** Initial subculture to a BAP is not required (see reference 11 for the recommended work flow for routine use of carrot broth).

B. Incubation
- **1.** LIM broth
  - **a.** Incubate selective direct plate at 35 to 37°C in 5% CO_2 (preferred) or ambient air. Alternatively, use other CO_2-generating system to provide the 5% CO_2.
  - **b.** Incubate broth at 35 to 37°C in ambient air or in 5% CO_2.
  - **c.** Subculture the broth after 18 to 24 h of incubation to BAP, if GBS have not been isolated on the direct plate.
  - **d.** Incubate subculture plate at 35 to 37°C in ambient air or 5% CO_2 for 48 h.
  - **e.** Optionally, in addition to subculture, test the broth after 18 to 24 h of incubation with either of the following.
    - (1) A streptococcal grouping test that includes an extraction step; this method is reported to be more sensitive than plate culture (21).
    - (2) AccuProbe GBS RNA probe assay or PCR method (4, 6) (Table 12.1.4)
- **2.** StrepB carrot broth
  - **a.** Incubate broth at 35 to 37°C in ambient air.

V. PROCEDURE *(continued)*

b. Subculture the carrot broth to a BAP after 18 to 24 h if there is no color change (see reference 11 for the recommended work flow for routine use of carrot broth).

C. Culture examination

1. Observe plates at 24 and 48 h for large, gray, translucent colonies with a small zone of beta-hemolysis or no zone of hemolysis.

2. Perform catalase test.

3. Perform Gram stain if catalase negative.

4. Identify gram-positive cocci in pairs and chains as *S. agalactiae* if they are catalase negative and demonstrate one of the following reactions.

a. Positive CAMP test

b. Positive with group B streptococcal grouping antisera

c. Positive GenProbe AccuProbe GBS test

d. Positive rapid hippurate

(1) If strain is nonhemolytic, a second test from the above list must be used for confirmation.

(2) If strain is hemolytic, perform PYR test (procedure 3.17.41). Enterococci are PYR positive but can be hippurate positive and hemolytic.

5. If the isolate is hemolytic and not identified as *S. agalactiae,* perform PYR and esculin or bile-esculin test. *S. pyogenes* is PYR positive, hemolytic, and esculin negative. Enterococci can be hemolytic and PYR positive but are esculin positive.

D. Perform disk diffusion testing for susceptibility to clindamycin and erythromycin, on request for penicillin-allergic patients (10) (*see* procedure 5.1). Place disks 15 to 26 mm apart.

E. If antimicrobial susceptibility testing (AST) is not performed, hold positive culture plates for several (3 to 7) days should AST be requested at a later date.

F. Perform a rapid commercial PCR test directly from vaginal-rectal swabs using the BD GeneOhm GBS or GeneXpert GBS assay according to the manufacturer's instructions.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. If the culture is negative, report "No group B streptococci isolated."

B. Positive reporting

1. Report the presence of *S. agalactiae* (group B) or *S. pyogenes* (group A) as soon as preliminary tests are completed.

2. Do not report any enumeration.

3. If the patient has been admitted to the hospital, notify the physician of positive results. For other patients, ensure that the results are easily available to the physician when the patient goes into labor.

◩ **NOTE:** A policy that may be useful for laboratories that lack a readily accessible computer system is to notify the patients themselves to be sure they tell their physicians when they go into labor.

4. Document notification of physician.

5. Include in the report the following: "If patient is penicillin allergic, contact laboratory for alternative susceptibility testing."

a. Report a zone size of ≥21 mm with the erythromycin disk as susceptible to erythromycin (12).

b. Report a zone size of ≥19 mm with the clindamycin disk (12) and no inhibition or flattening with the erythromycin disk as susceptible to clindamycin.

VII. INTERPRETATION

A. A positive culture indicates colonization with the organism, which may or may not indicate infection.

B. Urinary tract infections with *S. agalactiae* should be treated.

C. Virtually 100% of *S. agalactiae* strains are susceptible to penicillin (the drug of choice to treat colonization), negating the need for testing. If testing is performed, resistance should be confirmed in a reference laboratory (12).

D. For penicillin-allergic women, erythromycin or clindamycin is used. Approximately 6 to 15% of strains are resistant to clindamycin, and 20% are resistant to erythromycin (19).

VIII. LIMITATIONS

A. Although RT-PCR-based methods for the detection of GBS have been shown to be more rapid and sensitive than culture techniques, a major disadvantage of this technology is that the isolate is not retrieved in order to perform AST. Due to the concern about increasing rate of GBS resistance to both clindamycin and erythromycin, a subsequent culture may need to be performed so that AST can be done, particularly if there is treatment failure while the patient is on one of these antimicrobials.

B. False-negative cultures can result from contamination of the specimen with genital microbiota, especially *E. faecalis,* or from the inability to recognize nonhemolytic colonies.

C. False-positive results can be caused by misinterpretation of the confirmatory tests. Occasionally enterococci can have a positive hippurate test result, but it usually is not rapid.

D. *Listeria* colonies can be confused with *S. agalactiae,* if the Gram stain or catalase test is omitted. *Listeria* organisms are *catalase positive,* gram-positive *rods* that are hemolytic and CAMP and hippurate positive, but these are rarely seen in vaginal specimens.

E. For neonates with sepsis, blood and CSF cultures should be performed to diagnose GBS disease along with other microorganisms responsible for sepsis, such as *Listeria monocytogenes.*

F. While the methods presented here are designed to detect GBS colonization in pregnant females, disease in nonpregnant adults is increasing (9). The microbiologist must be aware of the organism's significance and report it when present in clinically significant specimens.

REFERENCES

1. **American Academy of Pediatrics.** 1992. Guidelines for prevention of group B streptococcal infection by chemoprophylaxis. *Pediatrics* **90:**775–778.
2. **American Academy of Pediatrics and COID/COFN.** 1997. Revised guidelines for prevention of early-onset group B streptococcal (GBS) infection. *Pediatrics* **99:**489–496.
3. **American College of Obstetricians and Gynecologists.** 1996. Prevention of early-onset group B streptococcal disease in newborns. *ACOG Comm. Opin.* **173:**1–8.
4. **Bergeron, M. G., D. Ke, C. Menard, F. J. Francois, M. Gagnon, M. Bernier, M. Ouellette, P. H. Roy, S. Marcoux, and W. D. Fraser.** 2000. Rapid detection of group B streptococci in pregnant women at delivery. *N. Engl. J. Med.* **343:**175–179.
5. **Block, T., E. Munson, A. Culver, K. Vaughan, and J. E. Hryciuk.** 2008. Comparison of carrot broth- and selective Todd-Hewitt broth-enhanced PCR protocols for real-time detection of *Streptococcus agalactiae* in prenatal vaginal/anorectal specimens. *J. Clin. Microbiol.* **46:**3615–3620.
6. **Bourbeau, P. P., B. J. Heiter, and M. Figdore.** 1997. Use of Gen-Probe AccuProbe group B streptococcus test to detect group B streptococci in broth cultures of vaginal-anorectal specimens from pregnant women: comparison with traditional culture method. *J. Clin. Microbiol.* **35:**144–147.
7. **Boyer, K. M., and S. P. Gotoff.** 1986. Prevention of early-onset group B streptococcal disease with selective intrapartum chemoprophylaxis. *N. Engl. J. Med.* **314:**1665–1669.

REFERENCES *(continued)*

8. **Centers for Disease Control and Prevention.** 1996. Prevention of perinatal group B streptococcal disease: a public health perspective. *MMWR Morb. Mortal. Wkly. Rep.* **45:**1–24.
9. **Centers for Disease Control and Prevention.** 1997. Adoption of hospital policies for prevention of perinatal group B streptococcal disease—United States. *MMWR Morb. Mortal. Wkly. Rep.* **47:**665–670.
10. **Centers for Disease Control and Prevention.** 2002. Prevention of perinatal group B streptococcal disease: revised guidelines from CDC. Recommendations and Reports. *MMWR Morb. Mortal. Wkly. Rep.* **51:**1–24.
11. **Church, D. L., H. Baxter, T. Lloyd, B. Miller, and S. Elsayed.** 2008. Evaluation of StrepB carrot broth versus Lim broth for detection of group B streptococcus colonization status of near-term pregnant women. *J. Clin. Microbiol.* **46:**2780–2782.
12. **Clinical and Laboratory Standards Institute.** 2008. *Performance Standards for Antimicrobial Susceptibility Testing.* Eighteenth informational supplement. Approved standard M100-S18. Clinical and Laboratory Standards Institute, Wayne, PA.
13. **Clinical and Laboratory Standards Institute.** 2004. *Quality Control for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
14. **Davies, H. D., M. A. Miller, S. Faro, D. Gregson, S. C. Kehl, and J. A. Jordan.** 2004. Multicenter study of a rapid molecular-based assay for the diagnosis of group B streptococcus colonization in pregnant women. *Clin. Infect. Dis.* **39:**1129–1135.
15. **Dunne, W. M., Jr., and C. Holland-Staley.** 1998. Comparison of NNA agar culture and selective broth culture for detection of group B streptococcal colonization in women. *J. Clin. Microbiol.* **36:**2298–2300.
16. **Fackrell, K. C., and N. K. Dick.** 2001. Comparison of LIM broth and Todd-Hewitt broth with gentamicin and nalidixic acid for recovery of group B streptococci, abstr. C-107. *Abstr. 101st Gen. Meet. Am. Soc. Microbiol.* American Society for Microbiology, Washington, DC.
17. **Farley, M. M.** 2001. Group B streptococcal disease in nonpregnant adults. *Clin. Infect. Dis.* **33:**556–561.
18. **Helali, E., A. Ly, J. C. Nguyen, Y. Giovangrandi, and E. Suvanet.** 2008. Intrapartum screening of group B streptococcus using GeneXpert real-time PCR versus late antenatal screening by culture, abstr. P1981. European Society of Clinical Microbiology and Infectious Diseases, Barcelona, Spain.
19. **Lin, F. C., P. H. Azimi, L. E. Weisman, J. B. Philips III, J. Regan, P. Clark, G. G. Rhoads, J. Clemens, J. Troendle, E. Pratt, R. A. Brenner, and V. Gill.** 2000. Antibiotic susceptibility profiles for group B streptococci isolated from neonates, 1995–1998. *Clin. Infect. Dis.* **31:**76–79.
20. **Montagne, N. S., T. J. Cleary, O. V. Martinez, and G. W. Procop.** 2008. Detection of group B streptococci by use of group B streptococcus peptide nucleic acid fluorescent in situ hybridization and selective and nonselective agars. *J. Clin. Microbiol.* **46:**3470–3472.
21. **Park, C. H., N. M. Vandel, D. K. Ruprai, E. A. Martin, K. M. Gates, and D. Coker.** 2001. Detection of group B streptococcal colonization in pregnant women using direct latex agglutination testing of selective broth. *J. Clin. Microbiol.* **39:**408–409.
22. **Schrag, S. J., S. Zywicki, M. M. Farley, A. L. Reingold, L. H. Harrison, L. B. Lefkowitz, J. L. Hadler, R. Danila, P. R. Cieslak, and A. Schuchat.** 2000. Group B streptococcal disease in the era of intrapartum antibiotic prophylaxis. *N. Engl. J. Med.* **342:**15–20.

SUPPLEMENTAL READING

Schuchat, A. 1998. Epidemiology of group B streptococcal disease in the United States: shifting paradigms. *Clin. Microbiol. Rev.* **11:**497–513.

3.9.3 *Neisseria gonorrhoeae* Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The fastidious and fragile nature of *Neisseria gonorrhoeae* requires careful consideration of proper methods of specimen collection and transport. A good selective medium is required for reliable isolation of gonococci from patient specimens, especially those that might contain a variety of microorganisms, including saprophytic *Neisseria* spp. Methods for the identification of *N. gonorrhoeae* from extragenital sites and cultures taken from children must be chosen and performed with the utmost care, since a positive report can have far-reaching psychosocial and medico-legal implications. In the United States, culture identification of *N. gonorrhoeae* is the only definitive method of diagnosis from a legal standpoint.

In women, the endocervix is the primary site of infection, but the organism can also be recovered from the urethra, rectum, oropharynx, conjunctiva, and the ducts of Bartholin's glands. A vaginal swab is not considered optimal for the recovery of gonococci from women but can be a valuable specimen for the diagnosis of gonorrhea in preteen-aged girls. Ascending genital infection in women can lead to pelvic inflammatory disease and, occasionally, perihepatitis. Transmission of *N. gonorrhoeae* from mother to newborn will often present as conjunctivitis (ophthalmia neonatorum).

The urethra is the primary site of infection in men, but extragenital sites, including the rectum, oropharynx, conjunctiva, and epididymis, can act as sources of *N. gonorrhoeae*, in addition to first-voided urine. Extragenital cultures of the rectum, conjunctivae, and pharynx for *N. gonorrhoeae* are performed only when specifically requested and samples are procured by the clinician. Such a request is not uncommon, however, as part of the overall diagnostic evaluation for gonorrhea.

Culture-independent nucleic acid probe hybridization and amplification assays for the detection of *N. gonorrhoeae* from urethral and cervical specimens are currently available that circumvent problems associated with specimen transport and loss of organism viability inherent in culture-based methods. These assays include the PCR AMPLICOR CT/NG assay and the PCR COBAS AMPLICOR CT/NG assay (Roche Molecular Diagnostics); the APTIMA COMBO 2 assay, which has a fully automated version that runs on the TIGRIS DTS system (Gen-Probe, Inc.); the BD ProbeTecET system (BD Diagnostic Systems); and the ligase chain reaction assay (Abbott Diagnostics). The two PCR assays are performed similarly except that the COBAS instrument automates the amplification and detection steps. All of these assays have been given clearance by the FDA for combined testing for *Chlamydia trachomatis* and *N. gonorrhoeae* in endocervical swabs, male urethral swabs, and female and male urine samples, except the two AMPLICOR assays, which have not been approved for female *N. gonorrhoeae* testing on urine and testing of urethral swabs from asymptomatic males. All of these assays have comparable performance for the detection of genital *C. trachomatis* and *N. gonorrhoeae* infection. (*See* section 12 for further information on these tests and reference 20 for evaluation of different systems for detection of *C. trachomatis*.)

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. Cervical
- **a.** Do not use lubricant during procedure.
- **b.** Wipe the cervix clean of vaginal secretion and mucus.
- **c.** Rotate a charcoal swab, and obtain exudate from the endocervical glands.
- **d.** If no exudate is seen, insert a charcoal swab into the endocervical canal, and rotate the swab.

2. Vaginal
- **a.** Insert a charcoal swab into the vagina.
- **b.** Collect discharge or vaginal secretions from the mucosa high in the vaginal canal.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

◩ **NOTE:** Vaginal specimens are not considered optimal for the diagnosis of gonorrhea in women and should be reserved only for the evaluation of preteen-aged girls with suspected sexually transmitted diseases due to presumed sexual abuse.

3. Urethral
- **a.** Collect specimen 1 to 2 h or more after patient has urinated.
- **b.** Female: stimulate discharge by gently massaging the urethra against the pubic symphysis through the vagina.
  - (1) Collect the discharge with a charcoal swab.
  - (2) If discharge cannot be obtained, wash external urethra with Betadine soap and rinse with water. Insert a urethrogenital swab 2 to 4 cm into the endourethra, gently rotate the swab, leave it in place for 1 to 2 s, and withdraw it.
- **c.** Male: insert a thin urethrogenital swab 2 to 4 cm into the endourethra, gently rotate it, leave it in place for 1 to 2 s, and withdraw it.

4. Epididymis

Use a needle and syringe to aspirate material from the epididymis.

5. Pharyngeal
- **a.** Depress tongue gently with tongue depressor.
- **b.** Extend charcoal swab between the tonsillar pillars and behind the uvula. Avoid touching other surfaces of the mouth.
- **c.** Sweep the swab back and forth across the posterior pharynx, tonsillar areas, and any inflamed or ulcerated areas to obtain sample.

6. Rectal
- **a.** Pass the tip of a charcoal swab approximately 2 cm beyond the anal sphincter.
- **b.** Carefully rotate the swab to sample the anal crypts, and withdraw it.

7. Urine
- **a.** Do not collect urine for culture of *N. gonorrhoeae.*

  ◩ **NOTE:** Urine cultures provide reasonable sensitivity for men with gonorrhea provided a first morning-voided specimen is obtained and plated on appropriate selective and nonselective culture media. Urine culture is not a sensitive alternative for the diagnosis of gonorrhea in women.
- **b.** Collect no more than 20 ml of first morning-voided urine for nucleic acid testing.

  ◩ **NOTE:** In contrast to routine urine cultures, the first part of the voided urine specimen, rather than the midstream urine, is collected for nucleic acid testing. The use of urine for the diagnosis of *N. gonorrhoeae* and/or *C. trachomatis* infection by nucleic acid amplification assays has been successfully adapted for several commercially available diagnostic kits. However, for females the sensitivity of the urine compared to an endocervical swab is generally 10% lower. Currently, the AMPLICOR CT/NG assay (Roche Molecular Diagnostics) is not FDA cleared for use with female urine samples (20).

8. Conjunctivae
- **a.** Collect purulent material on a swab.
  - (1) Roll charcoal swab over the conjunctiva before topical medications are applied.
  - (2) Culture both eyes with separate swabs.
- **b.** Alternatively, obtain material with a sterile spatula and inoculate directly onto culture media.

B. Specimen transport

1. Direct inoculation
- **a.** Inoculate specimen directly onto the surface of nonselective and selective medium plates. Roll the swab across one quadrant of the plate and streak for isolation, or roll swabs across the surface of the plate in a “Z” or “N”

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

figure. Plates are cross-streaked for isolation when received by the laboratory.

b. Place inoculated media in a holding jar or other system with a suitable CO_2-enriched (3 to 5%) atmosphere.

c. Transport systems that provide growth medium and an atmosphere-generating tablet in a self-contained transport pack can be substituted for direct medium inoculation and the holding-jar method. These include the following.

(1) JEMBEC (BD Diagnostic Systems)
(2) InTray GC (BioMed Diagnostics, San Jose, CA)
(3) Gono-Pak (BD Diagnostic Systems)

2. If direct medium inoculation is not practical, collect specimens on one of the following swab-based transport systems.

a. Difco Cultureswab (BD Diagnostic Systems)
b. Copan Venturi Transystem (Copan Italia, Bovezza, Italy)
c. BD Culturette EZ (BD Diagnostic Systems)
d. Starplex Starswab (Starplex Scientific, Inc., Etobicoke, Ontario, Canada)

◩ **NOTE:** The performance of each of these swab-based transport systems is comparable if specimens are submitted and cultured within 6 h of collection and either held at ambient temperature or refrigerated. However, recovery rates vary considerably if delivery is delayed (1, 15, 22). At 24 h, the Copan system held at either room temperature or 4°C and the Difco system held at refrigeration temperatures provide recovery rates of 96% (1). The addition of charcoal to Amies transport medium does not appear to increase recovery rates (22).

3. For Gram stain of male urethral discharge or conjunctival exudate, transfer some of the specimen to a slide and spread in a thin film. Allow to air dry.

◩ **NOTE:** Preparation of the smear at the time of specimen collection will avoid deterioration of the specimen, which results in false-negative smear reports. For children, a smear must be accompanied by a culture, since smears lack the specificity for accurate *N. gonorrhoeae* diagnosis.

4. For molecular detection assays, use the manufacturer's product-specific collection devices. These devices cannot be used when culture has been requested.

◩ **NOTE:** While cultures must be performed in all medico-legal cases and with specimens from prepubescent children, nucleic acid tests can be performed in addition to culture.

5. Once collected, submit specimens for culture to laboratory as soon as possible. If 24-h laboratory coverage is not provided, alert the laboratory that a specimen is in transit.

6. Store at ambient or refrigeration temperature after collection and during transport (1, 25).

7. Ensure that specimens are labeled with demographic information on the patient and the site and time of collection.

8. Record the patient diagnosis for improved processing of specimen.

C. Rejection criteria

1. Reject specimens on swabs in which significant desiccation has occurred or when instructions for correct use of the transport system have not been followed.

2. Consider rejection of specimens if significant delay (e.g., >24 h) in transportation and plating occurs. However, a more practical approach might include a provision for culture in all cases of delayed transportation with an accompanying comment for cultures negative for *N. gonorrhoeae* (*see* item VI below). Accompany such a provision with active education and revision of policies to improve transport.

III. MATERIALS

A. Media

◩ **NOTE:** For maximum recovery, culture media for isolation of *N. gonorrhoeae* should include both selective and nonselective agar formulations capable of supporting the growth of the organism. Selective media are designed to prevent overgrowth of contaminating normal microbiota. The addition of a nonselective medium such as CHOC allows for the growth of a small percentage of gonococci (approximately 2%) that are inhibited in the presence of vancomycin at concentrations of 4 µg/ml. Formulations such as GC-Lect reduce the concentration of vancomycin to 2 µg/ml (4, 14, 16, 17, 23, 25, 26). In selective medium formulations, anisomycin has a longer shelf life than nystatin to inhibit *Candida.* Trimethoprim is added to inhibit the growth of *Proteus* species. (For vendor information, see procedure 3.1.)

1. CHOC (omit for nasopharyngeal and rectal sites)

2. One of the following selective media

a. Modified Thayer-Martin medium (contains vancomycin, colistin, nystatin, and trimethoprim) is available from most medium manufacturers.

b. Martin-Lewis medium (contains vancomycin, colistin, and anisomycin) is available from most medium manufacturers. The vancomycin concentration is higher (400 µg/100 ml) in Martin-Lewis than in Thayer-Martin medium (300 µg/100 ml).

c. Thayer-Martin improved medium (TM improved) (contains vancomycin, colistin, anisomycin, and trimethoprim [Remel, Inc.])

d. TM improved without vancomycin, sold as a biplate with TM improved (Remel, Inc.)

e. Martin-Lewis medium with lincomycin (Hardy Diagnostics)

f. GC-Lect (contains lincomycin, vancomycin at 200 µg/100 ml, colistin, trimethoprim, and amphotericin B) (BD Diagnostic Systems)

g. New York City medium with the same antimicrobial agents as GC-Lect (BD Diagnostic Systems)

h. The following media provide both CO_2 generation and the media in a self-contained system that promotes survival of *N. gonorrhoeae* during transportation.

(1) JEMBEC (BD Diagnostic Systems; Remel, Inc.)

(2) Gono-Pak (BD Diagnostic Systems; Remel, Inc.)

(3) InTray GC (BioMed Diagnostics)

3. Biplate formulations containing both CHOC and selective agar are available from several manufacturers.

B. Gram stain reagents (procedure 3.2.1)

C. Direct amplification tests

Refer to reference 20.

D. Identification tests

1. Oxidase reagent (*see* procedure 3.17.39)

2. 30% Hydrogen peroxide (Sigma, St. Louis, MO) for superoxol test (2, 24)

3. Nutritionally basic media, such as nutrient agar, TSA, or Mueller-Hinton (MH) agar

4. *Neisseria* identification systems

a. Carbohydrate utilization assay: cysteine Trypticase agar (CTA) base with 1% carbohydrates (glucose, maltose, lactose, and sucrose) or carbohydrate degradation media with 20% carbohydrates (Remel, Inc.; *see* procedure 3.17.9)

b. Coagglutination

(1) Gonogen (New Horizons Diagnostics; BBL Microbiology, BD Diagnostic Systems)

(2) Phadebact GC monoclonal antibody test (Boule Diagnostics AB, Huddinge, Sweden)

c. Monoclonal antibody assay: GonoGen II (New Horizons Diagnostics; BBL Microbiology, BD Diagnostic Systems)

d. Substrate utilization

(1) RapID NH, BactiCard Neisseria, NET (Remel, Inc.)

(2) Gonochek II (EY Laboratories, Inc., San Mateo, CA)

(3) Neisseria Screen (Key Scientific Products, Round Rock, TX)

III. MATERIALS *(continued)*

(4) BBL CRYSTAL (BD Diagnostic Systems)
(5) *Neisseria/Haemophilus* ID kit, API NH (bioMérieux)

e. Fluorescent antibody: Syva MicroTrak *N. gonorrhoeae* culture confirmation test (Trinity Biotech plc., Co. Wicklow, Ireland)

f. Probe hybridization: AccuProbe *N. gonorrhoeae* culture confirmation test (Gen-Probe, Inc.)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Verify that media meet expiration date and QC parameters per Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22-A3 (10). See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

B. Test each lot of identification kits and reagents, according to package insert or procedure 14.2. Generally the following QC organisms are used for sugar or kit tests. See Table 3.9.3–1 for expected reactions.

1. *N. gonorrhoeae* ATCC 43069
2. *Neisseria meningitidis* ATCC 13077
3. *Neisseria lactamica* ATCC 23970
4. *Moraxella catarrhalis* ATCC 25240

C. Perform QC of CHOC and selective media by lot; incubate for 24 to 48 h in 5 to 7% CO_2 at 35°C.

CHOC

Test organism	Result
Neisseria gonorrhoeae ATCC 43069 or ATCC 43070	Growth
Haemophilus influenzae ATCC 10211	Growth

Selective media for pathogenic *Neisseria* spp.

Test organism	Result
Neisseria gonorrhoeae ATCC 43069 or ATCC 43070	Growth
Proteus mirabilis ATCC 43071	Partial inhibition—test only if medium contains trimethoprim
Staphylococcus epidermidis ATCC 12228	Partial inhibition

V. PROCEDURES

A. Direct test

1. Gram stain
a. Perform smears only on male urethra specimens.
b. Roll swab across slide after culture has been inoculated.
c. Fix smear with methanol. (See procedure 3.2.1 for staining and reading smear.)

2. Direct probe assays (20)

Observe standard precautions.

It is imperative that these cultures be handled in a biological safety cabinet.

B. Culture methods

1. Inoculation
a. Process specimen as soon as received.
b. Inoculate medium by rolling the swab over one quadrant of the plate and streaking for isolation. If medium is received that has already been inoculated (either in a "Z" streak or down the center of the plate), it is best to cross-streak for isolation to ensure separation of mixed colony types.

Table 3.9.3–1 Biochemical reactions of *Neisseria* and related oxidase-positive diplococci and rods that may grow on Thayer-Martin or similar selective agar[a]

Organism(s)	Superoxol, 30% H_2O_2	Growth on basic agar media at 35°C[c]	Colistin (10 μg) disk	Glucose	Maltose	Lactose, ONPG, or BGAL	Sucrose	PRO	GLUT	Butyrate[d]
Neisseria gonorrhoeae	4+	−	R	+⁻	−	−	−	+	−	−
Neisseria meningitidis	2–4+	V	R	+	+⁻	−	−	V	+	−
Neisseria lactamica	2+	+	R	+	+	+	−	+	−	−
Neisseria cinerea[b]	2+	+	V	V	−	−	−	+	−	−
Neisseria flavescens[b]	2+	+	S	−	−	−	−	+	−	−
Neisseria elongata[b]	2+	+	S	−	−	−	−	V	−	−
Other nonpathogenic *Neisseria* spp.[b]	2+	+	V	+	+	−	V	V	V	−
Moraxella catarrhalis	2−4+	+	V	−	−	−	−	V	−	+
Kingella species	−	+	R	+	V	−	−	V	−	−

[a] Abbreviations: PRO, prolyl-iminopeptidase; BGAL, β-galactosidase: GLUT, δ-glutamyl-aminopeptidase; V, variable reactions; +⁻, reaction is generally positive, but rare negative results occur, resulting in critical misidentifications if other tests are not also performed; R, resistant; S, susceptible. Polymyxin B can be substituted for colistin, or susceptibility can usually be determined by growth or lack of growth on Thayer-Martin or other selective agar with colistin or polymyxin B. Reactions are from package inserts, from http://www.CDC.gov/ncidod/dastlr/gcdir/neident/index.html, and from references 19 and 28.

[b] This organism(s) does not usually grow on selective media for *N. gonorrhoeae*. *N. subflava* and *N. flavescens* colonies are yellow; *N. subflava* is the only species other than *N. meningitidis* to be GLUT positive.

[c] Nutrient agar, MH agar, or TSA without blood at 35°C.

[d] See procedure 3.17.7. Do not read after time period in package insert, as this delay may result in false-positive reactions. Many *Moraxella* spp. and *Acinetobacter* spp. are butyrate positive. Isolate must be a diplococcus for identification of *M. catarrhalis* to be accurate.

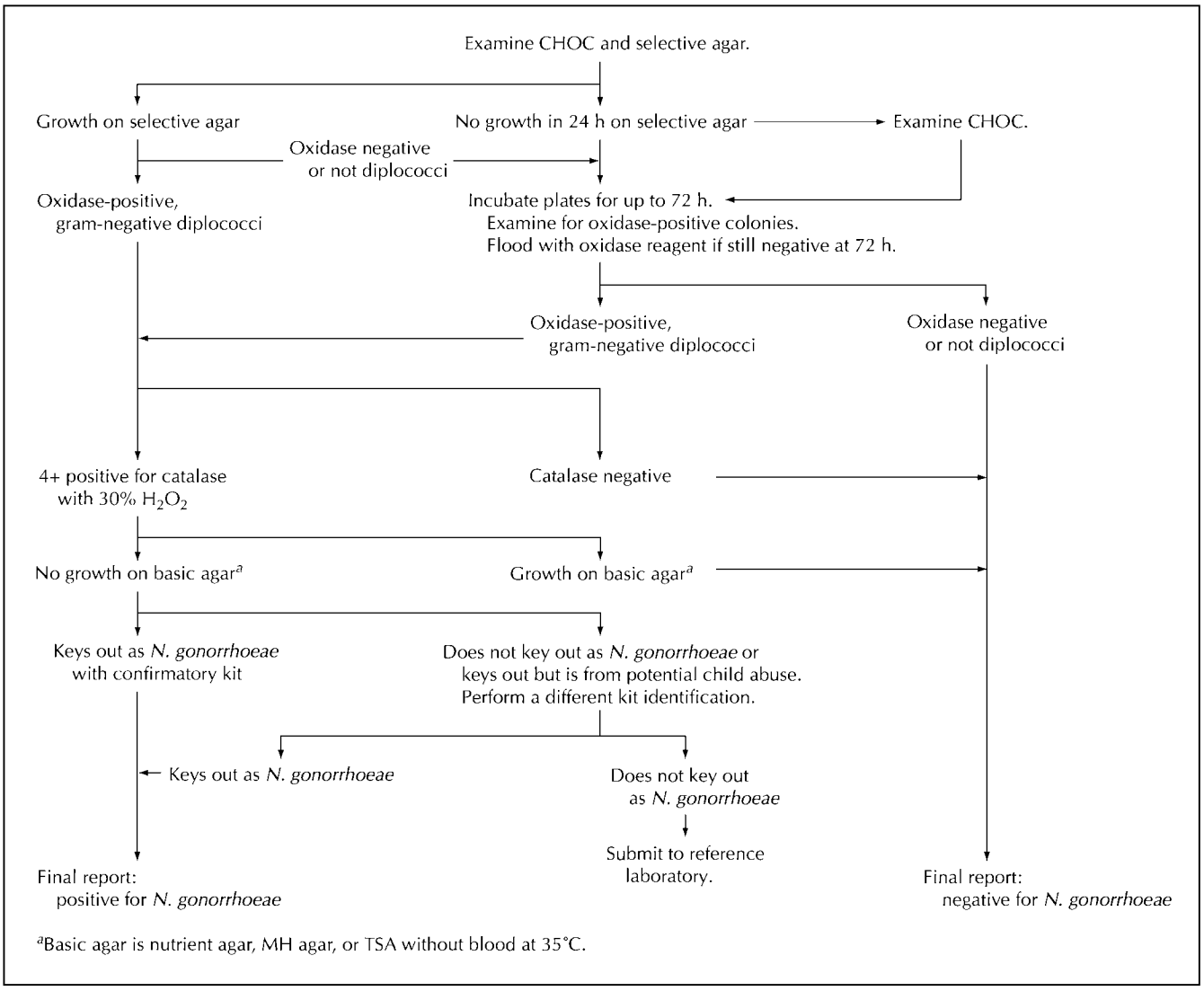

Examine CHOC and selective agar.
Growth on selective agar
No growth in 24 h on selective agar
Examine CHOC.
Oxidase negative or not diplococci
Oxidase-positive, gram-negative diplococci
Incubate plates for up to 72 h. Examine for oxidase-positive colonies. Flood with oxidase reagent if still negative at 72 h.
Oxidase-positive, gram-negative diplococci
Oxidase negative or not diplococci
4+ positive for catalase with 30% H_2O_2
Catalase negative
No growth on basic agar[a]
Growth on basic agar[a]
Keys out as *N. gonorrhoeae* with confirmatory kit
Does not key out as *N. gonorrhoeae* or keys out but is from potential child abuse. Perform a different kit identification.
Keys out as *N. gonorrhoeae*
Does not key out as *N. gonorrhoeae*
Submit to reference laboratory.
Final report: positive for *N. gonorrhoeae*
Final report: negative for *N. gonorrhoeae*
[a]Basic agar is nutrient agar, MH agar, or TSA without blood at 35°C.

Figure 3.9.3–1 Laboratory diagnosis of *N. gonorrhoeae* by culture.

V. PROCEDURES *(continued)*

2. Incubation
- **a.** Incubate plates at 35 to 37°C in 5% CO_2 contained system.
- **b.** Alternatively, use a CO_2-generating contained system to provide the proper atmosphere if a CO_2 incubator is unavailable.
- **c.** If no other method is available, place in a jar and light a candle. Seal the jar quickly. When the candle is extinguished, the atmosphere contains approximately 3 to 5% CO_2.

3. Save specimen in case confirmation of identification is needed.

C. Culture examination

1. Examine all plated media for macroscopic evidence of growth at 24 h.

2. If no visible growth is observed on the culture media
- **a.** Return cultures to incubator quickly to minimize loss of viability in the absence of CO_2.
- **b.** Read aerobic plates daily for a total of 72 h before reporting as negative.

V. PROCEDURES *(continued)*

c. Prior to discarding plates, flood plates with oxidase reagent. If a purple color colony is observed, immediately subculture to CHOC, since oxidase reagent is toxic to bacteria.

3. Cultures with growth (*see* Fig. 3.9.3–1)

a. Examine for typical colonies that are small, translucent, raised, gray, and mucoid with entire margins. When picked from the agar surface, they tend to come off as whole colonies.

b. Perform a Gram stain and oxidase test on suspicious colonies. If the Gram stain shows gram-negative diplococci with flattened adjacent sides or gram-negative cocci in clusters and the oxidase is positive, the isolate is presumed to be either a *Neisseria* sp. or *M. catarrhalis.*

c. If the specimen is sent for diagnosis of carriage of *N. meningitidis,* examine CHOC carefully and identify any oxidase-positive, gram-negative diplococci. These strains may not grow on selective agar.

d. Confirm identification to the species level. If the isolate is from an extragenital site or from a child, perform two confirmatory assays that utilize distinct identification mechanisms (e.g., monoclonal antibody and carbohydrate utilization).

(1) Superoxol test (references 2 and 24 and procedure 3.17.10)

(a) Drop 30% hydrogen peroxide reagent onto colony.

(b) *N. gonorrhoeae* will give immediate, explosive bubbling.

(c) Other species, except some *N. meningitidis* and *M. catarrhalis* strains, show weak reactions. A positive result should only be considered presumptive for the identification of *N. gonorrhoeae.*

(2) Subculture typical colonies to a nutritionally basic medium, such as nutrient agar, TSA, or MH agar, *and* to CHOC for proof of viability. Failure of viable organisms to grow on one of these basic media after overnight incubation at 35°C with growth on CHOC indicates a presumptive pathogenic *Neisseria* sp.

It is imperative that these cultures be handled in a biological safety cabinet.

D. Culture confirmation

Choose one method as the primary method for use in the laboratory.

1. Growth-dependent carbohydrate degradation (procedure 3.17.9)

a. CTA supplemented with 1% carbohydrates

(1) Inoculate tubes containing glucose, maltose, sucrose, and lactose (*o*-nitrophenyl-β-D-galactopyranoside [ONPG] may be substituted for lactose) and a control tube containing no additional carbohydrate source.

(2) Incubate at least overnight, depending on inoculum density. *N. gonorrhoeae* produces acid only in glucose.

b. Alternatively, use carbohydrate degradation media with 20% carbohydrates.

(1) Use a heavy inoculum and incubate for 1 to 4 h, since the test depends on the presence of preformed enzyme activity and uses a buffered low-peptone base with 2% glucose, lactose, and sucrose and 0.3% maltose (29).

(2) Use the same interpretation as for growth carbohydrate degradation tests.

2. BBL GonoGen

◩ **NOTE:** BBL GonoGen is a monoclonal antibody-based coagglutination test for the confirmatory identification of *N. gonorrhoeae* from culture. The assay is based on the recognition of a major outer membrane protein (protein I) of the organism.

V. PROCEDURES *(continued)*

a. Suspend several colonies of suspected *N. gonorrhoeae* from culture medium in a tube containing 0.5 ml of distilled water to the equivalent of a McFarland no. 3 standard (Appendix 3.16–1).

b. Heat the suspension at 100°C for 10 min, cool to room temperature, and vortex to remove aggregates.

c. Place 1 drop of reagent G on a separate circle of a glass slide for each test isolate and for positive and negative controls.

d. Add 1 drop of the heat-treated suspension of organism to the drop of reagent G and mix. Add 1 drop each of the positive and negative control suspension to separate drops of reagent G and mix.

(1) A positive reaction produces clumping of the reagents with partial or total clearing of the organism suspension in 2 min or less.

(2) Test positive reactions of test isolates against the staphylococcus control reagent (reagent S) to verify specificity.

3. Phadebact GC monoclonal test

◩ **NOTE:** The Phadebact GC monoclonal test is a coagglutination assay in which two pools of monoclonal antibodies directed against a gonococcus-specific membrane protein (protein I) are coupled to the protein A of nonviable staphylococci. When the monoclonal antibody reagent is mixed with a sample containing gonococci, a matrix is formed causing a visible agglutination reaction.

a. Prepare a slightly turbid suspension of cells in 0.5 ml of 0.9% saline from colonies presumptively identified as *N. gonorrhoeae.*

b. Heat the suspension in a boiling water bath for at least 5 min.

c. Cool the suspension to room temperature.

d. Place 1 drop of the gonococcal monoclonal reagent on a disposable slide.

e. Add 1 drop of the boiled colony suspension to the gonococcal reagent.

f. Mix the drops thoroughly but gently with a disposable loop.

g. Rock the slide and read the result within 1 min.

h. A positive and negative control should be included in each run.

i. A positive reaction is denoted by the appearance of visible agglutination.

4. GonoGen II

◩ **NOTE:** GonoGen II is a monoclonal antibody-based colorimetric test developed for the confirmatory identification of *N. gonorrhoeae* from culture. The assay is based on the recognition of a major outer membrane protein (protein I) of the organism.

a. Label a 12- by 75-mm test tube for each isolate to be tested and for a positive and negative control.

b. Add 500 µl of GonoGen II solubilizing buffer to each tube and prepare a suspension of the test isolates equivalent to a McFarland no. 1 standard (Appendix 3.16–1).

c. Add 1 drop of the GonoGen II murine monoclonal antibody solution (antibody against protein I surface antigens of *N. gonorrhoeae*) to each tube, mix well, and incubate at room temperature for at least 5 min.

d. Transfer 2 drops of each test and control suspension into a separate test reaction well and allow the solution to absorb into the reaction well matrix.

e. A pink to red residual dot in the test well or a color reaction more intense than the negative control should be interpreted as a positive result.

5. BactiCard Neisseria

◩ **NOTE:** BactiCard Neisseria relies on four chromogenic substrates impregnated in a test reaction card to detect preformed enzymes produced by pathogenic *Neisseria* species. Enzyme activity will presumptively differentiate *N. gonorrhoeae, N. meningitidis, N. lactamica,* and *M. catarrhalis* (27).

V. PROCEDURES *(continued)*

a. Rehydrate each substrate test circle with 1 drop of BactiCard rehydrating fluid.

b. From a pure culture 18 to 48 h old, growing on selective medium (not CHOC), smear several colonies of the isolate onto each substrate test circle with a wooden applicator stick.

c. Incubate at room temperature for 2 min.

d. Observe each test circle for the production of a blue-green color that indicates a positive test for enzyme activity or a pink to red color after addition of the color developer.

(1) *N. gonorrhoeae* is positive only for prolyl-iminopeptidase and negative for β-galactosidase, butyrate esterase, and δ-glutamyl-aminopeptidase (Table 3.9.3–1).

(2) Certain strains of *Kingella, Neisseria cinerea,* and *Neisseria subflava* could provide reactions consistent with *N. gonorrhoeae.* Care must be taken to only test isolates recovered from selective media that do *not* grow on nutrient agar (*see* Table 3.9.3–1).

6. Gonochek II, NET and Neisseria Screen

◩ **NOTE:** Gonochek II or NET or Neisseria Screen relies on three chromogenic substrates in a test tube to detect preformed enzymes produced by pathogenic *Neisseria* species. Enzyme activity will presumptively differentiate *N. gonorrhoeae, N. meningitidis,* and *N. lactamica* (28).

a. Dispense 4 drops of reagent buffer into reagent tube.

b. Remove 5 to 10 colonies and emulsify into tube.

c. Incubate at 37°C for 30 min.

d. Yellow color is indicative of *N. meningitidis* (positive for δ-glutamyl-aminopeptidase), and blue color (positive for indolyl-β-D-galactosidase) is indicative of *N. lactamica* (Table 3.9.3–1). If the yellow color is faint, incubate for 60 min to confirm identification.

e. If there is no color change, remove white cap and recap with red cap (Gonochek II). Invert to allow reagent in cap to mix with solution. (Alternatively, add reagent to the tube for Neisseria Screen and NET.) A red to pink color (positive for prolyl-iminopeptidase) when returned to the upright position indicates positivity for *N. gonorrhoeae.*

f. Certain strains of *Kingella, Neisseria cinerea,* and *Neisseria subflava* could provide reactions consistent with *N. gonorrhoeae.* Care must be taken to only test isolates recovered from selective media or from CHOC that do *not* grow on nutrient agar (*see* Table 3.9.3–1).

7. MicroTrak *N. gonorrhoeae* culture confirmation test

◩ **NOTE:** The MicroTrak test uses a fluorescein-labeled monoclonal antibody in a fluorescent microscopy assay to positively identify presumptive isolates of *N. gonorrhoeae.*

a. Perform the assay on 18- to 24-h colonies. Subculture colonies older than 24 h before testing.

b. Place 5 µl of distilled or deionized water in a 6-mm slide well.

c. Touch five colonies of the isolate to be tested with a loop and gently emulsify the cells into the drop of water to form an even suspension on the slide well. Prepare wells of known positive and negative controls in a similar fashion.

d. Allow the slide to air dry, and gently heat fix the smear.

e. Stain the slide with MicroTrak *N. gonorrhoeae* culture confirmation reagent (30 µl per well) for 15 min at 37°C in a humidified chamber.

f. Remove excess reagent and rinse the slide for 5 to 10 s in a gentle stream of water. Shake off the excess water, and air dry the smear.

g. Add a drop of mounting fluid and place a coverslip on the well.

V. PROCEDURES *(continued)*

h. Read the slide using a fluorescent microscope with a 100× oil objective. A positive test will demonstrate apple-green fluorescent diplococci along with appropriate reactions for the positive and negative control wells.

8. AccuProbe *N. gonorrhoeae* culture identification test

◩ **NOTE:** The AccuProbe system uses a single-stranded DNA probe with a chemiluminescent label that recognizes an rRNA sequence of *N. gonorrhoeae.* Following the release of the target RNA, the DNA probe forms a stable DNA:RNA hybrid. A selection reagent inactivates unhybridized probe and the labeled DNA:RNA hybrids are detected in a luminometer.

a. Label a sufficient number of probe reagent tubes to test the number of culture isolates and a positive *(N. gonorrhoeae)* and negative *(N. meningitidis)* control.

b. Pipette 50 µl of lysis reagent into all tubes.

c. Transfer a 1-µl loopful of cells or several small colonies (<48 h old) to the tubes and twirl the loop to remove the cells.

d. Pipette 50 µl of hybridization buffer into all tubes, and mix by vortexing.

e. Incubate tubes for 15 min at 60°C in a water bath or dry-heat block.

f. Remove and cool the tubes. Pipette 300 µl of selection reagent to each tube and mix by vortexing.

g. Incubate tubes for 5 min at 60°C in a water bath or dry-heat block.

h. Cool the tubes to room temperature and read results in the luminometer within 30 min. Cutoff values for positive and negative signals are established by the manufacturer for the type of luminometer used.

◩ **NOTE:** The performance of each confirmatory assay discussed above in terms of the definitive identification of *N. gonorrhoeae* varies considerably in the literature. For example, the reported sensitivity of the fluorescent MicroTrak *N. gonorrhoeae* culture confirmation test ranges from 76 to 100%, while the sensitivity of the AccuProbe *N. gonorrhoeae* culture identification test has consistently been found to be 100% (3, 5, 11, 18, 19, 27, 30).

Based on these findings, *a single negative result produced by any of the confirmatory tests does not rule out an identification of* N. gonorrhoeae. Repeat testing should be performed on a freshly subcultured isolate in conjunction with lack of growth on nutrient agar and/or carbohydrate utilization tests (*see* Table 3.9.3–1). Oxidase-positive, gram-negative diplococci that do not grow on nutrient agar but are repeatedly negative by any one assay or are uniformly negative for carbohydrate utilization should be referred to a reference laboratory for additional testing or evaluated by another identification method.

◩ **NOTE:** Biochemical and/or enzymatic bacterial identification systems that contain *N. gonorrhoeae* in their database but have not been specifically designed for culture confirmation of *N. gonorrhoeae* (e.g., API NH [bioMérieux], IDS RapID NH panel [Remel, Inc.], BBL CRYSTAL *Neisseria/Haemophilus* ID kit [BD Diagnostic Systems], Vitek NHI card [bioMérieux], and Microscan HNID [Dade Behring]) are reviewed elsewhere (13) but can be useful adjuncts for this purpose.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report the Gram stain results as soon as possible, usually within 1 h of receipt (procedure 3.2.1).

1. Interpret Gram stain for gram-negative intracellular diplococci.

2. Notify physician if smear is positive.

◩ **NOTE:** In contrast to almost all male urethral discharges with gram-negative diplococci, such morphotypes in vaginal, cervical, or other specimens are not diagnostic for *N. gonorrhoeae.*

VI. REPORTING RESULTS *(continued)*

B. Refer to procedure 3.3.2 for general reporting guidelines.

C. Report negative cultures as "No *Neisseria gonorrhoeae* organisms isolated." Additionally, a comment can be added as follows.

1. "Specimen overgrown by normal microbiota. Please submit another specimen."

2. "Specimen contaminated with yeast cells, which are inhibitory to *Neisseria gonorrhoeae*."

3. "Specimen received 24 h after collection. Rates of recovery of *Neisseria gonorrhoeae* from specimens with delayed transport can be significantly reduced."

D. Report positive cultures with probable genus and species as soon as preliminary tests are completed.

1. Document notification to physician of positive findings.

2. Generally report *N. gonorrhoeae* to the local health department.

3. In cases involving children or the possibility of legal intervention, isolates of *N. gonorrhoeae* should be frozen for long-term retrieval.

E. Antimicrobial susceptibility testing (AST)

1. Do not perform routine AST of *N. gonorrhoeae*. Perform AST only in cases of treatment failure (8) and for antimicrobial agents for which universal susceptibility has not been established. Refer to CLSI guidelines (9) for special media and conditions of AST.

2. Do not perform beta-lactamase test, because currently recommended therapies circumvent beta-lactamase production by *N. gonorrhoeae* (8).

VII. INTERPRETATION

A. A positive culture indicates infection with the organism.

B. Because of the labile nature of the organism, a negative culture does not rule out infection.

VIII. LIMITATIONS

A. False-positive reports can result from misidentification of the organism.

B. False-negative results can be caused by delayed or inappropriate transport.

C. A single negative result produced by any of the confirmatory tests does not rule out an identification of *N. gonorrhoeae*. Further confirmatory testing should be performed.

D. When carbohydrate degradation tests are used, a few strains of *N. gonorrhoeae* will be glucose negative and a few strains of *N. meningitidis* will be maltose negative. A second non-carbohydrate-based method should be used to identify these discrepant strains.

E. Use reagent-grade carbohydrates if you make your own media to avoid glucose contamination in other carbohydrate reagents.

F. The growth from a positive CTA glucose utilization test should be examined by Gram stain to ensure that the reaction is due only to *N. gonorrhoeae* and not a contaminant.

G. More than one confirmatory method for identification of *N. gonorrhoeae* is essential for potential cases of child abuse, since nearly every method has some errors.

H. Avoid use of candle jars, since superior products to generate CO_2 are available. If using candle jars, use only white wax candles. Colored candles yield toxic substances.

I. *N. cinerea*, usually a saprophyte found in the pharynx, can be an occasional pathogen. It has been reported in cases of conjunctivitis, bacteremia, and peritonitis. The microorganism gives a positive glucose reaction in some rapid sys-

VIII. LIMITATIONS *(continued)*

tems and is also positive for hydroxyprolylaminopeptidase. Hence, this organism can be mistaken for *N. gonorrhoeae* unless additional testing is performed (6, 12). Further, differentiation of *N. cinerea* from glucose-negative *N. gonorrhoeae* can be difficult when using substrate-based identification kits (7). *N. cinerea* is, however, susceptible to colistin and thus will likely not be recovered on media containing this agent. In addition, most strains will grow well on nutritionally basic agar media such as TSA, MH agar, or nutrient agar.

J. Monoclonal antibodies might not react with all strains of *N. gonorrhoeae*, and false-positive monoclonal antibody reactions have been reported with *N. meningitidis* (21).

REFERENCES

1. **Arbique, J. C., K. R. Forward, and J. LeBlanc.** 2000. Evaluation of four commercial transport media for the survival of *Neisseria gonorrhoeae. Diagn. Microbiol. Infect. Dis.* **36:**163–168.
2. **Arko, R. J., and T. Odugbemi.** 1984. Superoxol and amylase inhibition tests for distinguishing gonococcal and nongonococcal cultures growing on selective media. *J. Clin. Microbiol.* **20:**1–4.
3. **Beebe, J. L., M. P. Rau, S. Flageolle, B. Calhoon, and J. S. Knapp.** 1993. Incidence of *Neisseria gonorrhoeae* isolates negative by Syva direct fluorescent-antibody test but positive by Gen-Probe Accuprobe test in a sexually transmitted disease clinic population. *J. Clin. Microbiol.* **31:**2535–2537.
4. **Beverly, A., J. R. Bailey-Griffin, and J. R. Schwebke.** 2000. InTray GC medium versus modified Thayer-Martin agar plates for diagnosis of gonorrhea from endocervical specimens. *J. Clin. Microbiol.* **38:**3825–3826.
5. **Boehm, D. M., M. Bernhardt, T. A. Kurzynski, D. R. Pennell, and R. F. Schell.** 1990. Evaluation of two commercial procedures for rapid identification of *Neisseria gonorrhoeae* using a reference panel of antigenically diverse gonococci. *J. Clin. Microbiol.* **28:**2099–2100.
6. **Bourbeau, P., V. Holla, and S. Piemontese.** 1990. Ophthalmia neonatorum caused by *Neisseria cinerea. J. Clin. Microbiol.* **28:**1640–1641.
7. **Boyce, J. M., and E. B. Mitchell, Jr.** 1985. Difficulties in differentiating *Neisseria cinerea* from *Neisseria gonorrhoeae* in rapid systems used for identifying pathogenic *Neisseria* species. *J. Clin. Microbiol.* **22:**731–734.
8. **Centers for Disease Control and Prevention.** 1998. Guidelines for treatment of sexually transmitted diseases. *MMWR Morb. Mortal. Wkly. Rep.* **47**(RR-1)**:**70–73.
9. **Clinical and Laboratory Standards Institute.** 2006. *Performance Standards for Antimicrobial Susceptibility Testing.* Sixteenth informational supplement. Approved standard M100-S16. Clinical and Laboratory Standards Institute, Wayne, PA.
10. **Clinical and Laboratory Standards Institute.** 2004. *Quality Control for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
11. **Dillon, J. R., M. Carballo, and M. Pauze.** 1988. Evaluation of eight methods for identification of pathogenic *Neisseria* species: Neisseria-Kwik, RIM-N, Gonobio-Test, Minitek, Gonochek II, GonoGen, Phadebact Monoclonal GC OMNI Test, and Syva MicroTrak Test. *J. Clin. Microbiol.* **26:**493–497.
12. **Dossett, J. H., P. C. Appelbaum, J. S. Knapp, and P. A. Totten.** 1985. Proctitis associated with *Neisseria cinerea* misidentified as *Neisseria gonorrhoeae* in a child. *J. Clin. Microbiol.* **21:**575–577.
13. **Evangelista, A. T., A. L. Truant, and P. Bourbeau.** 2002. Rapid systems and instruments for the identification of bacteria, p. 22–49. *In* A. L. Truant (ed.), *Manual of Commercial Methods in Microbiology.* ASM Press, Washington, DC.
14. **Evans, G. L., D. L. Kopyta, and K. Crouse.** 1989. New selective medium for the isolation of *Neisseria gonorrhoeae. J. Clin. Microbiol.* **27:**2471–2474.
15. **Farhat, S. E., M. Thibault, and R. Devlin.** 2001. Efficacy of a swab transport system in maintaining viability of *Neisseria gonorrhoeae* and *Streptococcus pneumoniae. J. Clin. Microbiol.* **39:**2958–2960.
16. **Granato, P. A., J. L. Paepke, and L. B. Weiner.** 1980. Comparison of modified New York City medium with Martin-Lewis medium for recovery of *Neisseria gonorrhoeae* from clinical specimens. *J. Clin. Microbiol.* **12:**748–752.
17. **Greenwood, J. R., J. Voss, R. F. Smith, H. Wallace, C. Peter, M. Nachtigal, T. Maier, J. Wilber, and A. Butsumyo.** 1986. Comparative evaluation of New York City and modified Thayer-Martin media for isolation of *Neisseria gonorrhoeae. J. Clin. Microbiol.* **24:**1111–1112.
18. **Janda, W. M., L. M. Wilcoski, K. L. Mandel, P. Ruther, and J. M. Stevens.** 1993. Comparison of monoclonal antibody methods and a ribosomal ribonucleic acid probe test for *Neisseria gonorrhoeae* culture. *Eur. J. Clin. Microbiol. Infect. Dis.* **12:**177–184.
19. **Janda, W. M., and J. S. Knapp.** 2003. *Neisseria* and *Moraxella catarrhalis*, p. 585–608. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, DC.

REFERENCES *(continued)*

20. **Jerris, R. C., and C. M. Black.** 2002. *Chlamydia trachomatis*, p. 128–200. *In* A. L. Truant (ed.), *Manual of Commercial Methods in Microbiology.* ASM Press, Washington, DC.
21. **Kellogg, J. A., and L. K. Orwig.** 1995. Comparison of GonoGen, GonoGen II, and MicroTrak direct fluorescent antibody test with carbohydrate fermentation for confirmation of culture isolates of *Neisseria gonorrhoeae. J. Clin. Microbiol.* **33:**474–476.
22. **Olsen, C. C., J. R. Schwebke, W. H. Benjamin, Jr., A. Beverly, and K. B. Waites.** 1999. Comparison of direct inoculation and Copan transport systems for isolation of *Neisseria gonorrhoeae* from endocervical specimens. *J. Clin. Microbiol.* **37:**3583–3585.
23. **Reichart, C. A., L. M. Rupkey, W. E. Brady, and E. W. Hook III.** 1989. Comparison of GC-Lect and modified Thayer-Martin media for isolation of *Neisseria gonorrhoeae. J. Clin. Microbiol.* **27:**808–811.
24. **Saginur, R., B. Clecner, J. Portnoy, and J. Mendelson.** 1982. Superoxol (catalase) test for identification of *Neisseria gonorrhoeae. J. Clin. Microbiol.* **15:**475–477.
25. **Sng, E. H., V. S. Rajan, K. L. Yeo, and A. J. Goh.** 1982. The recovery of *Neisseria gonorrhoeae* from clinical specimens: effects of different temperatures, transport times, and media. *Sex. Transm. Dis.* **9:**74–78.
26. **Thayer, J. D., and J. E. Martin.** 1966. Improved medium selective for cultivation of *N. gonorrhoeae* and *N. meningitidis. Public Health Rep.* **81:**559–562.
27. **Turner, A., K. R. Gough, and A. E. Jephcott.** 1995. Comparison of three methods for culture confirmation of *Neisseria gonorrhoeae* strains currently circulating in the UK. *J. Clin. Pathol.* **48:**919–923.
28. **Yajko, D. M., A. Chu, and W. K. Hadley.** 1984. Rapid confirmatory identification of *Neisseria gonorrhoeae* with lectins and chromogenic substrates. *J. Clin. Microbiol.* **19:**380–382.
29. **Yong, D. C. T., and A. Prytula.** 1978. Rapid micro-carbohydrate test for confirmation of *Neisseria gonorrhoeae. J. Clin. Microbiol.* **8:**643–647.
30. **Young, H., and A. Moyes.** 1993. Comparative evaluation of AccuProbe culture identification test for *Neisseria gonorrhoeae* and other rapid methods. *J. Clin. Microbiol.* **31:**1996–1999.

SUPPLEMENTAL READING

Centers for Disease Control and Prevention. 2000. Gonorrhea—United States, 1998. *MMWR Morb. Mortal. Wkly. Rep.* **49:**538–542.

Chapel, T. A., M. Smelter, and R. Dassel. 1976. The effect of delaying incubation in a CO_2-enriched environment on gonococci. *Health Lab. Sci.* **13:**45–48.

Evangelista, A., and H. Beilstein. 1992. *Cumitech 4A, Laboratory Diagnosis of Gonorrhea.* Coordinating ed., C. Abramson. American Society for Microbiology, Washington, DC.

Evans, K. D., E. M. Peterson, J. I. Curry, J. R. Greenwood, and L. M. de la Maza. 1986. Effect of holding temperature on isolation of *Neisseria gonorrhoeae. J. Clin. Microbiol.* **24:**1109–1110.

Ingram, D. L., V. D. Everett, L. A. Flick, T. A. Russell, and S. T. White-Sims. 1997. Vaginal gonococcal cultures in sexual abuse evaluations: evaluation of selective criteria for preteenaged girls. *Pediatrics* **99:**E8.

Iwen, P. C., R. A. Walker, K. L. Warren, D. M. Kelly, J. Linder, and S. H. Hinrichs. 1996. Effect of off-site transportation on detection of *Neisseria gonorrhoeae* in endocervical specimens. *Arch. Pathol. Lab. Med.* **120:**1019–1022.

Koumans, E. H., R. E. Johnson, J. S. Knapp, and M. E. St. Louis. 1998. Laboratory testing for *Neisseria gonorrhoeae* by recently introduced nonculture tests: a performance review with clinical and public health considerations. *Clin. Infect. Dis.* **27:**1171–1180.

Palmer, H. M., H. Mallinson, R. L. Wood, and A. J. Herring. 2003. Evaluation of the specificities of five DNA amplification methods for the detection of *Neisseria gonorrhoeae. J. Clin. Microbiol.* **41:**835–837.

Ratner, J. B., H. Tinsley, R. E. Keller, and C. W. Stratton. 1985. Comparison of the effect of refrigerated versus room temperature media on the isolation of *Neisseria gonorrhoeae* from genital specimens. *J. Clin. Microbiol.* **21:**127–128.

Rosey, C. E., and E. M. Britt. 1984. Urine as a holding medium for *Neisseria gonorrhoeae. Sex. Transm. Dis.* **11:**301–303.

Shapiro, R. A., C. J. Schubert, and R. M. Siegel. 1999. *Neisseria gonorrhea* [sic] infections in girls younger than 12 years of age evaluated for vaginitis. *Pediatrics* **104:**e72.

3.9.4 *Haemophilus ducreyi* Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Haemophilus ducreyi is the etiologic agent of a sexually transmitted genital ulcer disease known as chancroid. The disease is common in Africa, Asia, and Latin America, but there are over 1,000 reported cases each year in the United States, mostly from the South and the southeastern region (12). Chancroid presents as an acute, localized infection with necrotizing ulcers. The soft chancre, which contains short streptobacillary gram-negative rods, can resemble the ulcers of herpes simplex or syphilis. Two lesions often appear together which are soft on palpation, having a deep ulcerative center (1). In most cases, the disease is diagnosed clinically, but reports of sensitivities using that method alone range from 40 to 80% (12). Ruling out syphilis and other genital infections can increase the sensitivity. Recently, it has been noted that human immunodeficiency virus infection prolongs the disease and can modify its presentation. Gram stains from the leading edge of the ulcer can be helpful early in the infection before the ulcer is contaminated with other microbiota, but culture is the definitive method of diagnosis. Unfortunately the organism is labile and difficult to grow without specialized media. West et al. (13) and others (12) have used PCR methods to detect the organism.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

◩ **NOTE:** Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

A. Specimen collection (1)

Refer to procedure 3.9.1 for further details on specimen collection.

1. Prior to collection, obtain two different selective agar plates (*see* item III.A) from the laboratory, if they are available.

2. Clean the surface of the lesion with 0.85% NaCl.

a. If there is a crust on the lesion, remove it.

b. Moisten swab with saline and collect specimen by vigorously rubbing the base of the lesion.

c. Alternatively, scrape the base of the ulcer with a sterile scalpel blade.

(1) Irrigate with sterile saline.

(2) Then rub the base vigorously with a sterile swab, or aspirate fluid with a flamed smoothed Pasteur pipette or needle and syringe.

3. Abscess

◩ **NOTE:** Intact bubo aspirates are rarely positive for the organism unless they have ruptured.

a. Disinfect skin with alcohol and iodine.

b. Aspirate fluid with a needle and syringe.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

B. Specimen transport

1. Plates

a. Inoculate selective agar plates (*see* item III.A) with specimen.

b. Submit to laboratory immediately for incubation.

2. Transport medium

a. Inoculate Amies transport medium or a specially formulated THIO-hemin-based medium containing L-glutamine and albumin (3).

b. Place immediately on ice or in the refrigerator until and during transport.

◩ **NOTE:** Dangor et al. (3) showed that at room temperature in any transport medium, the organisms do not survive 24 h. Organisms survived in Amies medium for 3 days at 4°C but were viable for longer periods in specially formulated THIO.

3. Label specimens with the patient's demographic information and the site of collection. Attach requisition listing the patient's name, address, medical record number, sex, age, collection location, site of collection, specific test request, and physician of record.

C. Rejection criteria

1. *H. ducreyi* is quite labile. Culture should not be attempted unless special medium is available or specimens can be transported in suitable transport medium on ice to a laboratory with specialized media.

2. Prior antimicrobial treatment reduces the ability to isolate the organism (4).

III. MATERIALS

A. Media

Inoculate any two selective media plus CHOC.

1. Selective media

◩ **NOTE:** Plates that are commercially available are from Remel, Inc., as CHOC with vancomycin and CHOC with fetal bovine serum and vancomycin. Other media listed below must be prepared in-house.

a. GC agar base with 1% IsoVitaleX (BD Diagnostic Systems), 5% fetal bovine serum, 1% hemoglobin, and 3 µg of vancomycin per ml (3, 4)

b. GC agar base with 5% Fildes enrichment, 5% horse blood, and 3 µg of vancomycin per ml

c. 5% Fresh rabbit blood agar with 3 µg of vancomycin per ml (11)

d. Mueller-Hinton agar with 5% chocolatized horse blood, 1% IsoVitaleX, and 3 µg of vancomycin per ml (3, 4)

◩ **NOTE:** Some isolates will grow on one medium but not others (12); some lots of fetal bovine serum are inhibitory. Use of two media and repeating cultures at 48 h increased the isolation rate to 92% for men with ulcers who had no prior antimicrobial use or evidence of syphilis (5). Oberhofer and Black (8) demonstrated that Mueller-Hinton base was clearly superior to TSA base, although there was little difference between 5% sheep blood and supplemented CHOC. Vancomycin is used to inhibit gram-positive microbiota but may inhibit some strains of *H. ducreyi.*

2. Nonselective media: CHOC

B. Identification methods

1. Gram stain (procedure 3.2.1)

2. Catalase test (procedure 3.17.10)

3. Oxidase test (procedure 3.17.39). Do not use *N,N-dimethyl-p*-phenylenediamine dihydrochloride.

4. Indole test (procedure 3.17.23)

5. Aminolevulinic acid (ALA) test for porphyrin synthesis (procedure 3.17.3)

6. Sodium polyanethol sulfonate (SPS) disk (procedure 3.17.45) (Remel, Inc.)

7. Optional: one of the following

a. RapID NH (Remel, Inc.) (6)

b. RapID ANA (Remel, Inc.) (10)

C. Other supplies

1. Incubator at 33°C

2. CO_2-generating bag for culture incubation

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that plate media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (formerly NCCLS) document M22. See procedures 14 and 3.3.1 for further procedures.

B. Perform QC with each lot of selective agar. Incubate aerobically overnight at 33°C in an airtight container with a CO_2-generating system.

Test organism	Result
Haemophilus ducreyi ATCC 33940	Growth in 48 h
Staphylococcus epidermidis ATCC 12228	Inhibition of growth if vancomycin in agar

V. PROCEDURE

Observe standard precautions.

A. Inoculation

◩ **NOTE:** Use of biosafety cabinet will avoid contamination of the culture or specimen as well as protect laboratory processing personnel.

If the specimen is not received on plates, inoculate plates from the swab in transport medium. Use CHOC without vancomycin and two selective media with vancomycin.

B. Direct smear

1. Prepare a Gram stain from the swab after plate inoculation.

2. Stain slide (procedure 3.2.1) and observe for gram-negative coccobacilli arranged in short chains, clumps, or whorls with occasional "rail track" or "school of fish" arrangements (7).

◩ **NOTE:** Direct exam has a sensitivity of about 60% (4).

C. Incubation

1. Incubate plates at 33 to 34°C in 5% CO_2, using a CO_2-generating system to provide the proper atmosphere and moisture. Keep plates moist using sterile-water-moistened filter paper.

◩ **NOTE:** Recovery is substantially increased if the incubation is at 33°C rather than 35°C (9).

2. Observe for growth after at least 48 h of incubation. Hold negative plates for 5 days.

D. Culture examination

1. Observe plates for variable-size, smooth, dome-shaped colonies that are buff-yellow to gray. Whole colonies move across the agar when touched with a loop (1, 7). Colonies are often pinpoint at 48 h and increase to 2 mm in diameter.

2. Perform Gram stain; colonies will clump in saline. Gram-negative coccobacilli should be identified further.

3. Rapidly perform the following tests from a young culture and determine if the reactions agree with those listed below (7).

a. No growth on MAC

b. Catalase negative

c. Oxidase positive—reaction may be slow and require 15 to 20 s

d. Indole negative

e. ALA negative (no fluorescence or porphyrin production)

4. If the above reactions are consistent with the identification, confirm the identification as follows.

a. Test with SPS on CHOC.

◩ **NOTE:** *H. ducreyi* is susceptible, with a zone ≥12 mm; no other *Haemophilus* species are susceptible. The addition of 0.002% Tween 80 may aid in dispersion of the cells (10). *Capnocytophaga* is another genus of

V. PROCEDURE *(continued)*

gram-negative coccobacillus that is reported to be SPS susceptible, but *Capnocytophaga* is ALA positive (10).

b. Optional: perform either the RapID NH (6) or the RapID ANA (10) for further confirmation using enzyme reactions. *H. ducreyi* is asaccharolytic.

c. Submit to a reference laboratory if further confirmation is desired, especially until proficiency in the recognition of the colony morphology is determined.

E. Hold positive culture plates for at least 7 days should antimicrobial susceptibility testing be needed. Since strains are usually beta-lactamase positive, there is no need to test for this enzyme (12).

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. If the culture is negative, report "No *Haemophilus ducreyi* isolated."

B. Positive reporting

1. *H. ducreyi* organisms are catalase-negative, oxidase-positive, gram-negative coccobacilli that are SPS sensitive and ALA negative.

2. Report the presence of *H. ducreyi* as soon as preliminary tests are completed.

3. Do not report any enumeration.

4. Generally report positive results to the physician and to the local health department.

5. Document notification of reporting to the physician and health department.

VII. INTERPRETATION

A. A positive culture indicates infection in a patient with an ulcerative lesion.

B. The CDC (2) recommends treating *H. ducreyi* with azithromycin, 1 g orally in a single dose; ceftriaxone, 250 mg intramuscularly in a single dose; or erythromycin base, 500 mg per os four times a day for 7 days.

C. Mixed infections with other agents known to cause ulcerative sexually transmitted diseases are not uncommon. The presence of *H. ducreyi* does not rule out these other infections, which should be considered in the evaluation of the patient.

VIII. LIMITATIONS

A. Methods that employ PCR for the detection of *H. ducreyi* are more rapid than culture techniques and may have an increased sensitivity (12).

B. False-negative cultures can result from prior antimicrobial therapy, strain growth variability, and sampling and transport techniques. Culture using one medium can have a sensitivity from 65 to 75% (12); using two media will increase the sensitivity to more than 84%.

C. False-negative reports can result from misreading the oxidase test as negative.

D. False-positive results can be caused by misinterpretation of the confirmatory tests.

REFERENCES

1. **Baron, E. J., G. H. Cassell, D. A. Eschenbach, J. R. Greenwood, S. M. Harvey, N. E. Madinger, E. M. Paterson, and K. B. Waites.** 1993. *Cumitech 17A, Laboratory Diagnosis of Female Genital Tract Infections.* Coordinating ed., E. J. Baron. American Society for Microbiology, Washington, DC.
2. **Centers for Disease Control and Prevention.** 2006. Sexually transmitted disease treatment guidelines. *MMWR Morb. Mortal. Wkly. Rep.* **55**(RR-11)**:**1–100.
3. **Dangor, Y., F. Radebe, and R. C. Ballard.** 1993. Transport media for *Haemophilus ducreyi*. *Sex. Transm. Dis.* **20:**5–9.
4. **Dieng Sarr, A., N. C. Toure Kane, N. D. Samb, C. S. Boye, I. K. Diaw, G. Diouf, I. N'Doye, and S. M'Boup.** 1994. Importance of culture media choice in the isolation of *Haemophilus ducreyi*. Experience in Senegal. *Bull. Soc. Pathol. Exot.* **87:**22–27. (In French.)

REFERENCES *(continued)*

5. **Dylewski, J., H. Nsanze, G. Maitha, and A. Ronald.** 1986. Laboratory diagnosis of *Haemophilus ducreyi:* sensitivity of culture media. *Diagn. Microbiol. Infect. Dis.* **4:**241–245.
6. **Hannah, P., and J. R. Greenwood.** 1982. Isolation and rapid identification of *Haemophilus ducreyi. J. Clin. Microbiol.* **16:**861–864.
7. **Lubwama, S. W., F. A. Plummer, J. Ndinya-Achola, H. Nsanze, W. Namaara, L. J. D'Costa, and A. R. Ronald.** 1986. Isolation and identification of *Haemophilus ducreyi* in a clinical laboratory. *J. Med. Microbiol.* **22:**175–178.
8. **Oberhofer, T. R., and A. E. Black.** 1982. Isolation and cultivation of *Haemophilus ducreyi. J. Clin. Microbiol.* **15:**625–629.
9. **Schmid, G. P., Y. C. Faur, J. A. Valu, S. A. Sikandar, and M. M. McLaughlin.** 1995. Enhanced recovery of *Haemophilus ducreyi* from clinical specimens by incubation at 33 versus 35°C. *J. Clin. Microbiol.* **33:**3257–3259.
10. **Shawar, R., J. Sepulveda, and J. E. Clarridge.** 1990. Use of the RapID-ANA system and sodium polyanetholesulfonate disk susceptibility testing in identifying *Haemophilus ducreyi. J. Clin. Microbiol.* **28:**108–111.
11. **Sottnek, F. O., J. W. Biddle, S. J. Kraus, R. E. Weaver, and J. A. Stewart.** 1980. Isolation and identification of *Haemophilus ducreyi* in a clinical study. *J. Clin. Microbiol.* **12:**170–174.
12. **Trees, D. L., and S. A. Morse.** 1995. Chancroid and *Haemophilus ducreyi:* an update. *Clin. Microbiol. Rev.* **8:**357–375.
13. **West, B., S. M. Wilson, J. Changalucha, S. Patel, P. Mayaud, R. C. Ballard, and D. Mabey.** 1995. Simplified PCR for detection of *Haemophilus ducreyi* and diagnosis of chancroid. *J. Clin. Microbiol.* **33:**787–790.

SUPPLEMENTAL READING

Albritton, W. L. 1989. Biology of *Haemophilus ducreyi. Microbiol. Rev.* **53:**377–389.

Dangor, Y., R. C. Ballard, S. D. Miller, and H. J. Koornhof. 1990. Antimicrobial susceptibility of *Haemophilus ducreyi. Antimicrob. Agents Chemother.* **34:**1303–1307.

Hammond, G. W. 1996. A history of the detection of *Haemophilus ducreyi*, 1889–1979. *Sex. Transm. Dis.* **23:**93–96.

Johnson, S. R., D. H. Martin, C. Cammarata, and S. A. Morse. 1995. Alterations in sample preparation increase sensitivity of PCR assay for diagnosis of chancroid. *J. Clin. Microbiol.* **33:**1036–1038.

Kilian, M. 2007. *Haemophilus*, p. 636–648. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.

Lagergard, T., A. Frisk, and B. Trollfors. 1996. Comparison of the Etest with agar dilution for antimicrobial susceptibility testing of *Haemophilus ducreyi. J. Antimicrob. Chemother.* **38:**849–852.

Morse, S. A. 1989. Chancroid and *Haemophilus ducreyi. Clin. Microbiol. Rev.* **2:**137–157.

3.10 Ocular Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Inflammatory eye conditions may be due to a variety of diseases, and microorganisms play a major role in both acute and chronic diseases (Table 3.10–1). The detection of infectious agents depends on knowledge of the site of infection and the severity of the process, because a variety of organisms cause infections of the eye. Unlike the procedures with other specimen types, it may be important for the physician to inoculate culture media at the bedside rather than transport the specimen to the laboratory for processing. This procedure describes the clinical syndromes associated with bacterial infections of the eye, the organisms associated with these syndromes, and the procedure for isolation of these infectious agents. In addition to aerobic bacterial culture, Table 3.10–2 indicates the media to inoculate for anaerobic, fungal, and mycobacterial cultures. Refer to the respective sections of the handbook for workup of the microorganisms that are not covered in this procedure.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

◩ **NOTE:** Most eye specimens are collected by an ophthalmologist. These specimens are inoculated onto culture media at the bedside, in the clinic or the physician's office. A variety of techniques are used to collect material from different parts of the eye. The conjunctiva is constantly contaminated by various bacteria from the environment and ocular adnexa. Therefore, specimens from the conjunctiva serve as a control when compared with specimens collected by more aggressive or invasive techniques. See section 2 for general principles of collection.

◩ **NOTE:** Refer to procedure 3.3.1 for additional details on specimen collection. The following can be copied for preparation of a specimen collection instruction manual for physicians and other caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

Observe standard precautions.

A. Provide fresh media to the clinical areas routinely collecting ocular cultures, and instruct physicians to immediately transport inoculated media and slides to the laboratory.

B. Obtain viral and chlamydial samples before topical anesthetics are instilled.

1. Obtain samples for chlamydial cultures with calcium alginate swabs.

2. For viral cultures, use Dacron or cotton swabs with nonwood shafts.

C. Collection by anatomic site

◩ **NOTE:** *Specimen collection must be performed by a qualified physician.*

1. Conjunctiva (bacterial conjunctivitis) and lid margin (if staphylococcal blepharoconjunctivitis is suspected)

a. Obtain the specimen with a sterile, premoistened cotton or calcium alginate swab.

b. Roll the calcium alginate or cotton swab over the conjunctiva before topical medications are applied.

Table 3.10–1 Clinical description of common ocular infections

Clinical syndrome	Location of infection	Predisposing factors	Clinical symptoms/signs	Specimen(s) collected
Conjunctivitis	Acute and chronic inflammation of the conjunctiva, the mucous membrane covering the sclera	Direct inoculation of organisms from fomites, hands, and the environment. Hematogenous spread may also occur but is rare.	Reddening of the conjunctival surface, increased tearing, and purulent discharge of one or both eyes	Swab of conjunctiva surface/pus
Dacryoadenitis/ dacryocystitis	Dacryoadenitis is infection of the lacrimal glands. Dacryocystitis is an infection of the lacrimal sac.	Obstruction of the nasolacrimal duct	Redness of the inner eyelid and corner of the eye with induration, pain, and increased tearing. Pus may be expressed from the lacrimal duct and a fistula may form.	Superficial wound cultures of the external lacrimal duct or pus
Canaliculitis	Inflammation of the canaliculus, the passage that connects the punctum to the lacrimal sac	Extension of dacryocystitis and dacryoadenitis	Swelling, pain, and tenderness at the corner of the eye. Patients may also have conjunctivitis and redness of the eyelid.	Superficial wound cultures of the external lacrimal duct or pus
Bacterial keratitis	Acute and chronic inflammation of the cornea. May be superficial infection of the corneal epithelium or deep stromal ulceration that may perforate and result in anterior chamber of deeper infection of the eye.	Prior ocular disease Contact lens wear Use of topical corticosteroids Burn patients Alcoholic patients Immunocompromised patients	Redness of the eye, inflammation of the conjunctiva, increased pain, decreased vision, and photophobia. Patients may feel like there is "sand" in the eye that results in increased tearing and formation of exudates. Bacterial keratitis requires prompt investigation and therapy to avoid permanent loss of vision.	Corneal scrapings
Bacterial endophthalmitis	Inflammation of the ocular cavities and intraocular tissue (uvea and retina)	Trauma or injury Surgery Corneal perforation due to keratitis Bacteremia with seeding of the eye	Postoperative infection usually manifest within 72 h after surgery. Endogenous infection on the eye occurs secondary to bacteremia. Endophthalmitis presents with pain, decreased vision, lid edema, conjunctival redness, severe iridocyclitis (uveitis), and hypopyon (pus in the eye). Endophthalmitis is a medical emergency that requires immediate surgical debridement, installation of intraocular antimicrobials, and prompt treatment with systemic antimicrobial therapy to minimize permanent loss of vision.	Aqueous and vitreous fluid is collected by aspiration.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

c. Culture both eyes with separate swabs.

d. Immediately inoculate the material at the bedside onto the media outlined in Table 3.10–2.

e. Inoculate the swab from the right conjunctiva in horizontal streaks, and inoculate the swab from the left conjunctiva in vertical streaks, each on one half of the same agar plate.

f. Inoculate specimens from the right and left lid margins, if collected, by making an R and an L to represent the respective sites on another agar plate.

Table 3.10–1 *(continued)*

Clinical syndrome	Location of infection	Predisposing factors	Clinical symptoms/signs	Specimen(s) collected
Preseptal cellulitis	Inflammation of the periorbital soft tissues	Trauma or injury Extension of facial cellulitis	Periorbital cellulitis must be clinically differentiated from orbital cellulitis. Periorbital cellulitis presents with redness, induration, and pain in the eyelids and soft tissues surrounding one or both eyes. Vision is usually preserved, except that the patient may not be able to fully open the eye due to swelling. Conjunctival edema and redness may be present, but there is no chemosis, proptosis, or extraocular muscle palsy as occurs in orbital cellulitis.	Superficial wound culture Blood cultures
Orbital cellulitis	Infection of the deeper orbital tissues that may extend to the retro-orbital space and brain, resulting in cavernous sinus thrombosis and/or other intracranial infection	Trauma and injury Surgery Extension of sinus or paranasal infection Extension of endophthalmitis	Patients are systemically unwell, with fever and leukocytosis, and have gross periorbital redness, induration, and severe pain in the eye. Eyelid edema and chemosis of the conjunctiva are extensive, and patients may not be able to open the eye. Limited ocular motility occurs due to swelling, and extension to the retro-orbital space results in proptosis. Palsy of one or more extraocular muscle movements suggests extension to the brain. Orbital cellulitis is a medical emergency that requires immediate investigation for underlying cause and extent of infection, and prompt treatment with systemic antibiotic therapy to minimize permanent loss of vision.	Superficial wound culture Blood cultures Sinus aspirates

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

g. Obtain conjunctival scrapings for a smear preparation as follows.
(1) Instill 1 or 2 drops of proparacaine hydrochloride.
(2) Using a Kimura spatula, gently scrape across the lower right tarsal conjunctiva.
(3) Smear the material in a circular area 1 cm in diameter on a clean glass slide.
(4) Prepare at least two slides.
(5) Immerse the slides in 95% methyl alcohol or 100% methanol for 5 min.
(6) Repeat steps for the left conjunctiva.

Table 3.10–2 Handling of specimens for diagnosis of ocular infections

Clinical condition	Primary isolation media[a]	Primary pathogen(s)	Comments
Conjunctivitis	BAP CHOC MAC (if hospitalized patient)	*Haemophilus influenzae* *Staphylococcus aureus* *Streptococcus pneumoniae* *Streptococcus pyogenes* *Moraxella* spp. *Neisseria gonorrhoeae*	*Enterobacteriaceae* and *P. aeruginosa* may be important in hospitalized and/or immunocompromised patients. DFA[b] should also be done for *Chlamydia trachomatis* in neonates.
Keratitis	BAP CHOC MAC Fungal media[c] AFB media[d] Viral culture	Corneal trauma/ulcer *Pseudomonas aeruginosa* *S. aureus* *S. pneumoniae* Viridans group streptococci *Moraxella* spp. AFB—rapid growers *Nocardia* spp. Herpes simplex and herpes zoster viruses Contact lens associated: Gram-negative bacteria, including *P. aeruginosa, Serratia* spp. *Bacillus* spp. *Acanthamoeba* spp.	Keratitis is caused by a variety of organisms, depending upon the mechanism of corneal injury. Fungi, AFB, and *Nocardia* spp. should be ruled out in chronic infection. Corneal ulcers should have viral cultures, particularly for patients with trigeminal herpes zoster infection.
Endophthalmitis	BAP CHOC MAC Anaerobic BAP Fungal media[c] AFB media[d] Viral culture	Postsurgical or traumatic *S. aureus* Coagulase-negative staphylococci Viridans group streptococci *Bacillus* spp. *P. aeruginosa* *P. acnes* Anaerobes, including *Clostridium* spp. Bacteremia *S. aureus* *H. influenzae* *S. pneumoniae* *N. gonorrhoeae* *Neisseria meningitidis* Gram-negative bacteria, including *P. aeruginosa* Any other bacteria or yeasts/fungi causing bacteremia or fungemia	Endophthalmitis is caused by a variety of organisms, depending upon the mechanism of infection. Fungi, AFB, and *Nocardia* spp. should be ruled out in chronic postsurgical and traumatic infections. Viral cultures should be done, particularly for patients with trigeminal herpes zoster infection. The inner eye chambers may be seeded by any bacterial or fungal organism causing bacteremia or fungemia, so blood cultures should be obtained.
Periorbital cellulitis	BAP CHOC MAC (if secondary to trauma) Anaerobic BAP (if secondary to trauma)	*S. aureus* *S. pyogenes* *H. influenzae*	Localized soft tissue infection around the eye. *H. influenzae* may be recovered in infants and young children.
Orbital cellulitis	BAP CHOC MAC Anaerobic BAP Fungal media[c]	*S. aureus* *S. pneumoniae* Gram-negative bacilli, including *P. aeruginosa* *H. influenzae* *S. pyogenes* Anaerobes	Mixed aerobic and anaerobic infection occurs secondary to trauma or sinusitis. Blood cultures should be obtained. Fungi, including *Aspergillus* spp. and *Mucor* spp., may be important in diabetic or other immunocompromised patients.

[a] Bacterial culture includes a Gram stain, fungal culture includes a calcofluor white stain, and AFB culture includes an auramine-rhodamine or Ziehl-Neelsen stain.
[b] DFA, direct fluorescent antibody testing.
[c] Fungal media may include IMA, BHI, or potato flake agar.
[d] AFB media may include Lowenstein-Jensen or Middlebrook agar and broth culture.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

2. Bacterial keratitis
- **a.** Instill 1 or 2 drops of proparacaine hydrochloride.
- **b.** Obtain conjunctival samples as described above, and then obtain corneal scrapings from the advancing edge of the ulcer by scraping multiple areas of ulceration and suppuration with a sterile Kimura spatula, using short, firm strokes in one direction. (Keep the eyelid open, and be careful not to touch the eyelashes.)
- **c.** Obtain approximately three to five scrapings per cornea.
- **d.** Inoculate each set of scrapings onto the media outlined in Table 3.10–2, using a C formation for each scraping.
- **e.** Prepare smears by applying the scrapings in a gentle circular motion over a clean glass slide or by compressing material between two clean glass slides and pulling the slides apart (*see* Fig. 3.2.1–1).

3. Bacterial endophthalmitis
- **a.** Collect an aspirate of the vitreous fluid or perform a paracentesis of the anterior chamber using a needle aspiration technique to collect intraocular fluid.
- **b.** Collect specimens for conjunctival cultures along with the fluid to determine the significance of indigenous microbiota.
- **c.** If a small volume of fluid is collected, inoculate cultures at the bedside by inoculating 1 or 2 drops of fluid onto culture media (Table 3.10–2).
- **d.** Alternatively, submit in anaerobic transport tube or capped syringe after replacing the needle with a Luer-Lok.

4. Preseptal cellulitis
- **a.** Cleanse the skin with alcohol and tincture of iodine or iodophor.
- **b.** In the absence of an open wound, the physician makes a stab incision in either the upper or lower lid with a no. 11 Baird-Parker blade.
- **c.** If there is an open wound, collect the purulent material with a syringe and needle.
- **d.** Inoculate media (Table 3.10–2), and prepare slides as described above for conjunctivitis.
- **e.** Inject some material into an anaerobic transport vial, and process specimens for anaerobes as described in section 4 of this handbook.

5. Orbital cellulitis
- **a.** Obtain aspirate or biopsy sample of the wound, and process as described above for preseptal cellulitis. Additionally, inoculate fungal media or submit to the laboratory for inoculation (Table 3.10–2).
- **b.** Collect blood cultures.

6. Dacryoadenitis
- **a.** Collect a specimen of the purulent discharge by using a swab, as described above for conjunctivitis. Inoculate media (Table 3.10–2).
- **b.** Do not perform a needle aspiration of the lacrimal gland.

7. Dacryocystitis
- **a.** Obtain conjunctival cultures.
- **b.** Press the lacrimal sac to remove exudate material for culture and smear, or collect exudate in a needle and syringe.
- **c.** Place aspirated material in a transport vial, and transport to the laboratory.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

8. Canaliculitis
- **a.** Compress the inner aspect of the eyelid to express pus.
- **b.** Follow procedure outlined above for conjunctivitis.
- **c.** Inoculate media (Table 3.10–2), and prepare slides as described above for conjunctivitis.
- **d.** Additionally, inoculate fungal media or submit to the laboratory for inoculation (Table 3.10–2).
- **e.** Inject some material into an anaerobic transport vial, and process specimens for anaerobes as described in section 4 of this handbook.

D. Rejection criteria
- **1.** Request that a swabbed specimen of the conjunctiva accompany any specimen collected by invasive technique.
- **2.** Even in cases of suspected unilateral conjunctivitis, indicate that bilateral bacterial cultures are mandatory.
- **3.** When inoculated plates are delayed in transit, notify the physician that the culture may be compromised or contaminated.

III. MATERIALS

A. Media
- **1.** BAP
- **2.** CHOC
- **3.** MAC
- **4.** Anaerobic BAP (*see* section 4 for options)
- **5.** THIO or other anaerobic broth for anaerobic culture
- **6.** Media for fungal and mycobacterial cultures as listed in Table 3.10–2 (*see* sections 7 and 8)

B. Gram stain reagents and slides

C. Specimen collection devices
- **1.** Kimura platinum spatula
- **2.** Cotton, polyester, or calcium alginate swab
- **3.** Needle and syringe
- **4.** Aerobic swab transport system
- **5.** Anaerobic transport system
- **6.** Topical anesthetic—proparacaine hydrochloride (0.5%)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that media meet expiration date and QC parameters per Clinical and Laboratory Standards Institute (formerly NCCLS) document M22-A3 (2). See procedures 14 and 3.3.1 for further procedures, especially for in-house-prepared media.

B. Swabbed specimens from the conjunctiva are used as a control along with other specimens collected by more aggressive or invasive techniques.

C. Conjunctival cultures are used to determine the presence of indigenous microbiota.

V. PROCEDURE

Observe standard precautions.

A. Direct smears
- **1.** Gram stain
  - **a.** Prepare two or three smears from the clinical material if glass slides do not accompany the specimen. If cheesy concentrations are present, crush them onto a slide for Gram stain.
  - **b.** Place slides in 95 or 100% methanol for 5 to 10 min to fix material.
  - **c.** Perform a Gram stain (*see* procedure 3.2.1).

V. PROCEDURE *(continued)*

d. Examine the stained smear for the presence of somatic cells and extra- and intracellular organisms.
(1) The presence of PMNs suggests a bacterial infection.
(2) The presence of mononuclear cells may indicate viral conjunctivitis. Refer to section 10 of this handbook for detailed viral procedures.
(3) Refer to the Gram stain procedure (3.2.1) for details on interpretation of various morphologies observed on the smear.
(4) Refer to section 9 for examination for microsporidia.

◩ **NOTE:** Pigment granules that resemble gram-positive cocci may be present on the Gram-stained smear. They can be differentiated from cocci because they are large, oval, and brown.

2. Giemsa stain
a. For suspected chlamydial infections, prepare smears and examine them for intracytoplasmic inclusions.
b. Alternatively, perform direct fluorescent-antibody test or culture for *Chlamydia* (procedures 10.6 and 10.7), PACE 2 nucleic acid probe (Gen-Probe, Inc.) (procedure 12.2.2, part 1), or a nucleic acid amplification assay (*see* section 12) according to the manufacturer's instructions. The manufacturer's package insert should be consulted to determine whether a specific assay has been approved for testing ocular specimens.

3. Calcofluor white stain
a. Prepare a calcofluor white stain if fungi or *Acanthamoeba* cysts are suspected.
b. For details, refer to procedure 9.3.8.

B. Culture inoculation, examination, and interpretation

1. Inoculate culture media according to Table 3.10–2, if plates are not received. Streak in quadrants for isolation of colonies.
a. If a scant specimen of intraocular fluid is submitted in a syringe, use broth to wash out the syringe by drawing up a small amount of broth.
b. Use the broth to inoculate plate media with 2 drops per plate.
c. Place the balance of the broth and specimen in broth culture tube. Avoid creating an aerosol.
d. For culture for *Acanthamoeba*, refer to procedure 9.9.2.

2. Incubate cultures at 35°C in 5 to 7% CO_2 for 72 h.

3. Refer to procedure 3.3.2 for instructions on incubation and examination of broth cultures. Hold broth cultures from invasively collected ocular specimens for 10 days (1, 4) to detect infections with *Propionibacterium acnes.*

4. Examine daily for the presence of microorganisms.

5. Estimate and report the number of each organism on each plate. The presence of moderate numbers of colonies or many colonies on one or more culture plates should indicate the bacterial etiology of the infection.

6. For quantitation of C streaks
a. 1+: less than half of the C streaks are positive per plate
b. 2+: more than half of the streaks, but not all, are positive
c. 3+: all streaks are positive for bacteria

7. Identify the organisms by using the tests recommended in procedures 3.3.2, 3.18.1, and 3.18.2.

V. PROCEDURE *(continued)*

8. Consult the physician prior to identifying rare colonies of indigenous microbiota from noninvasively collected specimens, including the following.
   - **a.** Coagulase-negative staphylococci
   - **b.** Diphtheroids
   - **c.** Viridans group streptococci
   - **d.** *Moraxella catarrhalis*
   - **e.** *P. acnes*
   - **f.** *Peptostreptococcus* spp.
9. Correlate culture with the Gram stain of the direct smear.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Telephone positive reports from invasively collected specimens to the physician as soon as possible.

B. Report the relative number and morphology of all microorganisms seen, the presence and numbers of somatic cells (especially PMNs), and whether the organisms were observed intracellularly as well as extracellularly.

C. Report the quantity and organism identity for each morphological type observed on culture media.

D. If indigenous microorganisms are present and it has been determined that they may be contaminants, include a comment such as "Possible contamination" and/or "Presence of indigenous conjunctival microbiota."

VII. INTERPRETATION

A. All organisms grown in any amount from critical eye specimens (i.e., aqueous and vitreous fluid) should be identified and have AST results reported.

B. All organisms present in the direct smear that grow on primary culture plates are considered clinically significant and should be worked up.

C. The following criteria may assist the laboratory to determine the clinical significance of bacterial isolates from critical eye specimens that may otherwise be considered indigenous microbiota.

1. Isolates of coagulase-negative staphylococci, diphtheroids, *P. acnes*, or viridans group streptococci that grow on more than one medium are generally considered significant.
2. Indigenous microbiota isolates may also be clinically significant when growth occurs only on one medium or in broth. In these cases a comment may be added to the final report indicating that the clinical significance of organisms that are part of the skin indigenous microbiota must be determined by the clinical picture.

VIII. LIMITATIONS

A. False-positive cultures can result from contamination of the specimen or the inoculated plates with skin microbiota.

B. Conversely, false-negative reports can result from considering corynebacteria as contaminating microbiota when they can be pathogens. For example, *Corynebacterium macginleyi* has been implicated in conjunctivitis and corneal ulcers (3).

C. False-negative results can occur if antimicrobial agents are given prior to collection of the specimens.

D. Even with the best techniques, culture often fails to yield the infecting organism. Currently, use of DNA probes is being investigated as a more sensitive alternative to culture.

REFERENCES

1. **Chern, K. C., D. M. Meisler, G. S. Hall, S. M. Myers, R. E. Foster, Z. N. Zakov, and C. Y. Lowder.** 1996. Bacterial contamination of anaerobic vitreous cultures: using techniques employed for endophthalmitis. *Curr. Eye Res.* **15:**697–699.
2. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
3. **Funke, G., M. Pagano-Niederer, and W. Bernauer.** 1998. *Corynebacterium macginleyi* has to date been isolated exclusively from conjunctival swabs. *J. Clin. Microbiol.* **36:**3670–3673.
4. **Hall, G. S., K. Pratt-Rippin, D. M. Meisler, J. A. Washington, T. J. Roussel, and D. Miller.** 1994. Growth curve for *Propionibacterium acnes. Curr. Eye Res.* **13:**465–466.

SUPPLEMENTAL READING

Armstrong, R. A. 2000. The microbiology of the eye. *Ophthalmic Physiol. Opt.* **20:**429–441.

Baum, J. 1995. Infections of the eye. *Clin. Infect. Dis.* **21:**479–486.

Brady, S. E., E. J. Cohen, and D. H. Fischer. 1988. Diagnosis and treatment of chronic postoperative bacterial endophthalmitis. *Ophthalmic Surg.* **19:**590–594.

Brook, I. 1980. Anaerobic and aerobic flora of acute conjunctivitis in children. *Arch. Ophthalmol.* **98:**833–835.

Brook, I. 1988. Presence of anaerobic bacteria in conjunctivitis associated with wearing contact lenses. *Ann. Ophthalmol.* **20:**397–399.

Donzis, P. B., B. J. Mondino, and B. A. Weissman. 1988. Bacillus keratitis associated with contaminated contact lens care systems. *Am. J. Ophthalmol.* **105:**195–197.

Friedlaender, M. H. 1995. A review of the causes and treatment of bacterial and allergic conjunctivitis. *Clin. Ther.* **17:**800–810.

Israele, V., and J. D. Nelson. 1987. Periorbital and orbital cellulitis. *Pediatr. Infect. Dis.* **6:**404–410.

Joondeph, B. C., H. W. Flynn, D. Miller, and H. C. Joondeph. 1989. A new culture method for infectious endophthalmitis. *Arch. Ophthalmol.* **107:**1334–1337.

Kinnear, F. B., and C. M. Kirkness. 1995. Advances in rapid laboratory diagnosis of infectious endophthalmitis. *J. Hosp. Infect.* **30**(Suppl)**:**253–261.

Klotz, S. A., C. C. Penn, G. J. Negvesky, and S. I. Butrus. 2000. Fungal and parasitic infections of the eye. *Clin. Microbiol. Rev.* **13:**662–685.

Kresloff, M. S., A. A. Castellarin, and M. A. Zarbin. 1998. Endophthalmitis. *Surv. Ophthalmol.* **43:**193–224.

Mandell, G. L., J. E. Bennett, and R. Dolin (ed.). 2005. *Principles and Practice of Infectious Diseases,* 6th ed. Churchill Livingstone, New York, NY.

McNatt, J., S. D. Allen, L. A. Wilson, and V. R. Dowell. 1978. Anaerobic flora of the normal human conjunctival sac. *Arch. Ophthalmol.* **96:**1448–1450.

Okhravi, N., P. Adamson, and S. Lightman. 2000. Use of PCR in endophthalmitis. *Ocul. Immunol. Inflamm.* **8:**189–200.

Perkins, R. E., R. B. Knudsin, M. V. Pratt, I. Abrahamsen, and H. B. Leibowitz. 1975. Bacteriology of normal and infected conjunctiva. *J. Clin. Microbiol.* **1:**147–149.

Smith, R. E., and J. R. Nobe. 1989. Eye infections, p. 213–232. *In* S. M. Finegold and W. L. George (ed.), *Anaerobic Infections in Humans.* Academic Press, Inc., New York, NY.

Smolin, G., K. Tabbara, and J. Witcher. 1984. *Infectious Diseases of the Eye.* The Williams & Wilkins Co., Baltimore, MD.

Weissgold, D. J., and D. J. D'Amico. 1996. Rare causes of endophthalmitis. *Int. Ophthalmol. Clin.* **36:**163–177.

Wilhelmus, K. R., T. J. Liesegang, M. S. Osato, and D. B. Jones. 1994. *Cumitech 13A, Laboratory Diagnosis of Ocular Infections.* Coordinating ed., S. C. Specter. American Society for Microbiology, Washington, DC.

3.11.1 Guidelines for Performance of Respiratory Tract Cultures

Specimens from the upper respiratory tract (throat specimens, nasopharyngeal swabs, nasal discharges) can be easily obtained but are contaminated with resident microbiota. In addition, many microorganisms present in the nares and throat are found in both the disease and the carrier states (2, 3, 4). It is estimated that 60% of children sporadically carry *Streptococcus pneumoniae* in their nasal passages by the age of 2 years (2). Because of this contamination, these specimens often do not provide accurate, clinically useful information for diagnosis of bacterial respiratory infection caused by organisms such as *S. pneumoniae, Haemophilus influenzae,* and *Moraxella catarrhalis.* On the other hand, these specimens are useful for the diagnosis of specific pathogens, whose presence in symptomatic patients most often indicates disease (i.e., *Streptococcus pyogenes, Bordetella pertussis, Corynebacterium diphtheriae,* and respiratory viruses). Nasal cultures are also performed as a part of the infection control of hospitalized patients to detect carriage of oxacillin-resistant *Staphylococcus aureus* or as part of a staphylococcal outbreak. In the latter case, nasal carriage by hospital employees may also be important (procedure 13.17). However, culture of nasopharyngeal specimens to detect carriage of potential pathogens such as *Neisseria meningitidis, S. pneumoniae,* and *H. influenzae* should be discouraged. Since these pathogens are all part of the normal oropharyngeal flora, the clinical relevance of culturing them from this site cannot be determined. In addition, reporting of normal oropharyngeal flora from this site may result in the patient receiving an unnecessary course of antibiotic therapy, thus fostering the development of resistance. Antibiotic prophylaxis of individuals in close contact with a patient with meningococcemia should be directed by the CDC guidelines, and should not be withheld based on the nasopharyngeal culture result (1). Likewise, nasopharyngeal specimen cultures for yeast or mold colonization in otherwise healthy asymptomatic individuals should be discouraged for similar reasons. Nasopharyngeal cultures for the detection of either *Aspergillus* spp., *Mucor* spp., or other fungi may be warranted for immunocompromised patients who are suspected of having an invasive infection.

Thus, a request for "routine" bacterial culture of noninvasively collected nasal or throat specimens, usually submitted to the laboratory on swabs or as a wash, aspirate, or discharge, should *not* be processed by the laboratory (refer to cystic fibrosis procedure [3.11.3] for exception for deep pharyngeal cultures from these patients). It is important that these cultures be done only when detection of a specific pathogen is sought and not be performed routinely to detect any organism that is present. When requests are received by the laboratory, the caregiver should be contacted to determine if the specimen was sent for detection of one of the specific pathogens (Table 3.11.1–1). If the specimen is being sent to diagnose a bacterial cause of lower respiratory disease, otitis media, or sinusitis, inform the caregiver that a more invasive specimen, such as endotracheal aspirate, maxillary sinus puncture, or tympanocentesis fluid, respectively, is needed to make the diagnosis (*see* Table 3.11.1–1 for specific specimens and procedural references). Procedures that follow in this portion present collection, processing, and interpretation to detect individual respiratory pathogens and procedures for bacterial etiology of upper and lower respiratory disease from specific anatomic sites. A procedure is also listed specifically for processing specimens from cystic fibrosis patients. Refer to Table 3.11.1–1 for the appropriate specimens and procedures.

REFERENCES

1. **Bilukha, O. O., and N. Rosenstein.** 2005. Prevention and control of meningococcal disease. Recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Morb. Mortal. Wkly. Rep.* **54**(RR-7)**:**1–21.
2. **Dagan, R., R. Melamed, M. Muallem, L. Piglansky, and P. Yagupsky.** 1996. Nasopharyngeal colonization in southern Israel with antibiotic-resistant pneumococci during the first 2 years of life: relation to serotypes likely to be included in pneumococcal conjugate vaccines. *J. Infect. Dis.* **174:**1352–1355.
3. **Faden, H., M. Heimerl, G. Goodman, P. Winkelstein, and C. Varma.** 2002. New technique (the NOW test) for rapid detection of *Streptococcus pneumoniae* in the nasopharynx. *J. Clin. Microbiol.* **40:**4748–4749.
4. **Robinson, D. A., K. M. Edwards, K. B. Waites, D. E. Briles, M. J. Crain, and S. K. Hollingshead.** 2001. Clones of *Streptococcus pneumoniae* isolated from nasopharyngeal carriage and invasive disease in young children in central Tennessee. *J. Infect. Dis.* **183:**1501–1507.

Table 3.11.1–1 Appropriate specimens for diagnosis of bacterial and yeast upper and lower respiratory diseases

Clinical condition	Primary pathogen(s)	Specimens	Procedure reference(s)
Candidiasis (oral thrush)	*Candida albicans*	Swab of buccal mucosa, tongue, or oropharynx	8.3 (KOH, calcofluor white stain)
Cystic fibrosis	*Pseudomonas aeruginosa* *S. aureus* *Burkholderia cepacia* *Aspergillus* spp.	Deep throat (young children) Lower respiratory[a]	3.11.3
Diphtheria	*C. diphtheriae*	Nasopharygeal swab[b]	3.11.7
Epiglottitis	*H. influenzae*	Blood culture	3.4.1
Esophagitis[c]	*C. albicans* Herpes simplex virus Cytomegalovirus	Esophageal biopsy samples should be sent for yeast and viral culture as well as histopathology	8.3, 8.4
Bacterial pharyngitis	Group A streptococcus (*S. pyogenes*) *N. gonorrhoeae*	Throat swab (use charcoal if for *Neisseria gonorrhoeae* culture)	3.9.3
Laryngitis, bronchiolitis	*Mycoplasma pneumoniae* Respiratory viruses	Lower respiratory[a] Nasopharyngeal swab for viruses	3.15
Lemierre's disease	*Fusobacterium necrophorum*	Blood culture	3.4.1
Otitis externa	*S. aureus* *S. pyogenes* *P. aeruginosa*	Swab of inner ear canal	3.11.5
Otitis media	*S. pneumoniae* *H. influenzae* *M. catarrhalis* *S. pyogenes* *Alloiococcus otitis*	Swab of myringotomy tube drainage Tympanocentesis fluid	3.11.5
Pertussis	*B. pertussis*	Nasopharyngeal swab or aspirate[b]	3.11.6
Chronic bronchitis	*S. pneumoniae* *S. aureus,* including MRSA[d] *H. influenzae* Gram-negative bacilli Respiratory viruses	Lower respiratory[a]	3.11.2
Pneumonia	Community-acquired pneumonia *S. pneumoniae* *S. aureus,* including MRSA *H. influenzae* *Klebsiella pneumoniae* Anaerobes (if aspiration) *Pasteurella* spp. *Legionella* spp. *M. pneumoniae* *Chlamydia pneumoniae* *Chlamydia psittaci* Respiratory viruses *Pneumocystis carinii* (PCP[e]) (HIV[f] risk factors or seropositive) Agents of bioterrorism, including *Bacillus anthracis, Brucella* spp., *Francisella tularensis, Yersinia pestis,* and *Burkholderia pseudomallei* *Mycobacterium tuberculosis*	Lower respiratory[a]	3.11.2, 3.15, 3.11.4

(continued)

Table 3.11.1–1 Appropriate specimens for diagnosis of bacterial and yeast upper and lower respiratory diseases *(continued)*

Clinical condition	Primary pathogen(s)	Specimens	Procedure reference(s)
Pneumonia (*cont.*)	Hospital-acquired pneumonia, including VAP[g] *S. aureus* Gram-negative bacilli, including *P. aeruginosa* and other nonfermenters Anaerobes (if aspiration) Immunocompromised patient Primary pathogen(s) listed above Unusual bacteria such as *Rhodococcus equi* *P. carinii* (PCP) Fungi AFB[h] Viruses	Lower respiratory[a]	3.11.2, 3.15, 3.11.4
Empyema	*S. aureus* *S. pyogenes* *S. pneumoniae* Anaerobes *M. tuberculosis*	Pleural fluid	3.5
Lung abscess	*S. aureus* *S. pyogenes* *K. pneumoniae* *P. aeruginosa* Anaerobes	Lung aspirate or biopsy sample	3.5, 3.11.2, 3.13.1
Sinusitis	Acute *S. aureus* *S. pneumoniae* *H. influenzae* *M. catarrhalis* Chronic Agents of acute sinusitis Gram-negative bacilli, including *P. aeruginosa* Anaerobes	Sinus aspirate	3.11.9
Vincent's angina	*Borrelia vincentii* (spirochetes) Anaerobes (fusiform rods)	Swab of gingiva	3.2.1 (Gram stain) or 7.2 (Ziehl-Neelsen stain but dilute 1:10 with water)
Staphylococcal carriage	*S. aureus*, including MRSA	Swab of nares	13.11

[a] Lower respiratory includes sputa, endotracheal tube aspirates, auger suction samples, lung aspirates, bronchial washings, brochoalveolar lavage specimens, protected brush specimens, and lung biopsy specimens. Procedures for detection of respiratory viruses (section 10), *M. tuberculosis* (section 7), aerobic *Actinomyces* (section 6), and fungi, such as *Histoplasma capsulatum* and *Coccidioides immitis* (section 8), should be included, depending on the patient's exposure history and clinical situation and condition.

[b] Specimen is also used to diagnose infection caused by respiratory viruses (section 10).

[c] Herpes simplex virus and cytomegalovirus are important causes, and biopsy samples should be sent for histopathology and virology testing.

[d] MRSA, methicillin-resistant *S. aureus*.

[e] PCP, *P. carinii* pneumonia.

[f] HIV, human immunodeficiency virus.

[g] VAP, ventilator-associated pneumonia.

[h] AFB, acid-fast bacilli.

3.11.2 Lower Respiratory Tract Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Infections of the lower respiratory tract are the sixth leading cause of mortality in the United States, with 2,000,000 to 3,000,000 cases per year, resulting in 500,000 hospitalizations (6). For community-acquired pneumonia (CAP) in adults and the elderly, *Streptococcus pneumoniae* is the cause in 6 to 10% of all cases and 60% of the bacterial cases (3, 7), hence the utility of the pneumococcal vaccine to prevent this disease.

Culture of lower respiratory secretions can be helpful, but in reality it is limited, with no agent isolated in 40 to 60% of cases (6). This lack of culture sensitivity may be due to the low sensitivity (50%) of sputum culture for *S. pneumoniae*, especially if specimens are not immediately processed. In addition, many agents of pneumonia are difficult to grow (e.g., *Legionella*, *Chlamydia pneumoniae*, and *Mycoplasma pneumoniae*). It is estimated that *C. pneumoniae* is the second most common cause of pneumonia and that *M. pneumoniae* accounts for most cases of ambulatory CAP identified by serologic methods (13). *Haemophilus influenzae* and *Legionella* are the third and fourth most common bacterial causes of CAP requiring hospitalization. In addition to the difficulty in culturing the pathogens, specimens are easily contaminated with upper respiratory secretions, which may obscure the presence of lower respiratory pathogens.

The Infectious Disease Society of America/American Thoracic Society (IDSA/ATS) has recently published new consensus guidelines on the management of CAP in adults (6). Routine diagnostic tests are not recommended for ambulatory patients with CAP prior to empirical antimicrobial treatment because of the overall low yield and infrequent positive impact on clinical care. However, the new guidelines include a list of clinical indications for more extensive diagnostic testing for patients admitted to hospital with more severe disease when the result is likely to have the highest yield and where it may change individual antimicrobial management. Hospitalized patients should have pretreatment cultures of expectorated sputum (if there is a productive cough) and blood if the clinical indications outlined in Table 5 of the guidelines are met (6). However, more extensive testing for identifiable pathogens that cause CAP is indicated for patients with severe CAP, particularly those who are critically ill and ventilated in intensive care units (ICUs). The new IDSA/ATS guidelines also provide recommendations for the empirical therapy of CAP based on the severity of illness and the disease presentation.

The sputum Gram stain has variability in sensitivity and specificity, depending on the specimen and the skill of the reader (11, 14). A great variability and a lack of reproducibility on repeat smear preparations were shown by Nagendra et al. (11); however, there was less variability for culture of the specimens. Generally smears are only of value if there are numerous inflammatory cells and a preponderance of gram-positive diplococci (57% sensitivity) or gram-negative rods suggestive of *Haemophilus* (82% sensitivity) (14). The specificity of the diagnosis by Gram stain can be increased if the Quellung test is performed on the direct specimen. The proper evaluation of the specimen by Gram stain is critical and is used to ensure that only appropriate specimens are processed. Refer to Appendix 3.2.1–2 of the Gram stain procedure for acceptability criteria.

Nosocomial pneumonia can be caused by a large number of pathogens, including anaerobes, as in aspiration pneumonia (7). The CDC has issued guidelines for the prevention of nosocomial pneumonia (1). *Staphylococcus aureus* and gram-negative rods are common causes of ventilator-associated pneumonia (VAP), particularly in patients that have had a prolonged hospital course. Patients with severe CAP, immunocompromised patients with progressive pneumonia, or those with other types of nosocomial pneumonia (e.g., postoperative or medial hypostatic) may also develop respiratory failure and require ventilator support. Diagnosis of the microbial pathogens causing VAP requires pulmonologists or intensivists to perform a bronchoalveolar lavage (BAL) for collection of samples from the alveoli of the lung, including protected brush specimens (PBS) for anaerobe culture. Quantitative bacterial culture of BAL samples has a sensitivity of 82 to 91% provided that a threshold of $\geq 10^4$ CFU/ml for primary pathogen identification is used (8). BAL samples from immunocompromised patients may also be tested for *Pneumocystis jiroveci* (formerly *P. carinii*) pneumonia (PCP; now PJB), fungal culture, acid-fast bacillus (AFB) culture, *Legionella* culture, and respiratory viruses. Culture of endotracheal tube (e.g., bronchial secretions and

sputa) samples from ventilated patients may be clinically misleading due to colonization of the bronchus by organisms that have not been inoculated into the deeper lung. Primary bacterial pathogens of nosocomial pneumonia are usually resistant to one or more commonly used antimicrobial agents, so microbiology culture results are essential for directing appropriate therapy for this serious infection.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

◩ **NOTE:** Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

A. Collection of sputa, ET tube aspirates, and auger suction specimens

1. Expectorated sputum

a. Do *not* have the patient rinse mouth and gargle with nonsterile water prior to sputum collection, since this can introduce contaminating microbiota.

◩ **NOTE:** Commensal mycobacteria from tap water can contaminate mycobacterial cultures but are rarely an issue for routine bacteriology culturing. For specialized cultures (e.g., mycobacteria and legionellae), supply sterile saline or water to gargle prior to collection.

b. Instruct the patient not to expectorate saliva or postnasal discharge into the container.

c. Collect expectorated sputum specimens into a sterile container with a screw cap that is tightly secured prior to transport.

d. The specimen should have a label that provides the sample type (i.e., ET tube aspirate, auger suction versus expectorated or induced sputa), date and time of collection, and the same patient demographic information as that provided on the requisition.

2. Induced sputum

◩ **NOTE:** Induced sputa should only be tested for the presence of *Mycobacterium tuberculosis* and other *Mycobacterium* spp. (6). Although PJP (formerly PCP) testing may also be performed by request of the physician, this test should only be done on induced sputa from human immunodeficiency virus (HIV)-infected/AIDS patients. PJP sputum testing is not diagnostic for other types of immunocompromised patients in whom there is a much lower burden of cysts in the alveoli.

a. Using a wet toothbrush and *sterile* water or saline, brush the buccal mucosa, tongue, and gums for 5 to 10 min prior to the procedure. Do not use toothpaste.

b. Rinse the patient's mouth thoroughly with *sterile* water or saline.

c. Using an ultrasonic nebulizer, have the patient inhale approximately 20 to 30 ml of 3% NaCl.

d. Collect induced sputum specimens into a sterile container with a screw cap that is tightly secured prior to transport.

e. The specimen should have a label that provides the sample type (i.e., ET tube aspirate, auger suction versus expectorated or induced sputa), date and time of collection, and the same patient demographic information as that provided on the requisition.

3. ET tube aspirates and auger suction specimen

a. Aspirate the specimen into a sterile sputum trap (i.e., Luken trap) and aseptically transfer the sputum sample to a sterile screw-cap container with the cap tightly secured before transport. Luken traps frequently leak during transport, which contaminates the sample.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

b. Neonates, infants, and small children cannot provide an expectorated sputum sample, so a sample is collected from the upper airway using an auger. Frequently a very small amount of secretions or sputum is obtained by this method, so the sample may be submitted in the catheter in a sterile container.

c. The specimen should have a label that provides the sample type (i.e., ET tube aspirate, auger suction versus expectorated or induced sputa), date and time of collection, and the same patient demographic information as that provided on the requisition.

B. Collection of lower lung samples by bronchoscopy—performed by pulmonologists, intensivists, or other trained physicians. Major clinical contraindications to bronchoscopy include severe hypoxemia, severe hypocapnia, and thrombocytopenia or other coagulopathy due to the risk of bleeding into the lung.

1. Bronchoscopy specimens include BAL samples, bronchial washings, PBS, and transbronchial biopsy specimens.

2. BAL specimens and PBS may be cultured semiquantitatively or quantitatively for bacterial pathogens. BAL samples and PBS should be quantitatively cultured if they are from an ICU patient suspected of having severe pneumonia, particularly VAP (*see* Appendix 3.11.2–1 for quantitative methods).

3. Precautions

a. To avoid excess blood in the recovered fluid, obtain bronchial wash and BAL specimens before brushing or biopsy specimens. Blood may alter the concentration of cellular and noncellular components.

b. Avoid suctioning through the working channel before retrieval of specimens to avoid contamination of the specimens.

c. Avoid the injection of topical anesthetic agents as much as possible, as the injection method may lead to contamination of the specimen. Aerosol application of anesthetic agents is preferred.

4. Bronchoscopy procedures

◩ **NOTE:** There may be variability in the way individual physicians or groups of physicians perform a bronchoscopy and collect samples from various locations in the lung. A standard procedure should be developed by physicians collecting bronchoscopy samples in consultation with the laboratory so that an adequate amount of sample is collected for the tests ordered, and the fractions are clearly labeled according to their lung locations and order of collection.

a. Bronchial wash samples

◩ **NOTE:** Bronchial wash samples are collected from the major bronchi, including the main bronchus, at the bifurcation and the right and left bronchi depending on the extent of disease. This procedure is most commonly done to diagnose cancer in patients who have bronchial lesions or masses. Diagnostic tests for infection may be done concomitantly to rule out infection, particularly if there is bronchial obstruction and pneumonia secondary to this complication.

(1) Pass the bronchoscope transnasally or transorally in nonintubated patients or via the ET tube in intubated patients.

(2) Inject sterile nonbacteriostatic 0.85% NaCl (generally 20- to 30-ml aliquots in adults and 5-ml aliquots in children) from a syringe through a biopsy channel of the bronchoscope.

(3) Gently suction the recovered bronchial wash specimen into a sterile container (e.g., sterile suction trap on the bronchoscope). Due to the smaller volumes of fluid injected into the bronchus during collection of a bronchial wash sample, a smaller sample is received by the laboratory than during a BAL collection.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

(4) Bronchial wash samples are frequently split between the cytopathology and microbiology laboratories.
(5) Bronchial wash samples from different lung locations (e.g., right and left bronchi) should not be pooled.

b. Transbronchial biopsy samples

Lung biopsy samples may be obtained during bronchoscopy by inserting a forcep on the biopsy channel of the bronchoscope. Individual samples are immediately placed into a sterile container with nonbacteriostatic saline for microbiological testing. Additional samples may be placed into a sterile contained with formalin for histopathological testing, or into a sterile container with RPMI 1640 for flow cytometry analysis.

c. BAL

◩ **NOTE:** The laboratory will not be able to distinguish a BAL from a bronchial wash based on the appearance of the container or the fluid in the container. BAL samples are collected from the distal bronchioles and alveoli after the bronchoscope has been wedged into the distal airway lumen in one or more lung segments (e.g., RUL, RML, RLL, LUL, and LLL) depending on the extent of the infection.

(1) Pass the bronchoscope transnasally or transorally in nonintubated patients or via the ET tube in intubated patients.
(2) Collect BAL samples by carefully wedging the tip of the bronchoscope into an airway lumen and instilling as large a volume of 0.85% nonbacteriostatic saline as possible per aliquot (i.e., 25 to 30 ml for adults and 5 to 10 ml for children). Several aliquots may be used in the same lung location to wash the alveoli in order to collect enough fluid to perform all of the required tests. Each BAL sample returned from separate lavage of the area contains secretions distal to the bronchioles and alveoli.
(3) Gently suction the recovered specimen into a sterile container before administering the next aliquot. (In general, 50 to 75% of the saline instilled is recovered in the lavage effluent.) Keep aliquots separate during collection.
(4) Discard the initial fluid as contaminated and submit the rest for culture and staining.

◩ **NOTE:** In the laboratory, aliquots from the same site may be combined for microbiology cultures and smears, but aliquots from separate sites (for example, right upper lobe and right lower lobe) should be combined only after consultation with the physician of record.

d. PBS

(1) Instill a brush to collect cellular material from the airway wall. This is the best specimen for viral culture and cytology studies.
(2) Only a PBS is acceptable for anaerobic bacterial culture. Obtain by inserting a telescoping double catheter plugged with polyethylene glycol at the distal end (to prevent contamination of the bronchial brush) through the biopsy channel of the bronchoscope.
(3) Place the PBS in a sterile vial containing 1 ml of nonbacteriostatic saline.

5. Lung aspirates

Lung aspiration is performed by a pulmonologist or interventional radiologist. Pulmonary lesions or abscess cavities that are accessible to aspiration are located in the periphery of the lung. This procedure must be performed with caution since a pneumothorax and/or bronchocutaneous fistula may be created. The presence of thrombocytopenia or a coagulopathy are contraindications to the procedure.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

a. A computed tomography scan is used to direct the aspiration needle through the chest wall directly into peripheral lung lesion or abscess cavity.

b. Material is aspirated from the lesion or cavity.

c. Multiple samples may be collected if the lesion is large or there are multiple lesions or cavities that are peripherally accessible.

d. Fluid or pus obtained by aspiration should be immediately transferred to a sterile container and promptly transported to the laboratory. If anaerobic culture is required, a portion of the sample should also be placed into a sterile tube containing prereduced anaerobically sterilized media.

◩ **NOTE:** Syringes with the needle attached are not acceptable for culture due to the sharp and biohazard risk to laboratory staff. Syringes with a capped Luer-Lok are also not acceptable because the sample may be contaminated if the sample leaks during transport or during handling of the sample by the laboratory (e.g., the sample may congeal in the syringe during transport and be very difficult or impossible to retrieve without contamination).

6. OLB samples

Open lung biopsy (OLB) samples are collected by thoracic surgeons during an operative procedure. Patients undergoing this procedure must be clinically able to withstand an operative procedure with a minimal risk of bleeding.

a. One or more pieces of tissue are collected from one or more lung segments depending on the extent of the infection. OLB pieces of 0.5 to 1.0 cm square are harvested. Larger biopsy samples increase the risk of pulmonary hemorrhage.

b. Multiple OLB pieces may be collected from a large area of disease in a particular lung segment, or individual OLB pieces may be harvested from multiple lesions or diseased lung areas.

c. OLB pieces should be immediately placed into a sterile container with nonbacteriostatic saline for microbiological testing. Individual OLB pieces should be placed into a sterile container with formalin for histopathological analysis.

7. Pleural fluid—see procedure 3.5

C. Transport of sputa, endotracheal aspirates, and auger suction specimens

1. Since the samples are not placed into buffered transport media, it is important that they be received and handled by the laboratory as soon after collection as possible.

2. Store sputum specimens at 2 to 8°C until samples can be transported or processed by the laboratory.

◩ **NOTE:** A delay in processing of more than 2 h may result in a decreased ability to recover fastidious pathogens, such as *S. pneumoniae* and *H. influenzae*, and/or overgrowth of upper respiratory microbiota.

3. Sputum samples should be placed into biohazard bags prior to transport to minimize contamination of the external environment if leakage occurs during transport.

D. Transport of bronchoscopy specimens

1. Bronchial wash specimens, BAL specimens, PBS, lung aspirates, and OLB specimens should be immediately transported and handled by the laboratory as soon as possible after collection, since saline may be toxic to the recovery of some primary pathogens.

2. Store specimens at 2 to 8°C until samples can be transported or processed by the laboratory.

◩ **NOTE:** A delay in processing of more than 1 to 2 h may result in a decreased ability to recover fastidious pathogens, such as *S. pneumoniae* and *H. influenzae*, and/or overgrowth of upper respiratory microbiota.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

3. Bronchial wash specimens, BAL specimens, lung aspirates, and OLB specimens should be placed into biohazard bags prior to transport to minimize contamination of the external environment if leakage occurs during transport.
4. For bronchial wash, BAL, and OLB specimens, the microbiology laboratory should assess whether enough sample has been collected to perform all of the requested tests. Also ensure that samples have been sent to cytopathology and histopathology if ordered.
5. Bronchial wash, BAL, and OLB specimens are acceptable for testing for other pneumonia pathogens, including *Legionella*, viruses, fungi, and mycobacteria.
6. BAL and OLB specimens are also acceptable for testing for PJP and mycoplasmas.
7. If insufficient sample has been collected to perform all of the microbiology tests ordered on a bronchial wash, BAL, or OLB specimen, then the laboratory must contact the physician to establish the testing priority. Tests should then be done according to the priority list established with the physician (i.e., start with the highest-priority test and distribute the sample in descending order of clinical priority so that as many tests as possible for the amount of submitted sample are performed).

E. Rejection criteria for sputum and endotracheal aspirate specimens

1. Reject duplicate specimens received on the same day unless the initial sample was inadequate for culture according to microscopic evaluation.
2. Do not accept repeat cultures at intervals of less than every 48 h.
3. Reject the following specimens for diagnosis of lower respiratory tract disease.
   - **a.** 24-h sputum collection
   - **b.** Contaminated sputum and endotracheal specimens per Gram stain rejection criteria (Appendix 3.2.1–2)
   - **c.** Specimens that are visually saliva only
   - **d.** Specimens that are visibly contaminated by toothpaste or other substances
   - **e.** Nasal washes and aspirates or swabs of nares (*see* Table 3.11.1–1 and procedure 3.11.9 for appropriate specimens to diagnose sinusitis)
   - **f.** Throat specimens, since they are not diagnostic of lower respiratory tract infection (5)
   - **g.** Anaerobic culture should not be done of sputum samples because they are highly contaminated with normal anaerobic microbiota of the upper respiratory tract.

F. Rejection criteria for bronchial wash specimens, BAL specimens, lung aspirates, and OLB specimens

1. Bronchial wash specimens, BAL specimens, lung aspirates, and OLB specimens should never be rejected by the laboratory, since the patient has undergone an invasive procedure in order to have them collected.
2. For specimens delayed in transit more than 2 h without refrigeration, indicate on the report that the delay in transit may compromise the culture results.
3. Bronchial wash samples should not be cultured quantitatively; only a semiquantitative bacterial culture result is required.
4. BAL samples should be cultured quantitatively for bacterial pathogens from patients with suspected VAP.
5. PBS is the preferred sample for anaerobic culture, since both bronchial wash and BAL samples and transbronchial biopsy samples are contaminated by respiratory anaerobic microbiota.
6. Anaerobic culture should be performed on lung aspirates, pleural fluid, and OLB specimens by request or when the original specimen Gram stain demonstrates morphotypes suggestive of anaerobic infection.

III. MATERIALS

A. Direct tests
- **1.** Gram stain (procedure 3.2.1)
- **2.** *S. pneumoniae* antisera (stored at 4°C). See Quellung procedure (3.17.42).
  - **a.** Methylene blue—0.3% in water
  - **b.** Normal rabbit serum
- **3.** NOW urinary antigen assay (Binax, Inc., Portland, ME) (9, 10)

B. Primary media
- **1.** BAP
- **2.** CHOC or horse blood agar (HBA), or HBA with 20,000 IU of bacitracin per liter (HBAB)
- **3.** MAC or EMB

C. Identification methods

Refer to procedure 3.3.2 for supplies for identification of the common agents of pneumonia.

D. Other supplies/equipment
- **1.** Incubator at 35°C with 5% CO_2 or other CO_2-generating system
- **2.** Inoculating sticks and loops
- **3.** Petri dishes and filter paper
- **4.** Bacitracin (10-IU disk) and optochin disks (optional)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Verify that media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

B. Test each lot of CHOC according to procedure 3.3.1.

C. QC HBA with bacitracin as indicated below; without bacitracin, test only with *H. influenzae.* Incubate at 35°C in 5% CO_2.

Test organism	Result
Haemophilus influenzae ATCC 10211	Growth
Staphylococcus epidermidis ATCC 12228	Partial to complete inhibition

D. If used on direct specimen plates, perform QC on each lot or shipment of bacitracin (10-IU disk) and optochin disks. Use CHOC for bacitracin and BAP for optochin. See procedure 3.17.38 for optochin QC. For bacitracin QC, incubate CHOC at 35°C in CO_2.

◩ **NOTE:** This is *not* the bacitracin disk historically used to detect group A beta-hemolytic streptococci.

Test organism	Bacitracin (10 IU) zone size
Haemophilus influenzae ATCC 10211	No zone
Staphylococcus epidermidis ATCC 12228	>12 mm

V. PROCEDURE

It is imperative that these cultures be handled in a biological safety cabinet.

A. Processing and culture examination of sputa, auger suctions, and ET aspirate specimens
- **1.** Inoculation and incubation
  - **a.** Process all specimens as soon after receipt as possible in order to maintain the viability of the primary pathogen(s).
  - **b.** Select the most purulent or blood-tinged portion of the specimen to microscopically examine.
  - **c.** Perform a Gram stain of the specimen as soon after receipt as possible to microscopically assess cellular and bacterial components.
  - **d.** For sputum samples collected from adults and older children, if the specimen is inadequate for culture based on microscopic criteria (*see* procedure 3.2.1, including Appendix 3.2.1–2, for details about the preparation

V. PROCEDURE *(continued)*

and reading of smears), reject the sample and immediately phone the physician or nursing unit to collect another sample.

e. Perform a Gram stain on auger suction and endotracheal aspirates. Although these types of specimens are analyzed like sputa, microscopic criteria for specimen adequacy have not been established.

f. If there is a predominance of gram-positive diplococci, perform either a direct bile test (procedure 3.17.6) or a direct Quellung test (procedure 3.17.42).

g. Process microscopically adequate sputum, auger suction, and endotracheal aspirate specimens in a BSL 2 cabinet, since aerosols can result in laboratory-acquired infections.

h. Using a sterile swab, stick, or pipette, inoculate specimen to BAP, CHOC (or HBA or HBAB), and MAC (or EMB). The following biochemical tests may also be performed at inoculation.

(1) Add a 10-U bacitracin disk (not Taxo A) to CHOC and HBA to inhibit upper respiratory microbiota and improve detection of *H. influenzae*.

(2) Add staphylococcal spots using *S. aureus* ATCC 25923 to BAP to demonstrate satelliting of *Haemophilus* on direct plates.

(3) Add an optochin disk to the second quadrant of BAP to demonstrate inhibition by *S. pneumoniae* for direct detection on primary plates.

◩ **NOTE:** Adding an optochin disk is not recommended routinely but may be helpful for laboratories with a large number of specimens from patients with CAP.

i. Incubate plates at 35 to 37°C in 5% CO_2 for a minimum of 48 h; 72 h is preferred.

2. Culture examination (*see* procedure 3.3.2 for identification tests and procedure 3.17 for biochemical procedures; additional identification charts are in procedures 3.18.1 and 3.18.2)

a. Observe plates at 24 h.

b. Incubate plates for an additional 24 to 48 h, which is useful to detect slow-growing, fastidious gram-negative rods, such as *Bordetella* spp. or molds such as *Aspergillus* spp.

c. Even if there was growth at 24 h, plates should be examined again at 48 h in order to detect additional isolates not found at 24 h.

d. The original specimen Gram stain should be used as a guide to interpret the culture.

(1) Primary pathogen(s) grown in culture should be present in the original specimen Gram smear.

(2) If one or more isolates grow on culture that were not reported from the original Gram stain, then the smear should be read a second time by a different person than the one who initially read the slide.

(3) Follow Table 3.11.2–1 for reporting bacterial morphotypes from the Gram stain that may be primary pathogens.

e. Identify the organisms that grow in significant amounts, defined as bacterial morphotypes that are potential primary pathogens that may or not be part of the normal respiratory microbiota. Table 3.11.2–1 provides guidelines for the reporting of primary pathogens from these types of specimens. Significant growth is defined as follows.

(1) Moderate to heavy growth of an isolate in the second or greater quadrant of the plate

(2) Small amounts of a primary pathogen in the culture that are consistent with the predominant morphotype seen in the original specimen Gram stain associated with PMNs

Table 3.11.2–1 Guidelines for reporting of primary pathogens for lower respiratory cultures

Organism	Amount (semiquantitative)[a]	Amount (CFU/ml) (quantitative PBS/BAL)[b]	Action	Comments
May be part of normal microbiota				
Group A streptococcus, *S. pyogenes*	Any	Any	Identification/no AST	AST should be performed for the penicillin-allergic patient due to the increasing resistance to erythromycin and clindamycin; D-test should be done to detect inducible resistance.
S. pneumoniae	≤Normal microbiota[c]	$<10^3$	No identification/report normal microbiota	AST should be done to detect resistance to penicillin and erythromycin. Levaquin-resistant strains have also been reported.
	Moderate to heavy and >normal microbiota	$\geq10^3$–10^4	Identification/AST	
S. aureus	≤Normal microbiota	$<10^3$	Rule out MRSA[d]/report normal microbiota if no MRSA	MRSA should be ruled out. Increasing disease due to invasive community-acquired MRSA.
	Moderate to heavy and >normal microbiota	$\geq10^3$–10^4	Identification/AST	
M. catarrhalis	≤Normal microbiota	$<10^3$	No identification/report normal microbiota	AST should be performed if strain is beta-lactamase negative.
	Moderate to heavy and >normal microbiota	$\geq10^3$–10^4	Identification/AST	
H. influenzae	≤Normal microbiota	$<10^3$	No identification/report normal microbiota	Type in neonates, infants, and small children.
	Moderate to heavy and >normal microbiota	$\geq10^3$–10^4	Identification/AST/beta-lactamase	
N. meningitidis	≤Normal microbiota	$<10^3$	No identification/report normal microbiota	Type on request.
	Moderate to heavy and >normal microbiota	$\geq10^3$–10^4	Identification/AST/beta-lactamase	
Group B, C, and G streptococci	≤Normal microbiota	$<10^3$	No identification/report normal microbiota	AST should be performed for the penicillin-allergic patient due to the increasing resistance to erythromycin and clindamycin; D-test should be done to detect inducible resistance.
	Moderate to heavy and >normal microbiota	$\geq10^3$–10^4	Identification/no AST	
Other normal microbiota[c]	NA[e]	$<10^3$	No identification	Reporting of normal microbiota from lower respiratory cultures may lead to inappropriate antimicrobial treatment. Coagulase-negative staphylococci, enterococci, and other normal microbiota should only be reported from BAL or PBS cultures if there is a heavy amount or pure growth.
		$\geq10^3$–10^4	Identification/AST of organism present in pure growth	
Anaerobes	NA	PBS only $<10^3$	Limited identification to list anaerobes present	Only PBS are acceptable for anaerobe culture. Anaerobes are an important cause of aspiration or obstructive pneumonia.
		$\geq10^3$–10^4	Identification/AST	

(continued)

Table 3.11.2–1 Guidelines for reporting of primary pathogens for lower respiratory cultures *(continued)*

Organism	Amount (semiquantitative)[a]	Amount (CFU/ml) (quantitative PBS/BAL)[b]	Action	Comments
Bioterrorism agents				
Brucella spp.	Any	Any	Limited identification according to CDC algorithm. Cultures should be handled in a BSL 2 cabinet.	Immediate referral for further analysis (*see* section 16)
F. tularensis	Any	Any	Limited identification according to CDC algorithm. Cultures should be handled in a BSL 2 cabinet.	Immediate referral for further analysis (*see* section 16)
Y. pestis	Any	Any	Limited identification according to CDC algorithm. Cultures should be handled in a BSL 2 cabinet.	Immediate referral for further analysis (*see* section 16)
Bacillus anthracis	Any	Any	Limited identification according to CDC algorithm. Cultures should be handled in a BSL 2 cabinet.	Immediate referral for further analysis (*see* section 16)
Gram-negative bacilli				
Pasteurella spp.	Moderate to heavy and >normal microbiota	$<10^3$ $\geq10^3$–10^4	Identification/AST	Patients usually have contact with animals, particularly horses
Enterobacteriaceae	One or two types ≤Normal microbiota	$<10^3$	No identification/AST unless ICU and/or immunocompromised patient	*Klebsiella* pneumonia more common in alcoholics; colonization of the upper airway with enterics more common in patients in hospitals. AST should be done to rule out ESBL[f]-producing or *ampC*-carrying strains.
	Moderate to heavy and >normal microbiota	$\geq10^3$–10^4	Identification/AST	
P. aeruginosa	Any	Any	Identification/AST	*P. aeruginosa* pneumonia has a high mortality rate. AST should be done to rule out antimicrobial resistance, including screening for metallo-beta-lactamase-producing strains in hospitalized patients.
Other nonfermenters (*Bordetella bronchiseptica, Stenotrophomonas maltophilia, Acinetobacter* spp., *B. cepacia,* etc.)	≤Normal microbiota	$<10^3$	Identification/AST	*B. bronchiseptica* is an unusual cause of CAP. Other nonfermenters are important causes of nosocomial pneumonia. These organisms may also be transmitted in the hospital, and infection control should be notified about positive cultures. *B. cepacia* is mainly found in CF[g] patients (*see* procedure 3.11.3).
	Moderate to heavy and >normal microbiota	$\geq10^3$–10^4		

Table 3.11.2–1 *(continued)*

Organism	Amount (semiquantitative)[a]	Amount (CFU/ml) (quantitative PBS/BAL)[b]	Action	Comments
Mixed gram-negative bacilli, >2 morphotypes	≤Normal microbiota Moderate to heavy and >normal microbiota	$<10^3$ $\geq10^3$–10^4	No identification/AST Limited identification unless patient is in ICU or immunocompromised	List all types of gram-negative bacilli present (e.g., "Growth of mixed gram-negative bacilli, including 2 lactose fermenters and a non-lactose fermenter that is not *P. aeruginosa*"). Include a comment that the plates will be held if further analysis is clinically required.
Gram-positive bacilli				
Corynebacterium spp.	≤Normal microbiota Moderate to heavy and >normal microbiota pure growth	$<10^3$ $\geq10^3$–10^4	No identification/report normal microbiota Identification/AST	Urea-positive strains such as *C. pseudodiphtheriticum* may cause pneumonia.
R. equi	Any	Any	Identification/AST	Important cause of CAP in immunocompromised patients, particularly those with HIV/AIDS. Patients may have contact with horses.
Aerobic actinomycetes (*Nocardia* spp., *Streptomyces*)	Any	Any	Identification/AST	Important cause of pneumonia in immunocompromised patients
Yeasts				
Cryptococcus neoformans	Any	Any	Identification	May cause pneumonia in otherwise healthy and immunocompromised patients. Isolates should be subtyped to rule out *C. neoformans* subtype *gattii.*
Fungi				
Molds not considered saprophytic contaminants (e.g., *Aspergillus* spp., *Mucor* spp., agents of systemic mycoses)	Any	Any	Identification	Important causes of pneumonia in immunocompromised patients. CF patients may be colonized with *Aspergillus* spp. (*see* procedure 3.11.3).

[a] The original specimen Gram stain should be correlated with the bacterial morphotypes grown on culture. Significant growth of a primary pathogen is defined as moderate to heavy growth into the second or greater quadrant of the plate that is more than the background normal microbiota. Smaller amounts of a primary pathogen may be clinically important if it is an almost pure growth (i.e., little or no growth of normal microbiota) and the bacteria are consistent with the morphotype seen in the Gram stain associated with PMNs.

[b] Counts of $\geq10^3$ CFU/ml are considered significant for quantitative PBS cultures, and counts of $\geq10^4$ CFU/ml are considered significant for BAL specimens.

[c] Normal respiratory tract microbiota is defined as viridans group streptococci, commensal *Neisseria* spp., *N. meningitidis*, diphtheroids, coagulase-negative staphylococci, *Rothia*, group F streptococci, anaerobes, *Haemophilus* spp. (not *H. influenzae*), enterococci, *Candida* spp., *Eikenella, Actinobacillus, Capnocytophaga*, and *Moraxella*.

[d] MRSA, methicillin-resistant *S. aureus.*

[e] NA, not applicable.

[f] ESBL, extended-spectrum beta-lactamase.

[g] CF, cystic fibrosis.

V. PROCEDURE *(continued)*

(3) Colonies in the first quadrant of the plate provided there is little or no other normal respiratory microbiota on the plate (i.e., pure growth of a primary pathogen) and the Gram smear shows purulence (i.e., PMNs).

f. Subculture to BAP and/or CHOC to obtain isolated colonies for accurate identification from mixed cultures.

B. Processing and culture examination of bronchial wash and BAL specimens

1. Inoculation and incubation

a. Process all bronchial wash and BAL specimens immediately after receipt in order to maintain the viability of primary pathogen(s).

b. Process bronchial wash and BAL specimens in a BSL 2 cabinet, since aerosols can result in laboratory-acquired infections.

c. Perform a Gram stain of the specimen as soon after receipt as possible to microscopically assess cellular and bacterial components. See procedure 3.2.1, including Appendix 3.2.1–2, for details on preparation and reading of smears. Use a cytocentrifuge to prepare BAL specimens for Gram stain.

d. Bronchial wash specimens should be inoculated and incubated using the same methods as for sputum specimens (*see* items V.A.1.a to V.A.1.g). Semiquantitative bacterial culture results may be reported for primary pathogens.

e. There are two methods for performing quantitative cultures on BAL samples: the serial dilution method and the calibrated loop method (1, 6). After incubation, the colonies are counted and multiplied by the appropriate dilution factor to determine the number of bacteria present per milliliter of fluid (*see* Appendix 3.11.2–1).

f. Identify organisms present in significant amounts in quantitative BAL culture (defined as $\geq 10^4$ CFU/ml) and in quantitative PBS (defined as $\geq 10^3$ CFU/ml) of a primary pathogen. See Table 3.11.2–1 for guidelines for reporting primary pathogens from these types of specimens.

g. The original Gram stain result should be used to guide further analysis of BAL and PBS cultures. A primary pathogen isolated in smaller amounts as pure growth on the primary plates should also be identified if a consistent bacterial morphotype was found in the Gram stain.

C. Processing and culture examination of other invasive specimens from the lower respiratory tract

1. See procedure 3.13 for processing and bacterial culture of lung aspirates.

2. See procedure 3.13 for processing and bacterial culture of lung tissue (e.g., transbronchial biopsy and OLB specimens).

3. See procedure 3.5 for processing and bacterial culture of pleural fluid.

D. Identification of primary pathogens of lower respiratory tract infection

The following species are important respiratory pathogens. See Table 3.3.2–5 for rapid identification methods.

1. *Streptococcus* species

a. Examine for beta-hemolytic colonies and identify catalase-negative cocci in chains and pairs.

(1) Use pyrrolidonyl-β-naphthylamide (PYR) test to identify *S. pyogenes,* which is reported in *any* amount.

(2) Examine for colonies with a small zone of hemolysis and identify group B streptococcus in pediatric patients, if present, in any amount.

(3) Identify other beta-hemolytic streptococci in significant amounts only if they are predominant. Do not report small colony types of beta-hemolytic streptococci or group F streptococci, as they are part of the upper respiratory microbiota.

V. PROCEDURE *(continued)*

b. Examine alpha-hemolytic colonies for morphology consistent with *S. pneumoniae*. These will be inhibited by optochin.
(1) Add a drop of 10% bile to colonies that resemble pneumococci.
(2) If colony dissolves, report *S. pneumoniae.*
(3) Quickly pick similar colonies. Perform antimicrobial susceptibility testing (AST) (*see* section 5). Confirm purity with optochin disk to detect contamination of AST results.
(4) If colonies are not dissolved by bile, but still resemble pneumococci, confirm identification with optochin susceptibility.

◩ **NOTE:** Some pneumococci are bile resistant and others are resistant to optochin (12). No one test is 100% accurate, and the combination of these two tests will prevent erroneous reporting. The DNA probe (Gen-Probe) is another accurate method to confirm identification when the optochin zone is less than 14 mm.

2. Fastidious gram-negative rods (these microorganisms grow slowly or not at all on MAC). Perform spot tests on significant numbers to differentiate them from normal respiratory microbiota.
a. *H. influenzae* organisms are coccobacilli that grow on CHOC but not on BAP, except with staphylococci or other microorganisms to demonstrate satelliting. Perform ALA (aminolevulinic acid) test (procedure 3.17.3) to confirm identification and beta-lactamase test for penicillin susceptibility for *H. influenzae* isolates.
b. *Francisella tularensis* organisms are coccobacilli that can grow on CHOC but do not grow on BAP, even with staphylococci for satelliting. They are weakly catalase positive and oxidase and urease negative. They are beta-lactamase positive.
c. *Legionella* organisms are gram-negative rods that may grow on CHOC but will not grow on BAP, even with staphylococci for satelliting. They are motile and have a characteristic colony and Gram stain morphology (*see* procedure 3.11.4)
d. The major significant *Bordetella* organisms which grow on BAP are catalase and urease positive. They may be visible only after 48 h. See Table 3.11.6–1.
e. *Pasteurella* organisms are indole and oxidase positive and represent normal mouth microbiota of animals.
f. Identify *Yersinia pestis* in any amount. It presents as a non-lactose-fermenting rod on MAC or EMB, but it may appear as pinpoint colonies on BAP at 24 h of incubation.
g. Do not identify most other fastidious gram-negative rods, such as *Eikenella,* unless they are *predominant and present in large amounts,* since they are part of the normal upper respiratory microbiota and rarely cause respiratory disease.

3. Gram-negative diplococci
a. Examine colonies present in significant amounts that move when pushed. Confirm as *Moraxella catarrhalis.* Since more than 90% of *M. catarrhalis* organisms are beta-lactamase positive, testing is not helpful to treatment.
b. Examine CHOC for any oxidase-positive colonies that do not grow or grow poorly on BAP. Confirm identification of *Neisseria gonorrhoeae* and *N. meningitidis* (*see* Table 3.9.3–1). Do not perform AST.

4. Gram-negative rods that grow well on either MAC or EMB
a. Identify and perform AST on enteric gram-negative rods, particularly *Klebsiella pneumoniae,* if there is only one morphology in *significant amounts with no other pathogens in greater amounts.*

V. PROCEDURE *(continued)*

b. For inpatients, regardless of the presence of other pathogens, examine for significant numbers of *Pseudomonas aeruginosa*, *Acinetobacter*, *Burkholderia*, and *Stenotrophomonas* organisms because they are typically resistant to multiple antimicrobials and can be implicated in nosocomial epidemics.

c. If more than one type of other gram-negative rods are present in equal numbers, perform minimal testing to be able to describe the organisms (e.g., indole, oxidase, odor and morphology on MAC, colony pigment, and reaction on Kligler's iron agar [KIA] or triple sugar iron agar [TSI]).

d. See procedure 3.11.3 for special requirements for cystic fibrosis patients.

5. Staphylococci

a. Identify *S. aureus* if present in significant amounts.

(1) Perform AST if the Gram stain shows predominant cocci in clusters associated with WBCs and no other pathogen in significant amounts.

(2) If the patient is an inpatient, based on infection control policy, check for resistance to oxacillin, even if organism is present in low numbers.

b. Identify coagulase-negative staphylococci, without species identification or AST, only if they are in 90% pure culture. Otherwise include in respiratory microbiota.

6. Do not report *Enterococcus* unless the culture is 90% pure and the identification is confirmed with extensive biochemical tests. See procedure 3.18.1. Many gram-positive cocci in the normal respiratory tract are PYR positive and even bile-esculin and LAP (leucine aminopeptidase) positive.

7. Gram-positive rods

a. Rule out *Nocardia* in *any* amount and *Rhodococcus equi* (mucoid and urease positive) from immunocompromised patients (*see* procedures 6.1 and 6.2 and Table 3.18.1–6).

b. Examine for large, spore-forming, gram-positive rods. If present, identify *Bacillus anthracis* and *Bacillus cereus.*

c. Limit identification of *Corynebacterium,* using a commercial kit for gram-positive rods, to those present in *numerous and predominant* amounts and when either of the following is true.

(1) The organism is rapid urea positive. (*Corynebacterium pseudodiphtheriticum* is urea positive.)

(2) The specimen is from an intubated patient from an ICU.

d. Generally, do not pursue other gram-positive rods, as they are unlikely to cause pneumonia.

It is imperative that these cultures be handled in a biological safety cabinet.

8. Identify molds (section 8), unless the organism is consistent with a laboratory or environmental contaminant (e.g., *Penicillium*).

◩ **NOTE:** Biphasic fungi *(Histoplasma capsulatum* and *Coccidioides immitis)* can be isolated from plates held for >48 h, even as the yeast-phase colony morphology. Use caution in examination of older cultures.

9. Rule out *Cryptococcus* (section 8) and do not identify other yeasts further.

◩ **NOTE:** *Candida* organisms are not a cause of pneumonia, except possibly in oncology (e.g., leukemia) or lung transplant patients or in neonates. Even in those cases, growth of *Candida* in lower respiratory specimens, regardless of species, does not correlate with disease (4). Yeasts are normal inhabitants of the mouth.

10. If bacteria were seen on smear but did not grow on culture, extend the incubation and inoculate other media (e.g., for *Legionella*, *Bordetella pertussis,* and *Mycobacterium*).

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report the presence of cells and bacteria from Gram stains of lower respiratory specimens as outlined in procedure 3.2.1. If significant bacterial morphotypes are found in the Gram stain, which do not grow in culture, a comment about this discrepancy should be provided on the report.

B. Report "No growth of pathogens or normal upper respiratory tract microorganisms" if there is no growth on any plates.

◩ **NOTE:** This may be indicative of antimicrobial inhibition of normal microbiota.

C. Positive reporting

1. Report preliminary and final results as "Isolates consistent with microorganisms encountered in the upper respiratory tract" if no pathogens are isolated.

2. Report all pathogens and susceptibility tests performed using Table 3.11.2–1 as a guide.

3. Provide preliminary reports as indicated in procedure 3.3.2.

4. Report AST in accordance with CLSI guidelines (3) and section 5.

VII. INTERPRETATION

A. A positive culture with *S. pneumoniae* or *H. influenzae* generally indicates infection with that organism, although carriage of these organisms may lead to false-positive results.

B. A positive culture with a predominant gram-negative rod or *S. aureus* generally indicates infection with that agent if the smear suggests an infectious process involving the corresponding morphology.

C. A negative bacterial culture does not rule out lower respiratory infection. The primary pathogen is frequently not recovered from patients with pneumonia, either because they have already been started on antimicrobial therapy when samples are taken or because they have infection with another type of organism (e.g., virus, parasite, fungus, mycoplasma, or mycobacterium) that will not be recovered by bacterial culture.

D. Although published guidelines by several expert groups are controversial about the clinical utility of sputum bacterial cultures for the diagnosis of CAP (6), lower respiratory tract specimens should be collected and analyzed to determine the microbial cause of infection in patients with nosocomial pneumonia or VAP.

E. CAP guidelines recommend treatment of ambulatory patients with either amoxicillin or amoxicillin-clavulanate or a macrolide. A fluoroquinolone with enhanced activity against penicillin-resistant strains of *S. pneumoniae* (i.e., levofloxacin [Levaquin]) should be used in patients with CAP, particularly those with severe infection who require admission to a hospital (2, 6).

F. Bacterial isolates from patients with nosocomial pneumonia and VAP are more likely to have resistance to one of the more commonly used agents. Primary bacterial pathogens should have AST reported to direct appropriate therapy.

G. Immunocompromised patients with progressive pneumonia are more likely to have infection due to *Legionella* or a nonbacterial cause of infection. Lower respiratory tract specimens (i.e., BAL and/or OLB specimens) should be collected early in the course of the infection in order to optimize the recovery of unusual pneumonia pathogens, including *Legionella, P. jiroveci* (formerly *P. carinii*), viruses, fungi, mycoplasmas, and mycobacteria. Laboratories should refer to the specific sections of this handbook that provide detailed procedures for the analysis of clinical specimens to detect these pathogens.

VIII. LIMITATIONS

A. Some primary pathogens of pneumonia do not grow in routine bacterial culture. Laboratories should refer to the specific sections of this handbook that provide detailed procedures for the analysis of clinical specimens to detect these pathogens (e.g., *Legionella,* viruses, fungi, mycoplasmas, and mycobacteria).

B. False-negative bacterial cultures usually result from improper collection and/or delayed transport of lower respiratory specimens to the laboratory. Routine bacterial culture may also be negative if the specimens were collected after the patient was started on antimicrobial therapy.

C. False-positive results are usually caused by contamination of the specimen by normal respiratory flora and its subsequent growth on culture and overinterpretation by the laboratory. Contamination of a lower respiratory sample may also occur by leakage during transport.

REFERENCES

1. **Centers for Disease Control and Prevention.** 1997. Guidelines for prevention of nosocomial pneumonia. *MMWR Morb. Mortal. Wkly. Rep.* **46**(RR-1)**:**1–79.
2. **Chow, A. W., C. B. Hall, J. Klein, R. B. Kammer, R. D. Meyer, and J. S. Remington.** 1992. General guidelines for the evaluation of new anti-infective drugs for the treatment of respiratory tract infections. *Clin. Infect. Dis.* **15**(Suppl. 1)**:**S62–S88.
3. **Clinical and Laboratory Standards Institute.** 2008. *Performance Standards for Antimicrobial Susceptibility Testing.* Eighteenth informational supplement. Approved standard M100-S18. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Kontoyiannis, D. P., B. T. Reddy, H. A. Torres, M. Luna, R. E. Lewis, J. Tarrand, G. P. Bodey, and I. I. Raad.** 2002. Pulmonary candidiasis in patients with cancer: an autopsy study. *Clin. Infect. Dis.* **34:**400–403.
5. **Korppi, M., M. L. Katila, R. Kalliokoski, and M. Leinonen.** 1992. Pneumococcal finding in a sample from upper airways does not indicate pneumococcal infection of lower airways. *Scand. J. Infect. Dis.* **24:**445–451.
6. **Mandell, L. A., R. G. Wunderink, A. Anzueto, J. G. Bartlett, G. D. Campbell, N. C. Dean, S. F. Dowell, T. M. File, Jr., D. M. Musher, M. S. Niederman, A. Terres, and C. G. Whitney.** 2007. Infectious Disease Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. *Clin. Infect. Dis.* **44**(Suppl. 2)**:**S27–S72.
7. **Marrie, T. J.** 2000. Community-acquired pneumonia in the elderly. *Clin. Infect. Dis.* **31:**1066–1078.
8. **Mayhall, C. G.** 2001. Ventilator-associated pneumonia or not? Contemporary diagnosis. *Emerg. Infect. Dis.* **7:**200–204.
9. **Michelow, I. C., J. Lozano, K. Olsen, C. Goto, N. K. Rollins, F. Ghaffar, V. Rodriguez-Cerrato, M. Leinonen, and G. H. McCracken, Jr.** 2002. Diagnosis of *Streptococcus pneumoniae* lower respiratory infection in hospitalized children by culture, polymerase chain reaction, serological testing, and urinary antigen detection. *Clin. Infect. Dis.* **34:**E1–E11.
10. **Murdoch, D. R., R. T. Laing, G. D. Mills, N. C. Karalus, G. I. Town, S. Mirrett, and L. B. Reller.** 2001. Evaluation of a rapid immunochromatographic test for detection of *Streptococcus pneumoniae* antigen in urine samples from adults with community-acquired pneumonia. *J. Clin. Microbiol.* **39:**3495–3498.
11. **Nagendra, S., P. Bourbeau, S. Brecher, M. Dunne, M. LaRocco, and G. Doern.** 2001. Sampling variability in the microbiological evaluation of expectorated sputa and endotracheal aspirates. *J. Clin. Microbiol.* **39:**2344–2347.
12. **Pikis, A., J. M. Campos, W. J. Rodriguez, and J. M. Keith.** 2001. Optochin resistance in *Streptococcus pneumoniae:* mechanism, significance, and clinical implications. *J. Infect. Dis.* **184:**582–590.
13. **Principi, N., S. Esposito, F. Blasi, L. Allegra, and the Mowgli Study Group.** 2001. Role of *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* in children with community-acquired lower respiratory tract infections. *Clin. Infect. Dis.* **32:**1281–1289.
14. **Rosón, B., J. Carratalà, R. Verdaguer, J. Dorca, F. Manresa, and F. Gudiol.** 2000. Prospective study of the usefulness of sputum Gram stain in the initial approach to community-acquired pneumonia requiring hospitalization. *Clin. Infect. Dis.* **31:**869–874.

SUPPLEMENTAL READING

Carroll, K. C. 2002. Laboratory diagnosis of lower respiratory tract infections: controversy and conundrums. *J. Clin. Microbiol.* **40:**3115–3120.

Centers for Disease Control and Prevention. 2000. Preventing pneumococcal disease among infants and young children: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Morb. Mortal. Wkly. Rep.* **49**(RR-9)**:**1–35.

Sharp, S. E., A. Robinson, M. Saubolle, M. Santa Cruz, K. Carroll, and B. Baselski. 2003. *Cumitech 7B, Lower Respiratory Tract Infections.* Coordinating ed., S. E. Sharp. American Society for Microbiology, Washington, DC.

APPENDIX 3.11.2–1

Quantitative Culture of Protected Specimen Brush and Bronchoalveolar Lavage Fluid Specimens

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Quantitative cultures increase the specificity of the diagnosis, but the sensitivity is dependent on the threshold chosen for a positive result. Many authors report a colony count of $\geq 10^4$ CFU/ml to be consistent with bacterial pneumonia, whereas counts below 10^4 CFU/ml likely indicate contamination with oronasal microbiota (1, 2, 5–7). This theory has been validated in numerous clinical studies, and quantitative culture of BAL fluid specimens has become the reference method for the diagnosis of pneumonia by BAL (3). For PBS, a colony count of $\geq 10^3$ CFU/ml is consistent with bacterial pneumonia. Taking the dilution factor of both specimen effluents into consideration, this represents a count of bacteria in the secretions of the lung of 10^5 to 10^6 CFU/ml (2).

There are two methods of performing quantitative cultures: the serial dilution method and the calibrated-loop method (1, 2). After incubation the colonies are counted and multiplied by the appropriate dilution factor to determine the number of bacteria present in 1 ml of fluid.

Observe standard precautions.

II. MATERIALS

A. Primary media
   1. BAP *or*
   2. CHOC

B. Other supplies/equipment
   1. Pipette method
      a. One 100-μl pipettor with sterile tips
      b. Two 16- by 125-mm snap-cap tubes containing 5 ml of sterile phosphate-buffered saline or suitable broth
   2. Loop method
      a. 0.001-ml (1-μl)
      b. 0.01-ml (10-μl) loop
      c. Disposable loops are color coded according to volume delivery.
   3. Sterile bent glass or plastic disposable sterile rods (hockey sticks) to spread inoculum (Excel Scientific, Wrightwood, CA, [760] 249-6371).
   4. Petri dish turntable to rotate plates (optional)
   5. Incubator at 35°C with 5% CO_2 or other CO_2-generating system

ANALYTICAL CONSIDERATIONS

III. QUALITY CONTROL

A. For calibrated-pipette QC, refer to Appendix 3.12–4. If calibrated loops are used, verify that they are accurate by one of the methods in Appendix 3.12–3.

◩ **NOTE:** Jacobs et al. (4) have indicated that actual BAL fluid should be used to validate the accuracy of the loops. Some, but not all, loops provide accurate results.

B. Refer to procedure 3.11.2 for medium QC testing.

It is imperative that these cultures be handled in a biological safety cabinet.

IV. PROCEDURES

A. Inoculation (*see* Fig. 3.11.2–A1).
   1. Vortex specimen vial vigorously for 30 to 60 s.
   2. Label a BAP or CHOC for each dilution. Do not use selective medium. If CHOC contains bacitracin, use BAP. Otherwise use CHOC for quantitative culture. Inoculate other media according to procedure 3.11.2 for other lower respiratory tract pathogens.

APPENDIX 3.11.2–1 *(continued)*

3. Pipette method
   a. Transfer 100 µl of PBS to each plate marked "× 10." Each colony from this plate = 10 CFU/ml.
   b. Transfer 10 µl of PBS or BAL specimen to each plate marked "× 100." Each colony from this plate = 100 CFU/ml.
   c. For BAL (and PBS, optional), do the following.
      (1) Label one tube of 5 ml of phosphate-buffered saline "1:100."
      (2) Transfer 50 µl of "undiluted" fluid from the vial to the tube labeled "1:100." Vortex.
      (3) Transfer 100 µl of "1:100" dilution to each plate marked "× 10^3." Each colony from this plate = 10^3 CFU/ml.
   d. For BAL fluid only, do the following.
      (1) Label one tube of 5 ml of phosphate-buffered saline "1:10,000."
      (2) Vortex.
      (3) Transfer 50 µl of "1:100" dilution to the tube labeled "1:10,000."
      (4) Transfer 100 µl of the "1:10,000" dilution to each plate marked "× 10^5." Each colony from this plate = 10^5 CFU/ml.
   e. *Spread the inoculum over the surface of each plate evenly by using a sterile glass or disposable plastic rod.*
      (1) Place plate on spinning turntable for even greater distribution of colonies.
      (2) Begin with the most dilute inoculum and proceed to the least dilute using the same sterile rod for each plate.
4. Loop method
   a. PBS
      (1) Vortex for 30 to 60 s.
      (2) Place 100 µl (2 drops) of specimen onto each plate marked "× 10." Each colony from this plate = 10 CFU/ml.
      (3) Using a 1:100 loop, place 10 µl on a plate labeled "× 100." Each colony from this plate = 100 CFU/ml.
   b. BAL fluid
      (1) Vortex for 30 to 60 s.
      (2) Using a 1:100 loop, place 10 µl on a plate labeled "× 100." Each colony from this plate = 100 CFU/ml.
      (3) Using a 1:1,000 loop, place 1 µl on a plate labeled "× 1,000." Each colony from this plate = 1,000 CFU/ml.
   c. *Spread the inoculum over the surface of each plate evenly by using a sterile glass or disposable plastic rod.*

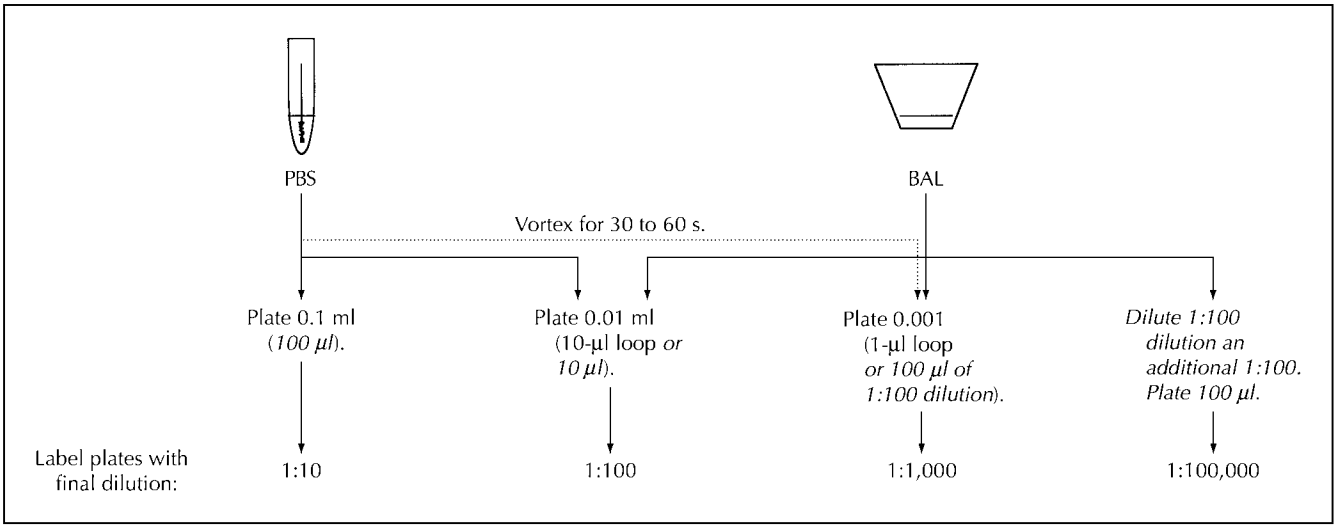


Figure 3.11.2–A1 Quantitative culture methods. Serial dilution method is in italics. Dashed line is optional. Adapted from reference 2.

APPENDIX 3.11.2–1 *(continued)*

(1) Place plate on spinning turntable for even greater distribution of colonies.
(2) Begin with the most dilute inoculum and proceed to the least dilute using the same sterile rod for each plate.
(3) Alternatively, spread the inoculum in only half the plate and use the second half to streak in two quadrants for isolation of large numbers of microorganisms.

B. Incubate the aerobic plates in a 5 to 10% CO_2, 35°C incubator.
C. Inoculate each additional medium with 100 µl of specimen (2 drops) and streak for isolated colonies, according to procedure 3.11.2 or procedure 3.11.3.
D. Prepare a Gram stain from the BAL sample, using the cytocentrifuge.
E. Centrifuge the rest of the BAL specimen for culture for viruses, mycobacteria, *Legionella,* and fungi and for smears for viruses, *Pneumocystis,* AFB, and fungi, on request. Alternatively, use the unprotected brush as the best specimen for smears, if it is available.

POSTANALYTICAL CONSIDERATIONS

V. INTERPRETATION

A. Count the colonies from the dilution with the greatest number of colonies without confluence. Multiply by the dilution factor listed for each dilution (e.g., 40 colonies on the "× 1,000" plate is 40×10^3 CFU or 4×10^4 CFU).
B. Count each morphotype individually.
C. If growth is too numerous to count on the highest dilution, report the count as greater than that dilution.
D. Counts for BAL fluid of less than 10^4 are considered to indicate contamination.
E. Counts for PBS of less than 10^3 are considered to indicate contamination.

VI. REPORTING RESULTS

A. Gram stain—report smear as indicated in procedure 3.2.1. Any organism seen in a cytocentrifuged smear of BAL fluid is considered indicative of bacterial pneumonia (6).
B. Quantitative culture: report the amount of each primary pathogen found as CFU per milliliter of specimen. If the culture count is lower than the threshold, identification and AST are not routinely performed unless there is a pure growth of a primary pathogen.
C. *Candida* species are most often contaminants of the procedure and generally should not be identified to the species level.

VII. LIMITATIONS

A. Counts near the thresholds may be difficult to interpret accurately.
B. Not all pathogens will grow on the media used and may result in a false negative culture.
C. Anaerobes are implicated in disease, especially in the elderly or those obtunded with aspiration pneumonia. Cultures are generally not cost-effective or helpful because they vary in sensitivity from 20 to 60%, possibly due to aeration during collection and dilution of the specimen.
D. Loop cultures are not as accurate as pipette cultures, but both are quite sensitive provided that a rod is used to spread the colonies on the plate. Spreading with the loop is *not* as sensitive a method and will underestimate the quantity of organisms present.
E. Even a single dose of antimicrobial therapy prior to specimen collection can negate the ability to isolate the causative agent of disease from the specimen.

APPENDIX 3.11.2–1 *(continued)*

References

1. **Baselski, V. S., M. El-Torky, J. J. Coalson, and J. P. Griffin.** 1992. The standardization of criteria for processing and interpreting laboratory specimens in patients with suspected ventilator-associated pneumonia. *Chest* **102:**571S–579S.
2. **Baselski, V. S., and R. G. Wunderink.** 1994. Bronchoscopic diagnosis of pneumonia. *Clin. Microbiol. Rev.* **7:**533–558.
3. **Ewig, S.** 1996. Diagnosis of ventilator-associated pneumonia: nonroutine tools for routine practice. *Eur. Respir. J.* **9:**1339–1341.
4. **Jacobs, J. A., E. I. G. B. De Brauwer, E. I. M. Cornelissen, and M. Drent.** 2000. Accuracy and precision of quantitative calibrated loops in transfer of bronchoalveolar lavage fluid. *J. Clin. Microbiol.* **38:**2117–2121.
5. **Kahn, F. W., and J. M. Jones.** 1988. Analysis of bronchoalveolar lavage specimens from immunocompromised patients with a protocol applicable in the microbiology laboratory. *J. Clin. Microbiol.* **26:**1150–1155.
6. **Thorpe, J. E., R. P. Baughman, P. T. Frame, T. A. Wesseler, and J. L. Staneck.** 1987. Bronchoalveolar lavage for diagnosing acute bacterial pneumonia. *J. Infect. Dis.* **155:**855–861.
7. **Torres, A., J. Puig de la Bellacasa, A. Xaubet, J. Gonzalez, R. Rodriguez-Roisin, M. T. Jimenez de Anta, and A. Agusti Vidal.** 1989. Diagnostic value of quantitative cultures of bronchoalveolar lavage and telescoping plugged catheters in mechanically ventilated patients with bacterial pneumonia. *Am. Rev. Respir. Dis.* **140:**306–310.

3.11.3 Respiratory Cultures from Cystic Fibrosis Patients

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Chronic lung infection is responsible for 75 to 85% of deaths in patients with cystic fibrosis (CF) (6, 8). Life expectancy has increased steadily over the past 50 years in large part because of improvements in management of lung disease in this patient population. The purpose of the CF culture is to provide a method for culturing respiratory secretions from patients with CF in order to isolate organisms associated with pulmonary disease in these patients. The number of microbial species associated with CF lung disease is relatively limited. Emphasis is placed on the recovery of those organisms, including mucoid and nonmucoid *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Burkholderia cepacia* complex (which includes *B. cepacia* genomovars I, III, and VI; *Burkholderia multivorans; Burkholderia stabilis; Burkholderia vietnamiensis; Burkholderia ambifaria; Burkholderia anthina;* and *Burkholderia pyrrocinia*), *Stenotrophomonas maltophilia* and other non-glucose-fermenting, gram-negative rods, and *Haemophilus influenzae* and *Streptococcus pneumoniae* (6, 8, 12, 14, 17). The vast majority of *B. cepacia* complex isolates that will be recovered from CF patients will be *B. cepacia* genomovar III, *B. multivorans*, and *B. vietnamiensis* (Table 3.11.3–1) (10). Nontuberculous mycobacteria and *Aspergillus* spp. may also play a role in CF lung disease and should be actively sought at the caregivers' request in these patients (8). Common respiratory viruses such as respiratory syncytial virus in children and influenza virus in all patients may also cause respiratory symptoms and should be considered during periods of disease activity in the community (8). In selected centers, lung transplantation is performed in CF patients. Special culture needs of this patient population should be applied to specimens obtained from lung recipients with CF disease since they continue to harbor the same types of organisms as they did pretransplantation (18). Patients with CF may be colonized or chronically infected with microbiota which may change little over a long period of time; therefore, an approach is adopted which is designed to maximize service to the patient while minimizing duplication in the laboratory and assisting in cost containment.

Table 3.11.3–1 Characteristics of *B. cepacia* complex and related organisms[a]

Test	*B. cepacia* genomovars I and III	*B. multivorans*	*B. stabilis*	*B. vietnamiensis*	*B. gladioli*	*Pandoraea*	*R. pickettii*
Oxidase	+[b]	+	+	+	−	−(+)[d]	+
Oxidation of:							
Glucose	+	+	+	+	+	− (+)	+
Maltose	(+)[c]	+	+	+	−	−	+
Lactose	+	+	+	+	−	−	+
Xylose	+	+	+	+	+	−	+
Sucrose	+	−	(+)	+	−	−	−
Adonitol	+	+	+	−	+	−	−
Lysine decarboxylase	+	(+)	+	+	−	−	−
Ornithine decarboxylase	(+)	−	+	−	−	−	−
ONPG	+	+	−	+	+	−	−

[a] Data are from reference 12; all isolates are colistin or polymyxin B resistant.

[b] +, >75% of isolates gave a positive result.

[c] (+), 20 to 75% of isolates gave a positive result.

[d] − (+), <20% of isolates gave a positive result.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. Collect the following specimens for culture of respiratory secretions from CF patients.

a. Deep pharyngeal (also referred to as "cough or gagged" throat specimens)

(1) Place a plastic shaft, Dacron- or rayon-tipped swab in the back of the throat and induce coughing.

(2) Remove the swab when coughed secretions have been collected.

◩ **NOTE:** This technique is used in children, usually <10 years of age, who are unable to produce sputum. *Do not use* this technique in children who are able to produce sputum.

b. Sputum

c. Endotracheal aspirates (on ventilated patients)

d. Bronchoscopically obtained specimens, including bronchoalveolar lavage (BAL) specimens, protected specimen brushings, and transbronchial biopsy specimens. See quantitative culture method (Appendix 3.11.2–1).

2. Use the same transport devices for these specimens obtained from CF patients that would be used for non-CF patients.

3. If the specimen cannot be transported and processed within 4 h, it may be held at 4°C for up to 24 h without affecting the recovery of the major pathogens of interest in CF patients.

B. In addition to specimen labeling which meets good laboratory practices, identify the specimen as being obtained from a "cystic fibrosis patient" to allow appropriate specimen processing and workup.

C. Rejection criteria

1. Since bronchoscopic specimens are obtained at significant expense and some degree of risk to the patient, all attempts should be made to process these specimens, even if they are compromised. However, on the final reports, note that specimen quality may have been compromised.

2. Process no more than one specimen per month from nontransplant CF patients who are outpatients without physician or other caregiver consultation.

3. Process no more than two specimens per admission for nontransplant CF patients who are inpatients without physician or caregiver consultation.

4. If a swab is received labeled as a sputum, contact caregiver prior to processing, to determine source of specimen. It is likely a deep pharyngeal specimen.

◩ **NOTE:** The microbiota responsible for chronic lung disease in CF patients is very stable, with patients being infected with organisms such as *S. aureus* or *P. aeruginosa* for months to years.

5. No guidelines currently exist for frequency of culture from CF lung transplant recipients. Process specimens by request.

6. Do not use culture rejection criteria for sputum or endotracheal aspirates based on Gram stain quality (Appendix 3.2.1–2), since they are of little value in CF patients. Specimens from CF patients will grow potential pathogens >90% of the time, although approximately 40% would be rejected based on the Gram stain evaluation (15).

III. MATERIALS

A. Media for culture

1. CHOC or horse blood agar with or without 20,000 U of bacitracin per liter (6) for recovery of *H. influenzae* (Remel, Inc.; BD Diagnostic Systems)

◩ **NOTE:** The addition of bacitracin will inhibit most strains of streptococci, staphylococci, and *Neisseria* and *Micrococcus* species, but *Escherichia coli,* some *Neisseria* species, and strains of *Candida, Klebsiella, Proteus,* and *Pseudomonas* spp. as well as other bacteria may grow on the medium.

2. Mannitol salt agar (MSA) for recovery of *S. aureus*

3. MAC

4. Columbia colistin-nalidixic acid agar (CNA)

5. *B. cepacia* selective agar (BCSA) (Remel, Inc.; Hardy Diagnostics) (11)

◩ **NOTE:** This medium contains lactose and sucrose with phenol red and crystal violet to differentiate colonies that oxidize these carbohydrates from those that do not, as well as polymyxin B, gentamicin, and vancomycin to inhibit most other bacteria.

B. Biochemical tests

1. See procedures 3.3.2 and 3.18.2 for standard laboratory tests and flowcharts helpful in identification of organisms recovered from CF patients.

2. For nonfermenting, gram-negative rods, use a commercial kit designed to identify them (e.g., Vitek GNI, GNI Plus, or API 20NE [bioMérieux, Inc.]; RapID NF Plus or N/F System [Remel, Inc.]; Microscan GNP [Dade-Behring Microscan]; Crystal E/NF [BD Diagnostic Systems]). Refer to procedure 3.1 for vendor contact information.

3. In addition, the following tests are helpful.

a. Lysine and ornithine decarboxylase (procedure 3.17.15)

b. *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) (procedure 3.17.37)

c. OF sugars (glucose, lactose, maltose, mannitol, adonitol, sucrose, and xylose [procedure 3.17.9])

d. Colistin or polymyxin B disks (procedure 3.17.4)

e. DNase (procedure 3.17.16)

4. Etests (AB Biodisk, Solna, Sweden) for trimethoprim-sulfamethoxazole, ticarcillin-clavulanic acid, and ceftazidime for susceptibility testing of *S. maltophilia*

5. Antimicrobial disks for susceptibility testing (procedure 5.1) or microdilution MIC method with overnight incubation (procedure 5.2)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that media meet expiration date and QC parameters per Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22-A3 (4). See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

B. QC the following media by lot when received.

1. *Haemophilus* media with bacitracin (*see* procedure 3.11.2 for details)

2. CHOC (*see* procedure 3.2.1 for details)

3. BCSA

Test organism	Incubation			Result
	Time (h)	Temp (°C)	Atmosphere	
B. cepacia ATCC 25416	24–72	35	Aerobic	Growth
P. aeruginosa ATCC 27853	24	35	Aerobic	Partial to complete inhibition
S. aureus ATCC 25923	24	35	Aerobic	Partial to complete inhibition

IV. QUALITY CONTROL *(continued)*

C. Perform standard QC for reagents, commercial identification systems, and antimicrobial susceptibility testing according to package inserts and CLSI documents (*see* sections 5 and 14).

V. PROCEDURE

Observe standard precautions.

A. Microscopic examination

1. Perform Gram stain on bronchoscopically obtained specimens and report results according to procedure 3.2.1.
2. Perform Gram stains on deep pharyngeal, sputum, and endotracheal aspirates by request only.

   ◩ **NOTE:** Screening for specimen quality is inappropriate since limited organisms are associated with CF lung disease and their presence on culture is considered clinically significant regardless of Gram stain findings. See Table 3.11.3–2 for further details.
3. Perform acid-fast bacillus stains on request for sputum, endotracheal aspirates, and bronchoscopically obtained specimens from CF patients.
4. Perform fungal stains on request from sputum, endotracheal aspirates, and bronchoscopically obtained specimens from CF patients.

Table 3.11.3–2 Processing of organisms

Organism	Oropharyngeal microbiota component	Identify	Special processing
Mold	NA[a]	Any amount	Identify *Aspergillus* spp. and other fungi to the species level on an annual basis.
Enterobacteriaceae	Not predominant or pharyngeal site	Predominant	Perform susceptibility testing.
S. aureus	NA	Any amount	Perform complete susceptibility testing yearly; perform oxacillin screening on each culture.
H. influenzae	Not predominant or pharyngeal site	Predominant	Perform beta-lactamase test. If colony is mucoid or wet, consult the lab director regarding possibly sending out for serotyping.
S. pneumoniae	Not predominant or pharyngeal site	Predominant	Screen for penicillin resistance with oxacillin disk. Perform penicillin Etest upon request.
P. aeruginosa, *B. cepacia*, other nonglucose-fermenting, gram-negative rods	NA	Any amount	Perform susceptibility testing.
Rapidly growing mycobacterium	NA	Any amount	Identify to species level.

[a] NA, not applicable.

V. PROCEDURE *(continued)*

B. Culture methods

1. Inoculate each of the five media listed above and incubate CHOC or horse blood agar and CNA in 5% CO_2 at 35°C.
- **a.** Incubate MAC, BCSA, and MSA without CO_2 at 35°C.
- **b.** Inoculate the CHOC or horse blood agar quantitatively, if from bronchoscopy, according to Appendix 3.11.2–1.
- **c.** For deep throat specimens, omit the CNA and CHOC or horse blood agar, since the specimen will only be examined for *P. aeruginosa, B. cepacia,* and *S. aureus.*

2. Because of the high rate of *P. aeruginosa* contamination, decontaminate all CF specimens in which the detection of acid-fast bacilli is requested using *N*-acetyl-L-cysteine–sodium hydroxide decontamination followed by 5% oxalic acid decontamination before inoculating standard mycobacterium isolation medium (19). See section 7 for general procedures.

3. Examine all plates at 24, 48, and 72 h of incubation. Examine BCSA plates also at 96 h for growth.
- **a.** BCSA medium—*B. cepacia* complex will appear as small to large, pink, yellow, or metallic colonies. The medium surrounding the colonies will change from red to yellow. Proceed with identification from growth as indicated in Fig. 3.11.3–1.
- **b.** MSA—for isolation of *S. aureus,* which will ferment the mannitol and produce a yellow zone around the colony
  - (1) Multiple strains of *S. aureus* may be present in one specimen; be alert to subtle differences in colonial morphology.
  - (2) In particular, thymidine-dependent strains of *S. aureus* may arise in patients treated with long-term trimethoprim-sulfamethoxazole; these strains frequently present with colonies which are somewhat smaller, flatter, and grayer than the parent strain (9).
  - (3) Pay close attention to colonial morphology of staphylococci on the CNA and horse blood agar or CHOC as well as the MSA. Strains of *S. aureus* which fail to ferment mannitol, or which do so only after many days of incubation, have been recovered from CF patients.
- **c.** MAC—identify lactose-positive organisms according to the criteria in Table 3.11.3–2 and the methods in procedure 3.18.2. Subculture various morphotypes of non-lactose-fermenting, gram-negative rods from their primary plate to BAP. This will enable evaluation of their probable species morphology based on the following general guidelines.
  - (1) *P. aeruginosa:* beta-hemolytic, metallic, grape odor or mucoid colony morphology, oxidase positive, and indole negative. Both mucoid and nonmucoid phenotypes of *P. aeruginosa* should be identified using odor and colistin susceptibility. Perform further identification using commercial kits or biochemical tests, if odor is lacking. Refer to procedures 3.3.2 and 3.18.2.
  - (2) *S. maltophilia:* non-hemolytic, slight yellow pigment, and oxidase negative, at least initially.

    ◩ **NOTE:** *S. maltophilia* isolates have been misidentified as *B. cepacia,* especially strains which are colistin resistant. *S. maltophilia* isolates growing on BCSA are colistin resistant. Key biochemical characteristics of *S. maltophilia* are an *inability* to oxidize mannitol, strong oxidation of maltose, and a positive reaction on DNase agar after a full 72 h of incubation. Unknown isolates should be considered DNase negative only after 72 h of incubation (7, 14).
  - (3) *Alcaligenes* and *Achromobacter* spp.: nonhemolytic, small colony type, oxidase positive, and not grape-like or metallic smelling; may be sweet smelling.

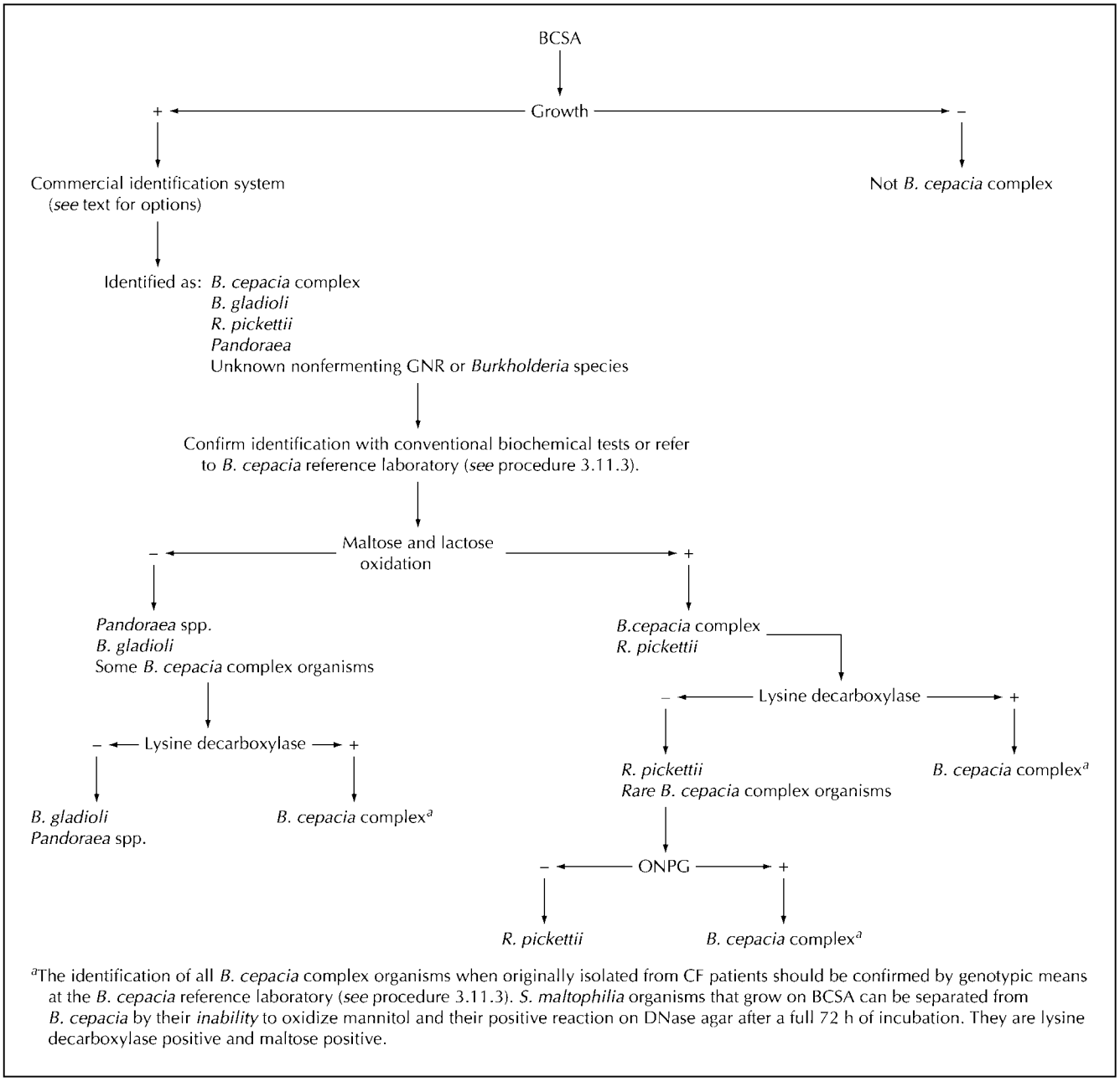

BCSA
Growth
+
–
Commercial identification system
(see text for options)
Not B. cepacia complex
Identified as: B. cepacia complex
B. gladioli
R. pickettii
Pandoraea
Unknown nonfermenting GNR or Burkholderia species
Confirm identification with conventional biochemical tests or refer to B. cepacia reference laboratory (see procedure 3.11.3).
Maltose and lactose oxidation
–
+
Pandoraea spp.
B. gladioli
Some B. cepacia complex organisms
B.cepacia complex
R. pickettii
Lysine decarboxylase
– Lysine decarboxylase +
B. gladioli
Pandoraea spp.
B. cepacia complex[a]
R. pickettii
Rare B. cepacia complex organisms
B. cepacia complex[a]
ONPG
– ONPG +
R. pickettii
B. cepacia complex[a]
[a]The identification of all B. cepacia complex organisms when originally isolated from CF patients should be confirmed by genotypic means at the B. cepacia reference laboratory (see procedure 3.11.3). S. maltophilia organisms that grow on BCSA can be separated from B. cepacia by their inability to oxidize mannitol and their positive reaction on DNase agar after a full 72 h of incubation. They are lysine decarboxylase positive and maltose positive.

Figure 3.11.3–1 Flowchart for the identification of *B. cepacia* complex. GNR, gram-negative rod.

V. PROCEDURE *(continued)*

(4) *B. cepacia* complex and related organisms: nonhemolytic, foul odor, oxidase variable. For details on identification of *B. cepacia* complex organisms to the species level by conventional methods, refer to Table 3.11.3–1.

◩ **NOTE:** Confirm the identity of any organism from a CF patient identified by a commercial system as *B. cepacia*, as a *B. cepacia*-like organism, including a *Burkholderia* sp., *B. gladioli*, a *Pandoraea* sp., and *Ralstonia pickettii*, or as an unidentified gram-negative rod by genotypic means (1, 5, 12, 13). The Cystic Fibrosis Foundation has

V. PROCEDURE *(continued)*

established a reference laboratory at the University of Michigan for this purpose. Call (734) 936-9769 or e-mail Jlipuma@umich.edu for details on submission of isolates.

d. Horse blood agar or CHOC—identify *Haemophilus*-like colonies from this medium by following criteria in Table 3.11.3–2 and methods in procedure 3.3.2.

e. CNA medium—identify *S. pneumoniae* isolates from this medium by following criteria in Table 3.11.3–2 and methods in procedure 3.3.2.

f. Molds may grow on BCSA, CNA medium, CHOC, or horse blood agar. Identify these organism by following criteria in Table 3.11.3–2 on an annual basis.

g. Rapidly growing mycobacteria may grow on CHOC/horse blood agar or CNA medium; identify if typical colony morphology is observed.

4. Further workup of positive cultures should follow guidelines in Table 3.11.3–2.

a. For quantitative cultures, identify all organisms and do susceptibility testing for all organisms listed in Table 3.11.3–2 which are found at concentrations of $>10^3$ CFU/ml in protected specimen brushings and $>10^4$ CFU/ml in BAL samples. If multiple potential pathogens (≥ 3) are found above the quantitative cutoff, consult with the physician or caregiver concerning further workup of the specimen.

b. Susceptibility testing guidelines

(1) *S. aureus:* complete susceptibility testing of *S. aureus* isolates should be done once a year at a minimum and on a more frequent basis as requested by the physician. Individual isolates should be screened for oxacillin resistance (procedure 13.17). Isolates should also be confirmed as either oxacillin resistant or susceptible (procedure 5.4).

(2) *H. influenzae:* perform beta-lactamase screening.

(3) *P. aeruginosa:* CF patients are colonized and may develop infection from multiple morphotypes of this organism. Each mucoid and nonmucoid morphotype should be separately identified and tested either by disk diffusion, Etest, or microdilution MIC determination (2). Patient isolates should be subjected to AST no more than once a month for outpatients, and weekly during acute respiratory exacerbations requiring hospital admission. Automated susceptibility test systems with an incubation time of less than 24 h should not be used for testing *P. aeruginosa* isolates from CF patients.

◩ **NOTE:** The laboratory should keep track of the different morphotypes of *P. aeruginosa* isolated from individual CF patients in order to recognize when new morphotypes that may be more resistant to antimicrobial agents emerge.

(4) *S. maltophilia:* test for MIC by either broth microdilution or Etest (6). The Etest panel can be limited to trimethoprim-sulfamethoxazole, ticarcillin-clavulanic acid, and ceftazidime.

◩ **NOTE:** Reliable disk diffusion breakpoints to trimethoprim-sulfamethoxazole, minocycline, and levofloxacin are available in CLSI document M100-S18 (3).

(5) *B. cepacia* complex, *Alcaligenes*, and related organisms: test either by disk diffusion or by microdilution MIC testing. Report disk diffusion results using *P. aeruginosa* breakpoints with the disclaimer "nonstandardized susceptibility results" (6).

◩ **NOTE:** Reliable disk diffusion breakpoints for *B. cepacia* to ceftazidime, minocycline, and meropenem are available in CLSI document M100-S18 (3).

V. PROCEDURE *(continued)*

5. Hold positive culture plates for 7 days at room temperature after laboratory results have been finalized.
6. Hold specimen for 7 days at 4°C to resolve any problem with the specimen.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. If no growth is observed, report "No growth."

B. If only normal microbiota is observed, report as such.

C. Report all potential pathogens according to criteria listed in Table 3.11.3–2.

1. For deep pharyngeal cultures, report the presence in any amount of *S. aureus, P. aeruginosa,* and *B. cepacia* only.
2. For quantitative cultures, report all potential pathogens (Table 3.11.3–2) that are in concentrations of $>10^3$ CFU/ml for protected specimen brushings and $>10^4$ CFU/ml for BAL samples.
3. For colistin-resistant gram-negative rods that are being referred to the CF reference laboratory, report as "probable XX," where XX is the most likely identification from the flowchart. Add a note: "Sent to *B. cepacia* reference laboratory for definitive identification." If the identification is truly in doubt, report the organism as "Unidentified gram-negative rod" and refer to the CF reference laboratory.

D. Document all results either as hard copy or by computerized work card.

VII. INTERPRETATION

A. The finding of *S. aureus, P. aeruginosa,* and *B. cepacia* complex regardless of quantity is always considered clinically significant (6, 8).

1. Sputum cultures from CF patients have been shown to correlate well with the presence of these organisms in the lower airways (6, 8, 16).
2. The presence of any of these three organisms in deep pharyngeal cultures should be reported. Other organisms in Table 3.11.3–2 should not be reported in pharyngeal cultures since they can be considered part of the oropharyngeal microbiota.

B. The finding of *B. cepacia* complex organisms in particular is viewed as important in the CF community, since patients with these organisms are often segregated from *B. cepacia*-negative CF patients both socially and medically. In addition, infection with these organisms may be considered a contraindication for lung transplantation, an important life-saving option in the terminal phase of this disease. Therefore, accurate identification of this complex of bacteria is critical for patient care, especially in light of the fact that there is not convincing evidence that the organisms with which it most frequently confused, *B. gladioli, R. pickettii,* and *Pandoraea* spp., play a significant role in CF lung disease (1, 5, 13).

C. *Aspergillus* spp., especially *Aspergillus fumigatus,* are associated with allergic bronchopulmonary aspergillosis (ABPA). ABPA is typically a clinical diagnosis with the finding of *Aspergillus* on culture supportive of the diagnosis of ABPA (8).

D. Other agents listed in Table 3.11.3–2 have been associated with pulmonary exacerbation of CF and should be reported according to criteria in Table 3.11.3–2.

VIII. LIMITATIONS

A. Patients with CF who are experiencing pulmonary exacerbation of their lung disease often respond to combinations of antimicrobials to which their organisms are resistant both individually and in combination by in vitro testing (6, 8).

VIII. LIMITATIONS *(continued)*

B. The interpretation of deep pharyngeal culture results is problematic. The absence of CF pathogens in these specimens is a good predictor of their absence in the lower airways. However, the presence of *S. aureus*, *P. aeruginosa*, and *B. cepacia* is not so strong a predictor of their presence in the lower airway. Nevertheless, caregivers of CF patients will treat the finding of these organisms in this specimen type as clinically significant (16). Other pathogens listed in Table 3.11.3–2 should not be reported from this specimen type.

REFERENCES

1. **Blecker-Shelly, D., T. Spikler, E. J. Gracely, T. Coeyne, P. Vandamme, and J. J. LiPuma.** 2000. Utility of commercial systems for the identification of *Burkholderia cepacia* complex from cystic fibrosis sputum culture. *J. Clin. Microbiol.* **38:**3112–3115.
2. **Burns, J. L., L. Saiman, S. Whittier, D. Larone, J. Krzewinski, A. Liu, S. A. Marshall, and R. N. Jones.** 2000. Comparison of agar diffusion methodologies for antimicrobial susceptibility testing of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *J. Clin. Microbiol.* **38:**1818–1822.
3. **Clinical and Laboratory Standards Institute.** 2008. *Performance Standards for Antimicrobial Susceptibility Testing.* Eighteenth informational supplement. Approved standard M100-S18. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Coenye, T., P. Vandamme, J. R. W. Govan, and J. J. LiPuma.** 2001. Taxonomy and identification of the *Burkholderia cepacia* complex. *J. Clin. Microbiol.* **39:**3427–3436.
6. **Cystic Fibrosis Foundation.** 1994. Microbiology and infectious disease in cystic fibrosis, p. 1–26. *In* Cystic Fibrosis Foundation, *Concepts of Care*, vol. V, section 1. Cystic Fibrosis Foundation, Bethesda, MD.
7. **Denton, M., and K. G. Kerr.** 1998. Microbiological and clinical aspects of infections associated with *Stenotrophomonas maltophilia. Clin. Microbiol. Rev.* **11:**57–80.
8. **Gilligan, P. H.** 1991. Microbiology of airway disease in patients with cystic fibrosis. *Clin. Microbiol. Rev.* **4:**35–51.
9. **Gilligan, P. H., P. A. Gage, D. F. Welch, M. J. Muszynski, and K. R. Wait.** 1987. Prevelance of thymidine-dependent *Staphylococcus aureus* in patients with cystic fibrosis. *J. Clin. Microbiol.* **25:**1258–1261.
10. **Heath, D. G., K. Hohneker, C. Carriker, K. Smith, J. Routh, J. J. LiPuma, R. M. Aris, D. Weber, and P. H. Gilligan.** 2002. Six-year analysis of *Burkholderia cepacia* complex isolates among cystic fibrosis patients at a referral center for lung transplantation. *J. Clin. Microbiol.* **40:**1188–1193.
11. **Henry, D. A., M. Campbell, C. McGimpsey, A. Clarke, L. Louden, J. L. Burns, M. H. Roe, P. Vandamme, and D. Speert.** 1999. Comparison of isolation media for the recovery of *Burkholderia cepacia* complex from respiratory secretions of patients with cystic fibrosis. *J. Clin. Microbiol.* **37:**1004–1007.
12. **Henry, D. A., E. Mahenthiralingam, P. Vandamme, T. Coenye, and D. P. Speert.** 2001. Phenotypic methods for determining genomovar status of the *Burkholderia cepacia* complex. *J. Clin. Microbiol.* **39:**1073–1078.
13. **Kiska, D. L., A. Kerr, M. C. Jones, J. A. Caracciolo, B. Eskridge, M. Jordan, S. Miller, D. Hughes, N. King, and P. H. Gilligan.** 1996. Accuracy of four commercial systems for identification of *Burkholderia cepacia* and other gram-negative nonfermenting bacilli recovered from patients with cystic fibrosis. *J. Clin. Microbiol.* **34:**886–891.
14. **LiPuma, J. L., B. J. Currie, G. D. Lum, and P. A. R. Vandamme.** 2007. *Burkholderia, Stenotrophomonas, Ralstonia, Cupriavidus, Pandoraea, Brevundimonas, Comamonas, Delftia,* and *Acidovorax*, p. 749–769. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
15. **Nair, B., J. Stapp, L. Stapp, L. Bugni, J. Van Dalfsen, and J. L. Burns.** 2002. Utility of Gram staining for evaluation of the quality of cystic fibrosis sputum samples. *J. Clin. Microbiol.* **40:**2791–2794.
16. **Ramsey, B. W.** 1996. What is the role of upper airway bacterial cultures in patients with cystic fibrosis? *Pediatr. Pulm.* **21:**265–266.
17. **Shreve, M. R., S. Butler, H. J. Kaplowitz, H. R. Rabin, D. Stokes, M. Light, and W. E. Regelmann for North America Scientific Advisory Group and Investigators of the Epidemiologic Study of Cystic Fibrosis.** 1999. Impact of microbiology practice on cumulative prevalence of respiratory tract bacteria in patients with cystic fibrosis. *J. Clin. Microbiol.* **37:**753–757.
18. **Steinbach, S., L. Sun, R.-Z Jiang, P. Flume, P. Gilligan, T. M. Egan, and R. Goldstein.** 1994. Persistent, clonal *Pseudomonas cepacia* infection in cystic fibrosis lung transplant recipients and clinical patients. *N. Engl. J. Med.* **331:**981–987.
19. **Whittier, S., R. L. Hopfer, M. R. Knowles, and P. H. Gilligan.** 1993. Improved recovery of mycobacteria from respiratory secretions of patients with cystic fibrosis. *J. Clin. Microbiol.* **31:**861–864.

3.11.4 *Legionella* Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Legionellosis is the collective name used to refer to (primarily pulmonary) infections caused by members of the genus *Legionella.* There are currently 45 named species in the genus, and several species contain more than one serotype. Many, but not all, species have caused human infection. Legionellae thrive in warm moist environments, both natural and artificial, and their multiplication is enhanced by the presence of free-living amoebae, in which the organisms multiply in these environments. Whether the few species which have not been isolated from humans are of lower virulence or simply live in environments with which humans do not come into contact is unknown. Transmission to humans typically occurs as aerosols. Legionellosis is not contagious.

Culture of respiratory secretions is among the most sensitive methods for the diagnosis of *Legionella* infection. The legionellae are intracellular pathogens, residing primarily in macrophages within the alveoli. Like other intracellular pathogens, they may not be present in every sputum sample and, when present, their numbers may be low. Since many patients with legionellosis do not produce sputum, collection of multiple respiratory specimens may be beneficial.

Urinary antigen testing has supplanted direct immunofluorescence antibody (DFA) testing of sputum for rapid diagnosis of legionellosis (*see* Appendix 3.11.4–1). Although urine antigen testing is designed to detect infections due to *Legionella pneumophila* serogroup 1, patients with infections due to other species and serogroups may sometimes have a positive test (1, 4). While *L. pneumophila* serogroup 1 represents the most commonly recognized cause of legionellosis, it may account for as little as 50% of cases (8, 9); thus, a negative urinary antigen test is not sufficient to presume that a patient does not have legionellosis.

Testing for antibodies to *Legionella* is not useful in the care of critically ill patients and is not recommended for routine use. Most patients with culture-confirmed legionellosis do not develop detectable antibodies until >3 weeks after infection, and some highly immunosuppressed individuals never produce detectable antibodies (3). Likewise, high titers in acute-phase serum samples are not, in themselves, diagnostic. Many persons have preexisting antibody from prior (subclinical) *Legionella* infection. Antibody tests using paired serum samples may be useful, however, as a tool in tracking nosocomial and community outbreaks.

The procedures presented here include the detection of legionellae by culture of clinical specimens. Refer to procedure 13.5 for culture of environmental samples for legionellae.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection (*see* procedure 3.11.2 for details on collection of lower respiratory specimens)

◩ **NOTE:** *L. pneumophila* survives in up to 3% salt solutions at temperatures below 30°C; in fact, small amounts of salt (0.1 to 0.5%) enhance survival (5). Saline is not toxic to the organism, as previously thought.

1. Respiratory secretions (sputum, bronchial and tracheal aspirates, and bronchial washings)

a. Place expectorated specimen in a sterile screw-cap cup.

b. Specimens collected by bronchoscopy or aspiration may remain in the Luken trap for transport so long as the free ends of the tubing are securely joined together to prevent leakage of the specimen during transport.

c. Collect at least 3 ml of specimen for *Legionella* culture.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

2. Bronchoalveolar lavage (BAL) fluid
   Submit a minimum of 50 ml of fluid in a sterile container.
3. Sterile body fluids (pleural, pericardial, and peritoneal)
   a. Submit at least 5 ml in a sterile tube or in the syringe used for collection.
   b. Remove the needle and cap the syringe with a Luer-Lok before transport.
4. Bronchial brushings
   a. Cut the brush off at a point about 30 to 40 mm from the end.
   b. Place the brush into a small tube containing no more than 0.5 ml of sterile saline or TSB.
5. Lung tissue
   a. Place a piece of tissue approximating the size of a dime onto a gauze square moistened with sterile nonbacteriostatic saline.
   b. Place the gauze square and the tissue into a sterile specimen cup with a screw cap.
6. Other tissues and wound specimens (including prosthetic heart valves)
   a. Perform culture for legionellae on such specimens, especially if routine bacterial cultures prove to be negative.
   ◩ **NOTE:** Legionellae survive in the specimen when stored in the refrigerator for long periods of time. However, they may also be present in a backup broth used for culture but will not grow or be detected in the broth. If the routine culture is negative, and the specimen is no longer available, subculture the broth to selective medium for legionellae. Postsurgical wound infections due to use of contaminated water for wound care have been reported.
   b. Collect tissues as for lung tissues.
   c. Collect external wound specimens on swabs after cleansing the site with sterile saline.
7. Blood
   There are no reliable methods for recovering legionellae from blood. "Blind subculture" of "negative" standard blood culture bottles sometimes results in recovery of the organism. The sensitivity of this method is not sufficient for routine use.

B. Timing and transport

1. Submit samples in the acute phase of infection, preferably before the beginning of antimicrobial therapy.
2. Transport to laboratory quickly. While legionellae may survive extended transport, their isolation may be compromised by overgrowth of commensal bacteria in the specimens.
3. If specimens are being transported to a remote laboratory, place samples on wet ice for transport.
4. For extended transport times (>1 day), freeze samples (-70°C) and transport on dry ice.

C. Rejection criteria

1. Respiratory secretions (sputum, etc.) submitted for routine bacterial culture are screened for adequacy by Gram stain; *do not* apply these criteria for specimens submitted for *Legionella* culture (6).
   ◩ **NOTE:** Patients with Legionnaires' disease typically produce sputum which is thin and watery and may contain few WBCs. Additionally, only normal oral bacterial morphotypes may be seen in Gram stains because legionellae do not stain with Gram stain reagents in clinical specimens.
2. BAL specimens should never be rejected, since they are collected by an invasive procedure. Since *Legionella* organisms are present in very low numbers, as large a volume of sample as possible (i.e., ideally up to 25 to 30 ml) should be concentrated and centrifuged before culture to optimize the recovery of *Legionella*.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

3. Do not perform "quantitative BAL cultures" for legionellae; their concentration in such specimens is always low.
4. Pleural fluid specimens of less than 5 ml should be cultured only after alerting the physician that such specimens are unreliable for the recovery of *Legionella.*
5. Reject "test-of-cure" cultures, since they should not be used to monitor a patient's response to therapy.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Media

1. Inoculate two media for each culture.
   a. Buffered charcoal-yeast extract agar (BCYE-α)
      ◩ **NOTE:** This nonselective medium contains ferric pyrophosphate, cysteine, α-ketoglutarate, and charcoal.
   b. BCYE-α with polymyxin, anisomycin, cefamandole, and α-ketoglutarate (BMPA-α)
      ◩ **NOTE:** While useful for specimens containing other microorganisms, this medium may be inhibitory to *Legionella* species (such as *Legionella micdadei*) which are susceptible to cefamandole. It should *never* be used alone.
2. Store at 4°C in sealed bags to keep it moist. Do not store for any period out of the bags.

B. Other supplies

1. Inoculating loop
2. Pasteur pipettes
3. Tissue homogenization apparatus (stomacher or other mechanical homogenizer) and TSB
4. Centrifuge to allow for 1,500 × *g* and centrifuge tubes for BAL samples (50 ml) and body fluids (10 to 15 ml)

C. Reagents

1. KCl acid-wash solution, pH 2.2
   a. Hydrochloric acid (0.2 M): add 2 ml of 1 M HCl to 8 ml of distilled water.
   b. Potassium chloride (0.2 M): dissolve 1.5 g of KCl in 100 ml of distilled water.
   c. Add 3.9 ml of 0.2 M HCl to 25 ml of 0.2 M KCl. Adjust pH to 2.2 ± 0.1 at 25°C with HCl or KCl; filter sterilize.
   d. Aseptically dispense 1-ml volumes into sterile screw-cap tubes containing a few glass beads. Store at room temperature. Shelf life is 1 year.

   ◩ **NOTE:** KCl solution (pH 2.2, 0.2 M) may be purchased from Remel, Inc. (20 tubes/pack, catalog no. 062621; 100 tubes/case, catalog no. 062620)
   Formula is HCl (0.2 M) at 135 ml plus potassium chloride (0.2 M) at 865 ml.
2. BCYE without cysteine (Remel, Inc.)
3. 5% Solution of powdered skim milk in sterile distilled water
4. Beta-lactamase test (procedure 5.3)
5. Hippurate test (procedure 3.17.21)
6. DFA reagents (*see* Appendix 3.11.4–2)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that media are within expiration period. Do not use if media appear to have dried, even if they have not passed the expiration date.
◩ **NOTE:** In particular, the effectiveness of BMPA-α declines with age as the antimicrobial agents degrade. Media which are stored for extended periods may dry out, causing the concentrations of ingredients to rise and making the media inhibitory.

B. Source of QC organisms
◩ **NOTE:** True QC of *Legionella* media is difficult, since the organisms rapidly become adapted to growth on artificial media within one or two passages. Media which pass internal QC procedures may not be presumed always to be of adequate quality.

IV. QUALITY CONTROL *(continued)*

1. Aliquot respiratory or tissue samples from a patient with Legionnaires' disease, for the best source of QC. Store at −70°C with approximately a 1-year expiration date. Lacking a clinical specimen, obtain a low-passage isolate of *L. pneumophila* from a reference laboratory.
2. Suspend the isolate in a 5% solution of sterile skim milk and store in small aliquots at −70°C.

C. Preparation of QC cultures. Use the methods outlined in Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) standard M22-A3 (2) and procedure 4.2.

1. Prepare a culture of the stored *Legionella* isolate on BCYE-α.
2. Suspend colonies from this first stock culture in sterile TSB to match the turbidity of a no. 1.0 McFarland standard.
3. Mix this adjusted culture with an equal volume of 10% (wt/vol) skim milk solution. Divide this final suspension into small aliquots and freeze at −70°C.

D. QC testing

1. Remove one of the frozen aliquots from the freezer, thaw, and dilute 1/100 in TSB.
2. Inoculate the volume of a 10-μl inoculating loop onto the surface of the media to be tested.
3. Incubate for 48 h at 35°C and count the colonies. Depending on the exact concentration of the original suspension, there should be between 50 and 200 colonies on each inoculated plate.
4. Keep detailed records of the colony counts for each lot of medium. Over time, the viability of the stored inoculum may decline, and a decrease in the number of colonies on the tested plates could be the result of decline in viability of the inoculum rather than a decrease in the quality of the media. In order to assess this possibility, test the lot in question again with a fresh inoculum which has not been frozen.
5. Verify that BMPA-α is able to inhibit growth of similar suspensions containing *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Candida albicans.*

V. PROCEDURES

It is imperative that these cultures be handled in a biological safety cabinet.

A. Commercial multiplex PCR is in development for the simultaneous detection of *L. pneumophila, Mycoplasma pneumoniae,* and *Chlamydia pneumoniae* (ProPneumo2; Prodesse, Inc.) (7); however, this assay is for research use only.

B. Culture inoculation

1. For all specimens, inoculate BCYE-α and BMPA-α plates.
   - **a.** Label plates with patient name and identifying number.
   - **b.** Record the date and time of inoculation.
   - **c.** Streak plates in several sectors to produce isolated colonies after incubation.
   - **d.** After initial processing, save a portion of specimen at 4°C for 7 days in case the specimen is contaminated and acid treatment is indicated.
2. Respiratory secretions
   - **a.** Touch a swab to the most purulent portions of the specimen containing blood or mucus, if present.
   - **b.** Use separate swabs to inoculate each plate.

V. PROCEDURES *(continued)*

c. If the specimen is from a patient with cystic fibrosis or a patient known to have colonization with gram-negative rods, acid treat the specimen and inoculate an additional BAP and BCYE-α plate.
(1) Place 0.1 ml of sputum sample into 0.9 ml of KCl solution, and vortex.
(2) Allow sample to incubate for 5 min at room temperature.
(3) Inoculate 0.1-ml portions of the treated specimen onto BCYE-α and BMPA-α.

3. BAL specimen
a. Centrifuge 30 to 50 ml or more of specimen at 1,500 × *g* for 20 min.
b. Decant all but 0.3 to 0.5 ml of the supernatant. Vortex to resuspend sediment into the remaining supernatant.
c. Use a sterile Pasteur pipette to dispense 2 drops of the specimen onto each plate.

4. Sterile body fluids
a. Concentrate at least 10-fold, when possible, by centrifuging at least 5 ml at 1,500 × *g* for 20 min.
b. Decant all but 0.5 ml of the supernatant. Resuspend any sediment in the remaining supernatant by vigorous shaking or vortexing.
c. Use a sterile Pasteur pipette to dispense 2 drops of the specimen onto each plate.

5. Bronchial brushings
a. Vigorously agitate the brush in the TSB to remove the cellular material adherent to it.
b. Use a sterile Pasteur pipette to dispense 2 drops of the specimen onto each plate.

6. Tissue
a. Place a piece of tissue no larger than a dime into 5 ml of TSB.
b. Homogenize the tissue using a mechanical tissue grinder or a stomaching device.
c. Use a sterile Pasteur pipette to dispense 2 drops of the specimen onto each plate.

C. Examination of cultures

1. Incubate cultures on buffered media in ambient air *(i.e., without increased CO_2)* at 35 to 37°C for 7 days with adequate humidity (50 to 70% relative humidity) to prevent drying of media during incubation.
◩ **NOTE:** Dried media are inhibitory to growth of *Legionella.* If low incubator humidity is a problem, place sterile gauze moistened with sterile water in bottom of jar and place plates on gauze in the jar.

2. Examine culture plates *daily* with the lids removed.
a. Examine plates early each morning so that suspicious colonies can be tested with FA reagents that day for early diagnosis of disease.
b. It is imperative to examine the plates using a dissecting microscope with a magnification of ×20 to ×50. Even colonies not yet visible to the naked eye will have the colony morphology typical of *Legionella.*
c. Use a focused light source aimed at a high oblique angle to illuminate the surface of the plate.
d. Avoid exposure to contaminating airborne molds during examination. Work in a biological safety hood, if possible.

3. At 24 h, if there is heavy growth of non-*Legionella* bacteria on the BCYE-α and BMPA-α plates, reprocess the original specimen using acid treatment, as described above for respiratory secretions containing gram-negative rods.
◩ **NOTE:** Reprocessing of the culture may not be required if the "routine" culture of the sample yields organisms consistent with the patient's diagnosis.

V. PROCEDURES *(continued)*

Consultation with the patient's physician regarding the likelihood of legionellosis may be appropriate prior to undertaking acid treatment of the specimen.

4. When viewed as described above, *Legionella* organisms appear as circular colonies with an entire edge, having a distinctive "ground-glass" appearance (*see* Fig. 3.11.4–1) and an iridescent blue, green, or pink hue. Only a few other bacteria of medical importance have this appearance. Older colonies are opaque, with a white center and opalescent perimeter.

◩ **NOTE:** Colonies resembling *Legionella* may not be present on both media. *L. micdadei* and a few other species may be inhibited by the cefamandole in BMPA-α. On BCYE-α, growth of large amounts of other gram-negative bacteria and yeasts may inhibit the growth of *Legionella.*

Caution: *Exercise caution when examining non-*Legionella *gram-negative bacteria present on BCYE-α. Some highly infectious organisms, such as* Francisella tularensis, *may grow preferentially on BCYE-α. Additionally, fungi such as* Coccidioides immitis *and* Histoplasma capsulatum *may also grow on BCYE-α.*

D. Preliminary identification of isolates

1. Gram stain suspicious colonies. Legionellae will appear as small gram-negative rods, which may stain faintly. Typically they are about the size of *Haemophilus* spp.; however, it is not unusual for there to be long filamentous forms present as well.

2. Examine plates with presumptive legionella morphology immediately using a long-wave UV lamp (Wood's lamp) in a darkened room, since some legionellae exhibit autofluorescence.

a. Bright blue-white fluorescence

(1) *Legionella bozemanii*
(2) *Legionella dumoffii*
(3) *Legionella gormanii*
(4) *Legionella anisa*
(5) *Legionella tucsonensis*
(6) *Legionella cherrii*
(7) *Legionella parisiensis*
(8) *Legionella steigerwaltii*

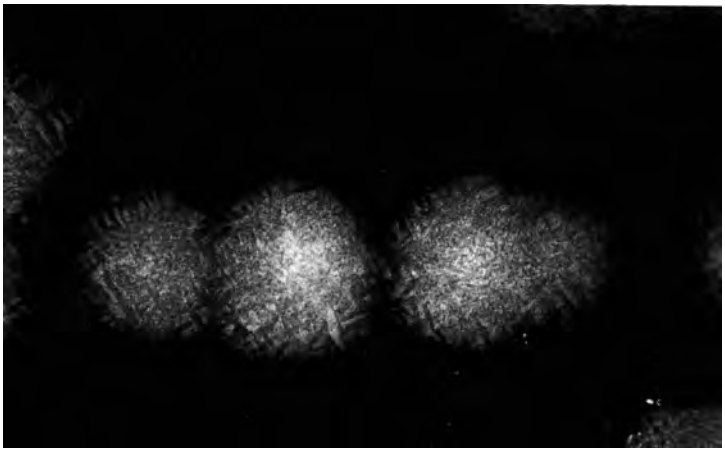


Figure 3.11.4–1 Colonies of *L. pneumophila* on BCYE-α showing ground-glass appearance.

V. PROCEDURES *(continued)*

b. Yellow-green fluorescence
(1) *Legionella wadsworthiii*
(2) *Legionella birminghamensis*

c. Red fluorescence
(1) *Legionella erythra*
(2) *Legionella rubrilucens*

3. Subculture suspicious colonies to BCYE-α and either a BAP or a plate of BCYE-α from which cysteine has been omitted. Do not use CHOC, since some *Legionella* isolates will grow on this medium.

a. Incubate plates at 35 to 37°C for 24 to 48 h.

b. Isolates which grow on BCYE-α but not on BAP or on the unsupplemented BCYE plate should be considered as presumptive *Legionella* spp. However, since rapid diagnosis is important to patient care, do not wait for these subculture results to perform confirmatory FA testing on typical colonies.

4. Perform rapid hippurate test and beta-lactamase test on suspicious colonies.

◩ **NOTE:** There are few biochemical tests which can be used to identify legionellae. Testing for the ability to hydrolyze hippurate may be of routine value, especially for laboratories that do not perform DFA testing. All serotypes of *L. pneumophila* hydrolyze hippurate, but a few less common species (e.g., *Legionella spiritensis* and *Legionella waltersii*) may also. Nevertheless, since *L. pneumophila* is the predominant species isolated from human infections, the finding that an isolate hydrolyzes hippurate is reasonably powerful suggestive evidence of its identity.

5. Use the beta-lactamase test to separate *L. pneumophila* (positive) from other species, such as *L. micdadei* (negative), as an aid in choosing which antisera to test for identification.

E. Definitive identification of isolates

1. For definitive identification, refer isolates to the local or state health department laboratory or a reference laboratory.

◩ **NOTE:** Definitive identification of *Legionella* to the species level requires testing not generally available in routine clinical microbiology laboratories, including the determination of the major branched-chain fatty acids contained in the cells and/or genetic analysis.

2. Identify the most prevalent species, *L. pneumophila* and *L. micdadei,* by testing the isolated colonies with DFA or indirect FA reagents, which are available commercially.

a. Perform FA tests as soon as suspicious colonies are isolated and preliminary rapid tests are performed.

b. Do not wait for subculture to confirm that the isolate does not grow on BAP, as delays may affect patient care.

c. See Appendix 3.11.4–2 for procedure.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Negative cultures

1. Preliminary report: "No *Legionella* isolated to date."

2. Final report: "No *Legionella* isolated after 7 days."

B. Positive cultures

1. Report "Positive for *Legionella* spp." if isolate has only been identified with polyvalent DFA reagents.

2. If serogroup or species has been determined, then report using the full species and/or serogroup designation, e.g., "Positive for *Legionella micdadei*."

3. Notification

a. For hospitalized patients, notify the patient's physician immediately. Notify infection control immediately also.

b. For outpatients or recently discharged patients, notify the patient's physician immediately.

c. Report positive cultures and urinary antigen tests to local or state health departments using whatever means is customary, following local disease reporting guidelines.

4. Storage of isolates

Molecular methods for typing *Legionella* isolates are available from some reference laboratories. Since an individual patient may be a part of a developing outbreak, it is prudent to save clinical *Legionella* isolates in 5% sterile skim milk at −70°C for possible future typing studies.

C. Contaminated cultures

If culture is overgrown with bacteria, yeast, or mold, report "Culture terminated due to growth of contaminating microbiota."

VII. LIMITATIONS

A. Legionellae are reliably susceptible to macrolides (azithromycin and erythromycin), quinolones (e.g., levofloxacin), and rifampin. Acquisition of resistance during therapy does not occur. In vitro susceptibility testing is not useful for selecting the "best" antimicrobial agent or for any other therapeutic purpose and should not be performed.

B. Legionellae are intracellular pathogens and may persist in sputum for several weeks after the institution of antimicrobial therapy. Test-of-cure cultures should not be used to monitor a patient's response to therapy, since patients who are recovering from Legionnaires' disease may continue to shed organisms. The patient's clinical response is the best indicator of a response to therapy.

C. Culture may be the most sensitive indicator of disease, but a negative culture does not rule out infection. Inhibitors of growth, such as the presence of yeasts, sampling error in collection, and prior treatment with antimicrobial agents, can affect the sensitivity of culture.

D. Legionellae have been isolated on CHOC in rare instances.

E. *F. tularensis, Bordetella pertussis,* and *Afipia* will grow on BCYE-α and not on BAP. The former two species are not motile and the latter does not have the colony morphology of *Legionella* spp.

F. Some species of *Legionella* will not react with current antisera. Suspicious colonies should be submitted to a reference laboratory for further identification.

REFERENCES

1. **Benson, R. F., P. W. Tang, and B. S. Fields.** 2000. Evaluation of the Binax and Biotest urinary antigen kits for detection of Legionnaires' disease due to multiple serogroups and species of *Legionella. J. Clin. Microbiol.* **38:**2763–2765.
2. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
3. **Edelstein, P. H., R. D. Meyer, and S. M. Finegold.** 1980. Laboratory diagnosis of Legionnaires' disease. *Am. Rev. Respir. Dis.* **121:**317–327.
4. **Helbig, J. H., S. A. Uldum, P. C. Luck, and T. G. Harrison.** 2001. Detection of *Legionella pneumophila* antigen in urine samples by the BinaxNOW immunochromatographic assay and comparison with both Binax *Legionella* Urinary Enzyme Immunoassay (EIA) and Biotest *Legionella* Urin Antigen EIA. *J. Med. Microbiol.* **50:**509–516.
5. **Heller, R., C. Holler, R. Sussmuth, and K. O. Gundermann.** 1998. Effect of salt concentration and temperature on survival of *Legionella pneumophila. Lett. Appl. Microbiol.* **26:**64–68.
6. **Ingram, J. G., and J. F. Plouffe.** 1994. Danger of sputum purulence screens in culture of *Legionella* species. *J. Clin. Microbiol.* **32:**209–210.
7. **Khanna, M., J. Fan, K. Pehler-Harrington, C. Waters, P. Douglass, J. Stallock, S. Kehl, and K. J. Henrickson.** 2005. The Pneumoplex assays, a multiplex PCR-enzyme hybridization assay that allows simultaneous detection of five organisms, *Mycoplasma pneumoniae, Chlamydia pneumoniae, Legionella pneumophila, Legionella micdadei,* and *Bordetella pertussis,* and its real-time counterpart. *J. Clin. Microbiol.* **43:**565–571.
8. **Marston, B. J., H. B. Lipman, and R. F. Breiman.** 1994. Surveillance for Legionnaires' disease. Risk factors for morbidity and mortality. *Arch. Intern. Med.* **154:**2417–2422.
9. **Reingold, A. L., B. M. Thomason, B. J. Brake, L. Thacker, H. W. Wilkinson, and J. N. Kuritsky.** 1984. *Legionella* pneumonia in the United States: the distribution of serogroups and species causing human illness. *J. Infect. Dis.* **149:**819.

SUPPLEMENTAL READING

Benin, A. L., R. F. Benson, and R. E. Besser. 2002. Trends in Legionnaires disease, 1980–1998: declining mortality and new patterns of diagnosis. *Clin. Infect. Dis.* **35:**1039–1046.

Edelstein, P. 2007. *Legionella,* p. 835–849. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.

Edelstein, P. H. 1998. Antimicrobial chemotherapy for Legionnaires disease: time for a change. *Ann. Intern. Med.* **129:**328–330.

Edelstein, P. H. 1998. Legionnaires' disease. *N. Engl. J. Med.* **338:**200–201.

Grant, W. W., V. S. Baselski, and R. G. Wunderink. 2001. *Legionella* and community-acquired pneumonia: a review of current diagnostic tests from a clinician's viewpoint. *Am. J. Med.* **110:**41–48.

Stout, J. E., and V. L. Yu. 1997. Legionellosis. *N. Engl. J. Med.* **337:**682–687.

Winn, W., Jr., S. Allen, W. Janda, E. Koneman, G. Procop, P. Schreckenberger, and G. Woods (ed.). 2006. *Koneman's Color Atlas and Textbook of Diagnostic Microbiology,* 6th ed., p. 549–565. Lippincott Williams & Wilkins, Baltimore, MD.

APPENDIX 3.11.4–1

Observe standard precautions.

Legionella Urinary Antigen Test

I. PRINCIPLE

Urinary antigen testing has supplanted direct immunofluorescence testing of sputum for rapid diagnosis of legionellosis. While urinary antigen tests are generally of excellent specificity, the sensitivity (70 to 90%) is less than that of culture. These tests detect infections due to *Legionella pneumophila* serogroup 1 and perhaps some other serogroups as well (1, 2). When possible, antigen tests should be performed in conjunction with culture or respiratory secretions to enable the recovery of species and serotypes not detected by the urinary antigen test and also to provide organisms for molecular typing in the event that the patient is part of an outbreak of Legionnaires' disease. (*Also see* procedure 11.4.)

II. SPECIMEN

A. Collect at least 5 ml of urine by clean catch or from a catheter.
B. Submit in a sterile screw-cap container. Storage and transport in urine preservative tubes containing boric and formic acids are also acceptable.
C. Store samples at 4°C until tested.
D. Allow patient samples to come to room temperature before testing.

III. MATERIALS

A. NOW *Legionella* urinary antigen test kit (Binax, Inc., Portland, ME)
B. For larger-volume laboratories, use similar tests available in EIA or ELISA format.
   1. *Legionella* urinary antigen EIA kit (Binax, Inc.)
   2. Bartels *Legionella* urinary antigen ELISA (Intracel Corp., Rockville, MD)
   3. Urine *Legionella* antigen ELISA (Wampole Laboratories, Cranbury, NJ)

IV. QUALITY CONTROL

A. Remove control swabs (positive and negative) from pouch.
B. Place each swab into a test card as described for patient specimens.
C. Unlike for test specimens, add *6 drops* of reagent A to the control specimens.
D. Seal the test cards, as described for the assay, and incubate for 15 min.

V. ASSAY PROCEDURE

A. Open test card pouch just before use. Label the test card with the sample accession number and other appropriate information.
B. Dip a test swab (supplied as part of the test kit) into the urine sample, completely covering the head of the swab. Remove the swab from the urine.
C. If the swab drips, touch the swab to the side of the container to remove the excess sample.
D. Insert the swab through the bottom hole on the test card and push it forward until the top of the swab is visible in the top hole of the card.
E. Hold the vial of reagent A vertically, 1 to 1.5 in. above the test card. Add 2 drops of reagent A to the bottom hole of the card.
F. Peel off the adhesive tape from the right side of the card. Close and seal, pressing down on the edge of the test card. Incubate at room temperature.
G. Start timer and read result after 15 min of incubation.

APPENDIX 3.11.4–1 *(continued)*

VI. INTERPRETATION

A. Determine results after 15 min of incubation. Reading results beyond this point may produce inaccurate results.

B. If no pink lines are seen in the test window, the assay is invalid and should be repeated.

C. A negative sample will produce a pink upper control line. The lower test line in the window will not be visible.

D. A positive sample will display two pink lines. Both the upper control line and the lower test line will be visible.

VII. REPORTING RESULTS

A. Positive urinary antigen
Report "Positive for urinary antigen of *Legionella pneumophila* serogroup 1."

B. Negative urinary antigen

1. Report "Negative for urinary antigen of *Legionella pneumophila* serogroup 1."
2. Also add the following comment: "Other serogroups and species of *Legionella* which may cause human disease are not detected by this test. Culture of respiratory secretions is recommended if infection with *Legionella* is still suspected."

VIII. LIMITATIONS

A. This test detects primarily *L. pneumophila* serogroup 1 and should not be relied on solely for the diagnosis of legionellosis. A negative test does not rule out the possibility of legionellosis caused by other serogroups and species.

B. *Legionella* urinary antigen may persist in the urine of successfully treated patients for weeks to months following recovery. Sequential urinary antigen tests should not be performed to monitor response to therapy.

C. In one study comparing two EIAs and the NOW test, the sensitivities of the assays were 88 to 100% for severe cases of pneumonia but dropped to 40 to 53% for milder cases of the disease (3).

References

1. **Benson, R. F., P. W. Tang., and B. S. Fields.** 2000. Evaluation of the Binax and Biotest urinary antigen kits for detection of Legionnaires' disease due to multiple serogroups and species of *Legionella. J. Clin. Microbiol.* **38:**2763–2765.
2. **Helbig, J. H., S. A. Uldum, P. C. Luck, and T. G. Harrison.** 2001. Detection of *Legionella pneumophila* antigen in urine samples by the Binax NOW immunochromatographic assay and comparison with both Binax *Legionella* Urinary Enzyme Immunoassay (EIA) and Biotest *Legionella* Urin Antigen EIA. *J. Med. Microbiol.* **50:**509–516.
3. **Yzerman, E. P. F., J. W. den Boer, K. D. Lettinga, J. Schellekens, J. Dankert, and M. Peeters.** 2002. Sensitivity of three urinary antigen tests associated with clinical severity in a large outbreak of Legionnaires' disease in The Netherlands. *J. Clin. Microbiol.* **40:**3232–3236.

APPENDIX 3.11.4–2

Detection of *Legionella* by Fluorescent Antibody

I. PRINCIPLE

Rabbit antibodies prepared against *Legionella* species and serogroups are conjugated with fluorescein isothiocyanate (FITC). The antibodies will bind to the homologous *Legionella* species, making them visible when viewed under UV light. Direct immunofluorescence is most commonly used for the identification of *Legionella* isolates from cultures.

Direct immunofluorescence may also be used to detect legionellae in clinical specimens; however, the sensitivity of this procedure may be low, depending on the skill and experience of the operator (1). It should be used cautiously by inexperienced laboratorians for critical specimens such as lung tissue. Some laboratories use it as an adjunct to examination of highly contaminated sputum specimens in conjunction with acid decontamination and culture (J. Stout, personal communication). (*Also see* procedure 11.3.)

APPENDIX 3.11.4–2 *(continued)*

Observe standard precautions.

II. SPECIMENS

A. Tissue, fresh

1. Use a sterile scalpel to cut a fresh face on the surface of the tissue. Homogenize the tissue in sterile water to make an approximately 10% suspension.
2. Alternatively, use a sterile scalpel to cut a fresh face on the surface of the tissue. Grasping the tissue with forceps, press the newly exposed face of the tissue to a clean slide.
3. Make one slide for each antibody conjugate employed and one slide for a negative control conjugate.
4. Air dry and heat fix.
5. Fix smears in 1% formalin for 10 min. Drain and rinse off formalin with a gentle stream of filtered water.

B. Tissue, formalin fixed

1. Use a scalpel to cut a fresh face on the tissue. Place the tissue in a petri dish and use the scalpel to scrape a fine puree of debris and tissue fluids from the newly cut surface of the tissue.
2. Use the scalpel blade to transfer portions of the puree to clean microscope slides.
3. Prepare one slide for each antibody conjugate employed and one slide for the negative control conjugate.
4. Air dry and heat fix.

C. Respiratory secretions

1. If present, select purulent-appearing portions of the sample. Use a swab to prepare thin smears of the sample on clean microscope slides. Prepare one slide for each antibody conjugate employed and one slide for the negative control conjugate.
2. Air dry and heat fix.
3. Fix in 1% formalin for 10 min. Drain and rinse with a gentle stream of filtered water.

D. Any bacterial colony with morphology resembling *Legionella* spp.

1. Emulsify colonies into 1% formalin to match the turbidity of a no. 0.5 McFarland standard in saline or phosphate-buffered saline (PBS [Appendix 3.11.4–3]). Dilute the suspension 1/100 in PBS. *Note:* Making these suspensions too dense may quench the fluorescence.
2. Use a separate slide for each antibody conjugate to be tested.
3. Fix in 1% formalin for 10 min. Drain and rinse with a gentle stream of filtered water.

III. MATERIALS

A. Reagents

1. *Legionella* antisera, conjugated with FITC. Minimally, use conjugates reactive with *L. pneumophila* serogroups 1 to 6 and *L. micdadei,* except where local experience indicates an increased prevalence of other *Legionella* species. In these latter instances, antibody conjugates reactive with a broader range of species or serogroups are available.
   a. SciMedx, Inc., Danville, NJ
      (1) Polyvalent *L. pneumophila* serogroup 1 to 6 reagent and monovalent *L. micdadei* reagent *or*
      (2) Polyvalent *L. pneumophila* serogroups 1 to 6, *L. bozemanii, L. dumoffii, L. gormanii, Legionella longbeachae* serogroups 1 and 2, *L. micdadei, Legionella jordanis*
   b. Zeuss Scientific, Raritan, NJ
      Polyvalent *L. pneumophila* serogroup 1 to 6 reagent
2. FITC-conjugated negative control serum (normal rabbit globulins)
3. PBS, pH 7.5, filtered (*see* Appendix 3.11.4–3)
4. Buffered glycerol mounting fluid, pH 9.0
5. Formalin (1%) in 0.85% saline (10% formalin diluted 1/10 with saline)
6. Distilled water, filtered (*see* below)
7. Low-fluorescence immersion oil

◩ **NOTE:** Filter all aqueous reagents made with locally produced deionized water through a 0.22-µm-pore-size filter before use. Legionellae may colonize water-deionizing systems and produce false-positive tests.

APPENDIX 3.11.4–2 *(continued)*

B. Alternative methods—indirect FA staining

◩ **NOTE:** Test kits employing indirect FA methods for staining of *Legionella* are also available and have performance similar to that of the DFA staining method described above. Contact the vendors for additional information, other supplies needed, and specific instructions for testing.

1. Remel, Inc. (Lenexa, KS)—Legionella Poly-ID test kit
   L. pneumophila serogroups 1 to 6, *L. dumoffii, L. longbeachae* serogroups 1 and 2, *L. bozemanii, L. gormanii, L. micdadei, L. wadsworthii* serogroup 1, *Legionella oakridgensis, Legionella feeleii* serogroup 1, *Legionella sainthelensi* serogroup 1, *L. jordanis* serogroup 1, *Legionella anisa* serogroup 1, *L. spiritensis* serogroup 1, *Legionella hackeliae, Legionella maceachernii, Legionella jamestownensis, L. cherrii* serogroup 1, *L. steigerwaltii* serogroup 1, *L. parisiensis, L. rubrilucens* serogroup 1, *L. erythra* serogroup 1.
2. Bio-Rad Laboratories (Hercules, CA)—MONOFLUO monoclonal immunofluorescence test kit contains monoclonal antibody reactive with all known serogroups of *L. pneumophila.*
3. Zeuss Scientific—Legionella IFA test system
   Legionella pneumophila serogroups 1 to 4

C. Supplies

1. Clean plastic wash bottles
2. Microscope slides suitable for FA testing. FA slides with delineated circles are recommended (Precision Laboratory Products, Middleton, WI, http://www.precisionslides.com).
3. Tuberculin syringes or 50-µl pipettor to dispense antiserum
4. Wooden applicator sticks
5. Humidified staining chamber: a large covered petri dish lined with filter paper or covered glass baking dish lined with a paper towel. Chamber should contain a rack (e.g., glass rods) so that the slides do not sit directly on the moistened paper.

IV. QUALITY CONTROL

A. Prepare bacterial control slides containing *Legionella* for use with each test.

1. *L. pneumophila* serogroup 1 may be used as a positive control for conjugates containing antibody against *L. pneumophila* and as a negative control for *L. micdadei* antibody conjugates.
2. *L. micdadei* may be used as a positive control for conjugates containing *L. micdadei* antibody and as a negative control for conjugates containing *L. pneumophila* antibody.
3. For polyvalent conjugates containing *L. micdadei* antibody conjugates, use another species of *Legionella* or *Escherichia coli* (e.g., ATCC 25922) as a negative control.
4. Prepare suspensions of the control organisms as described above for cultures, or purchase formalin- or heat-killed suspensions of control organisms from vendor that supplies antibody reagents.

B. Place a drop of the diluted suspension of each control organism on one slide for each conjugate used and on one slide for the negative antibody conjugate. Thus, each control slide should contain both *L. pneumophila* and *L. micdadei* or another species if other species are being tested.

C. Prepare an extra slide for each specimen being tested, to use as a negative specimen control.

D. Do not report patient results if positive control slides do not produce a 3+ or greater fluorescence or the negative control slides produce more than 1+ fluorescence. See item VI for definitions of degree of fluorescence.

V. PROCEDURE

A. Place the slides in a humidified chamber on racks.

B. Add 25 to 50 µl of the *L. pneumophila* polyvalent conjugate to each circle on one of the specimen slides and one of the bacterial control slides. Only one conjugate should be applied to any one slide, although one slide may contain several specimens.

APPENDIX 3.11.4–2 *(continued)*

C. Use a wooden applicator stick to spread the conjugate drop over the entire area delineated by the circles on all of the slides. Use caution to avoid carrying over conjugate or bacteria between the circles. *Use a separate applicator stick for each circle or break off the end of the applicator before spreading the conjugate in the next circle.*

D. Similarly, add the *L. micdadei* conjugate to the specimen slides and a bacterial control slide.

E. Add 25 to 50 μl of the negative antibody conjugate to each circle on the control slide prepared from each of the specimens being tested and to a bacterial control slide. Spread within each circle, as described above.

F. Place the slides (test slides and control slides) into the humidified chamber, making certain that they do not touch each other. Cover the chamber to exclude light and allow the slides to incubate at room temperature for 20 to 30 min.

G. Rinse the antibody off of the slides with a gentle stream of PBS from a squirt bottle. While rinsing, hold the slide horizontally and allow the PBS to run across one circle at a time to minimize the chance that stained bacilli will be transferred between the circles.

H. Place the slides back onto the rack in the chamber or on a staining rack in a sink. Fill each circle with PBS and allow the slides to sit for 10 min. Do not allow the PBS to run between the circles, since stained bacilli from one sample may be carried to the next circle. Rinse with deionized water.

I. Wash and rinse the slides one more time with PBS and deionized water as described above. Allow slides to air dry.

J. Place a drop of buffered glycerol mounting fluid and a no. 1 coverslip on each slide. Examine immediately.

K. Examine slides using transmitted or incident UV light. Check with your microscope supplier for the proper combination of barrier and exciter filters needed for use with FITC.

VI. INTERPRETATION

A. Scan the slides with a 40× objective. Legionellae from cultures may appear as rods or as long filaments which stain apple green on their periphery. Examine the bacterial control slides, where the bacteria will be easily visualized, first.

B. Examine positive areas of the slides with a 100× oil immersion objective to quantitate the fluorescence.

1. 4+: cell wall appears as a brilliant yellow-green ring around a less bright central area of the bacilli.
2. 3+: cell wall stains bright yellow-green.
3. 2+: cell wall is dull yellow-green, barely discernible from central area of cell.
4. 1+: diffuse faint staining of cells; cell center is indistinguishable from cell wall.

C. Fluorescence in the "test" conjugate of ≥3+ and no fluorescence in the control conjugate is a positive test as long as there is ≥3+ fluorescence of the appropriate bacterial control suspension.

D. Similarly examine the negative control slides containing the negative conjugate. They should display no more than 1+ fluorescence.

E. Examine slides made from clinical specimens (especially respiratory secretions) for at least 5 min, since they may contain few bacilli. Bacteria in clinical specimens may appear either intra- or extracellular.

VII. REPORTING RESULTS

A. Report positive or negative results on direct tests as "Positive (or negative) for *Legionella* (add the antiserum name that produced the positive result) by direct fluorescent-antibody test."

B. For cultures, use the positive results of the species-specific antiserum to definitively identify the species present.

VIII. LIMITATIONS

A. Dim fluorescence (≤2+) of the test conjugate and dim fluorescence of the control conjugate indicate probable cross-reaction with a non-*Legionella* bacterium.

B. Dim fluorescence of the test conjugate and no fluorescence in the control conjugate indicate possible cross-reaction with a species or serotype of *Legionella* other than the ones against which the test conjugate was produced. This result may also be

APPENDIX 3.11.4–2 *(continued)*

produced by some cross-reacting non-*Legionella* organisms, such as *P. aeruginosa, Pseudomonas fluorescens, B. pertussis,* and *Bacteroides* spp. These cross-reacting bacteria can be recognized because the size and shape of the fluorescent cells will be different from those of the fluorescent cells of *Legionella* spp. Compare the morphologies of bacteria in these slides to those of the positive bacterial control slides.

C. The sensitivity of direct immunofluorescence testing on clinical samples may range from <50% to about 75%, depending on the skill and experience of the operator.

Reference

1. **Edelstein, P. H., R. D. Meyer, and S. M. Finegold.** 1980. Laboratory diagnosis of Legionnaire's disease. *Am. Rev. Respir. Dis.* **121:**317–332.

APPENDIX 3.11.4–3

Phosphate-Buffered Saline, pH 7.5 ± 0.1

A. Stock buffer (pH will not be 7.5)

Na_2HPO_4 (anhydrous reagent grade) ……	13.8 g
$NaH_2PO_4:H_2O$ (reagent grade) ……	1.8 g
NaCl (reagent grade) ……	85.0 g
Distilled water to make final volume of ……	1,000.0 ml

B. Working solution (pH 7.5 ± 0.1; 0.01 M buffer; 0.85% NaCl)

concentrated stock solution ……	100.0 ml
distilled water to make final volume of ……	1,000.0 ml

C. When commercially available reagent packets are used
1. Add one envelope of the powdered mixture to a 1-liter volumetric flask, and fill the flask to 1 liter with freshly deionized water.
2. Mix until all powder is dissolved. Buffer should be clear and colorless.
3. Measure the pH. Adjust to pH 7.5 ± 0.1 with HCl or NaOH.
4. Assign a 1-month expiration date. Store at 2 to 8°C.

D. Filter buffer if made with locally produced deionized water through a 0.22-µm-pore-size filter before use.

3.11.5 Otitis Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Otitis media is an infection of the middle ear and accounts for more than 30 million office visits per year for a cost of over $2 billion in the United States (1). More than 80% of children have one or more episodes of otitis media by age 6 years, with the highest number of cases between 2 and 6 years of age (18). Hearing loss and deficits in learning are a few of the complications (15). The most common agents of otitis media are *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis*, and *Alloiococcus otitis* (2, 3, 10, 15), although *Streptococcus pyogenes* is found on a seasonal basis (13, 15). (*Note:* Although the name *Alloiococcus otitidis* is in common usage [7], that name has not been officially adopted; *Alloiococcus otitis* is the official name.) Amoxicillin and amoxicillin-clavulanate are the drugs of choice for initial treatment (16). Although *S. pneumoniae* organisms are becoming increasingly more resistant, the concentrations of amoxicillin that can be achieved in the middle ear fluid are sufficient to eliminate all but the most resistant strains (13). Treatment generally resolves the infection, but treatment failures occur and surgical intervention can be necessary (4, 12, 13, 16). Tympanocentesis and culture of the middle ear fluid constitute a valuable tool for definitive diagnosis, to guide therapy, to evaluate treatment failures, and for research studies to determine the efficacy of antimicrobials against the most common agents. However, the diagnosis is usually made on clinical grounds, because of the invasive nature of tympanocentesis. Culture is usually reserved for persistent infections. Recently, multiplex PCR methods for the detection of common agents have been used to improve sensitivity in the diagnosis (9, 17). Historically pediatricians cultured the nasopharynx to predict the pathogens in the middle ear fluid. This practice is no longer recommended, since the presence of pathogens in the nasopharynx does not predict the pathogens present in the middle ear (8). However, lack of isolation of any pathogen has a 96% negative predictive value for lack of a pathogen in the middle ear fluid (8).

Otitis externa is an infection of the external auditory canal. Unique problems occur with this infection because of the narrow and tortuous nature of the canal and its tendency to trap foreign objects, wax, and water. Infections are classified as acute and chronic. Acute infections are often referred to as "swimmer's ear." *Pseudomonas aeruginosa* is a frequent cause of freshwater otitis, and *Vibrio alginolyticus* is a cause in oceanic swimmers, although other aerobic bacteria can be involved. Localized infections with *Staphylococcus aureus* or *S. pyogenes* can also occur. Contaminating skin bacterial microbiota (corynebacteria and staphylococci) can be present, which are not significant. More invasive infections are caused by extension of bacteria into the adjacent soft tissues and bone, with the formation of a cholesteatoma. Chronic otitis is usually caused by bacterial infection, and although *P. aeruginosa* may be predominant, a variety of anaerobes may also be present. Tissue and bone samples from the inner ear should also be cultured for fungi, *Nocardia*, and mycobacteria, which may also be etiologic agents of chronic infection (13). Underlying diseases such as tertiary syphilis may also cause chronic otitis, but this presentation is rare in the developed world (13).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

NOTE: Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and other caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

Observe standard precautions.

A. Specimen collection

1. External ear

a. Insert sterile swab into ear canal until resistance is met.

b. Rotate swab and allow fluid to collect on swab.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

2. Tympanocentesis fluid

◩ **NOTE:** Because of the invasive nature of the collection process, these specimens are usually submitted primarily to diagnose middle ear infections only if previous therapy has failed.

a. Clean the external canal with mild detergent.

b. Using a syringe aspiration technique, the physician will obtain the fluid from the ear drum.

c. Send the specimen in a sterile container or in the syringe capped with a Luer-Lok and with the needle removed.

◩ **NOTE:** Establish a policy for the proper collection and transport of clinical specimens not collected on swabs. Educate the physicians that needles must be removed from the syringe and the syringe cap secured prior to transport to avoid leakage.

d. Syringes that have been capped with a Luer-Lok (with needle removed) prior to transport may be accepted for culture provided the specimen has not clotted inside the syringe and there is no leakage during transport which could result in contamination of the culture. The laboratory may reject specimens that have clotted in a capped syringe because they cannot be processed for culture without inadvertently contaminating the specimen.

e. If the eardrum is ruptured, collect exudate by inserting a sterile swab through an auditory speculum.

B. Specimen transport

1. Submit to laboratory

a. Submit swabs in tube of transport medium or in BD Culturette EZ.

b. Submit aspirates in a sterile container or in the original syringe capped with a Luer-Lok to prevent leakage (*see* items II.A.2.c and II.A.2.d above).

2. Label specimens with demographic information, date and time of collection, and site of collection.

3. List the diagnosis of otitis media, chronic otitis, or otitis externa.

III. MATERIALS

A. Primary media

1. BAP

2. CHOC

3. MAC or EMB

4. BHI agar prepared with 5% rabbit blood for detection of *A. otitis* (the following companies supply defibrinated rabbit blood and dehydrated BHI agar for preparation of media: Remel, Inc.; BD Diagnostic Systems; and Hardy, Inc.)

◩ **NOTE:** *A. otitis* occasionally grows on BAP with 5% sheep blood in 5 days but does not grow on CHOC, Mueller-Hinton agar (MH) with lysed horse blood, buffered charcoal-yeast extract agar, brucella agar, or Columbia colistin-nalidixic acid agar (3). Growth generally takes 72 h on rabbit blood agar (3). Routine testing for this organism is beyond the capabilities of most laboratories, but awareness of the species may be important to communicate to the physician.

B. Identification methods

1. Gram stain (procedure 3.2.1)

2. Refer to procedure 3.3.2 for identification of the common agents of otitis.

C. Other supplies

1. Incubator at 35°C with 5% CO_2 or a CO_2-generating system

2. Inoculating sticks and loops

3. Petri dishes and filter paper

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that plate media meet expiration date and QC parameters per Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22-A3 (6). See procedures 14.2 and 3.3.1 for further procedures.

B. Test each lot of CHOC per procedure 3.3.1.

V. PROCEDURE

Observe standard precautions.

A. Inoculation

1. Inoculate specimen to BAP, CHOC, and MAC.

2. Firmly roll swab over one-sixth (no more) of the agar surface, or aspirate 3 or 4 drops of fluid onto agar. Streak carefully for isolation in four quadrants to minimize overgrowth by other microorganisms.

B. Incubation

1. Incubate plate at 35 to 37°C in 5% CO_2 for a minimum of 48 h.

2. For cultures of invasively collected ear fluid samples, incubation may be extended to 4 days, if results are negative. Anaerobic cultures may be indicated (*see* section 4).

C. Gram stain

1. Perform a Gram stain from the swab or fluid (procedure 3.2.1).

2. Note the presence of WBCs and bacteria.

D. Culture examination

1. External otitis: observe plates at 24 and 48 h for growth of enteric gram-negative rods, pseudomonads, vibrios, streptococci, coryneforms, and *S. aureus.*

a. Since only one pathogen is generally responsible for otitis externa, mixed cultures of gram-negative rods should be minimally identified.

b. For gram-negative rods, perform spot oxidase and indole tests to avoid misidentifications.

(1) *V. alginolyticus* is an oxidase- and indole-positive, gram-negative rod that grows well on MAC. It will grow on MH with 4% salt (11). Place a colistin or polymyxin B disk on MH. It is further identified by most kit systems, although the reactions are improved if the inoculum is made in saline rather than water (*see* Table 3.18.2–8).

(2) *P. aeruginosa* is oxidase positive and indole negative and often has a characteristic odor or blue-green or brown pigment, for definitive identification. If the characteristic odor or these pigments are lacking, it can be definitively identified by positive growth at 42°C and production of a fluorescent pigment on agar media (procedure 3.17.17).

(3) Other gram-negative rods are less common and are usually identified by commercial kits. Exception: swarming *Proteus* spp. are identified by indole, ampicillin susceptibility, and ornithine (*see* Table 3.3.2–5).

(4) Perform antimicrobial susceptibility testing (AST) on the predominant microorganism. Evaluate swarming *Proteus* organisms on MAC or EMB, where they generally do not swarm, to be sure they are the predominant microorganism before performing AST; they can be an important pathogen from a patient with diabetes mellitus.

c. Examine cultures for predominant gram-positive cocci. Generally *S. aureus* and *S. pyogenes* are the most common gram-positive cocci involved in otitis externa.

(1) Identify *S. pyogenes* and other beta-hemolytic streptococci (Table 3.3.2–5).

V. PROCEDURE *(continued)*

(2) Identify and perform AST on *S. aureus* (Table 3.3.2–5 and section 5, respectively)

d. Identify yeasts and molds, if present. *Aspergillus* and *Candida albicans* have been implicated in chronic infections.

e. Since resident cutaneous microbiota (coagulase-negative staphylococci and coryneforms) are normal in the external ear canal, they should not be further evaluated.

2. Otitis media: observe plates at 24 h and up to 4 days for cultures of middle ear fluid. Pursue all organisms present, since the specimen is collected by an invasive procedure and any microorganism can be considered the agent of disease.

a. Fastidious gram-negative rods and diplococci

(1) Identify *H. influenzae* and *M. catarrhalis* according to Table 3.3.2–5 and perform beta-lactamase test on *H. influenzae.* Since more than 90% of *M. catarrhalis* organisms are beta-lactamase positive, testing is not helpful to treatment.

(2) *Bordetella trematum* is an oxidase-negative, catalase-positive, gram-negative rod that has been implicated in ear infections (19). It is motile, frequently reduces nitrate, and may or may not grow on MAC (*see* Table 3.11.6–1).

b. Observe for growth of gram-positive cocci. Refer to Table 3.3.2–5 for rapid identification tests to identify the following.

(1) *S. pyogenes* and other beta-hemolytic streptococci

(2) *S. pneumoniae*

(a) Confirm negative bile solubility test with optochin disk.

(b) Perform AST, using standardized methods (reference 5 and section 5), per laboratory protocol and physician policy.

c. Normal skin microbiota (coagulase-negative staphylococci and corynebacteria) are not generally identified to the species level unless they are the only predominant species in the culture and are present in large numbers.

(1) Identify *Turicella otitidis,* a long coryneform rod implicated in otitis media (7). It is catalase positive, asaccharolytic, and CAMP test positive.

(2) Examine for *Nocardia* in chronic infections (*see* procedure 6.1).

3. Examine, on request, for *A. otitis* from middle ear fluid. Prepare BHI agar with 5% rabbit blood and inoculate with specimen. Incubate plates for 5 days; increased CO_2 is not required.

◩ A few strains have been reported to grow on sheep blood agar, but it is not optimal (3).

a. *A. otitis* is slow growing and produces pinpoint colonies that are moist and slightly yellow (3). No hemolysis is observed. Eventually the colonies adhere to the agar.

b. No growth is seen on CHOC (3).

c. On Gram stain, *A. otitis* organisms are gram-positive cocci in clusters and tetrads without chains and cannot be distinguished from staphylococci.

d. Perform the following tests to confirm the identification. Note the expected reactions (14).

(1) Catalase negative or weak

(2) Pyrrolidonyl-β-naphthylamide (PYR) positive

(3) Leucine aminopeptidase (LAP) positive

(4) Vancomycin susceptible

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Gram stain—report smear as indicated in procedure 3.2.1.

B. Negative results

1. Report preliminary and final results as "No growth."

2. Indicate the number of days the culture was incubated.

3. If bacteria were seen on smear but did not grow on culture, extend the incubation and make a notation on the report to indicate the discrepancy.

C. Positive reporting

1. Indicate the presence of skin microbiota, without identification.

2. If the culture is mixed but with no predominating pathogen, indicate the genera and do not report further: e.g., "Mixed microbiota present, consisting of three morphologies of gram-negative rods, molds, and skin microorganisms; contact laboratory if further testing is clinically indicated."

3. Report all pathogens and susceptibility tests performed, using preliminary reports as indicated in procedure 3.3.2 and CLSI guidelines (5). Also refer to section 5.

4. For *Vibrio*, report that the organism is resistant to colistin or polymyxin B if there was no zone around the disk, since ear drops often contain this antimicrobial.

VII. INTERPRETATION

A. A positive external ear culture with a predominant gram-negative rod, beta-hemolytic streptococci, or *S. aureus* generally indicates infection with that agent.

B. A positive middle ear culture with *S. pneumoniae, H. influenzae, M. catarrhalis*, and *A. otitis* generally indicates infection with that organism.

C. A negative culture cannot rule out otitis media. In fact, in chronic infections, a pathogen is often not isolated.

D. Controversy regarding the need for treatment of otitis media has been found in recent literature, but most physicians agree on its benefits (16). *A. otitis* is resistant to sulfamethoxazole-trimethoprim and often to erythromycin (3), but it does not have a beta-lactamase.

E. Otitis externa is usually treated with ear drops containing a variety of agents, including 2% acetic acid, hydrocortisone, and antimicrobial agents, such as quinolones, neomycin, and either gentamicin or polymyxin B.

VIII. LIMITATIONS

A. Methods that employ PCR for the detection of pathogens responsible for otitis media are more sensitive than culture techniques and increase the rate of detection of a pathogen to 75% (17).

B. False-negative cultures can result from overgrowth of the culture with normal cutaneous microbiota.

C. False-positive results can be caused by overinterpretation of the culture results.

D. *T. otitidis*, a coryneform rod, has been infrequently isolated from ear fluid and may be a cause of otitis media (7).

E. *A. otitis* is difficult to culture and may not be detected.

REFERENCES

1. **Bluestone, C. D.** 1989. Modern management of otitis media. *Pediatr. Clin. N. Am.* **36:**1371–1377.
2. **Bluestone, C. D., J. S. Stephenson, and L. M. Martin.** 1992. Ten-year review of otitis media pathogens. *Pediatr. Infect. Dis. J.* **11:**S7–S11.
3. **Bosley, G. S., A. M. Whitney, J. M. Pruckler, C. W. Moss, M. Daneshvar, T. Sih, and D. F. Talkington.** 1995. Characterization of ear fluid isolates of *Alloiococcus otitis* from patients with recurrent otitis media. *J. Clin. Microbiol.* **33:**2876–2880.
4. **Chow, A. W., C. B. Hall, J. Klein, R. B. Kammer, R. D. Meyer, and J. S. Remington.** 1992. General guidelines for the evaluation of new anti-infective drugs for the treatment of respiratory tract infections. *Clin. Infect. Dis.* **15**(Suppl. 1)**:**S62–S88.
5. **Clinical and Laboratory Standards Institute.** 2008. *Performance Standards for Antimicrobial Susceptibility Testing.* Eighteenth informational supplement. Approved standard M100-S18. Clinical and Laboratory Standards Institute, Wayne, PA.
6. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
7. **Funke, G., S. Stubbs, M. Altwegg, A. Carlotti, and M. D. Collins.** 1994. *Turicella otitidis* gen. nov., sp. nov., a coryneform bacterium isolated from patients with otitis media. *Int. J. Syst. Bacteriol.* **44:**270–273.
8. **Gehanno, P., G. Lenoir, B. Barry, J. Bons, I. Boucot, and P. Berche.** 1996. Evaluation of nasopharyngeal cultures for bacteriologic assessment of acute otitis media in children. *Pediatr. Infect. Dis. J.* **15:**329–332.
9. **Hendolin, P. H., A. Markkanen, J. Ylikoski, and J. J. Wahlfors.** 1997. Use of multiplex PCR for simultaneous detection of four bacterial species in middle ear effusions. *J. Clin. Microbiol.* **35:**2854–2858.
10. **Hendolin, P. H., U. Kärkkäinen, A. Markkanen, T. Himi, and J. Ylikoski.** 1999. High incidence of *Alloiococcus otitis* in otitis media with effusion. *Pediatr. Infect. Dis. J.* **18:**860–865.
11. **Janda, J. M., C. Powers, R. G. Bryant, and S. L. Abbott.** 1988. Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. *Clin. Microbiol. Rev.* **1:**245–267.
12. **Klein, J. O.** 1993. Microbiologic efficacy of antibacterial drugs for acute otitis media. *Pediatr. Infect. Dis. J.* **12:**973–975.
13. **Klein, J. O.** 2005. Otitis externa, otitis media, mastoiditis, p. 766–771. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), *Principles and Practice of Infectious Diseases,* 6th ed. Churchill Livingstone, New York, NY.
14. **LaClaire, L. L., and R. R. Facklam.** 2000. Comparison of three commercial rapid identification systems for the unusual gram-positive cocci *Dolosigranulum pigrum, Ignavigranum ruoffiae,* and *Facklamia* species. *J. Clin. Microbiol.* **38:**2037–2042.
15. **McCarthy, J. M.** 1995. Bacterial susceptibility and tympanocentesis in acute otitis media. *Pediatr. Infect. Dis. J.* **14:**S45–S50.
16. **Pichichero, M. E.** 2000. Recurrent and persistent otitis media. *Pediatr. Infect. Dis. J.* **19:**911–916.
17. **Post, J. C., R. A. Preston, J. J. Aul, M. Larkins-Pettigrew, J. Rydquist-White, K. W. Anderson, R. M. Wadowsky, D. R. Reagan, E. S. Walker, L. A. Kingsley, A. E. Magit, and G. D. Ehrlich.** 1995. Molecular analysis of bacterial pathogens in otitis media with effusion. *JAMA* **273:**1598–1604.
18. **Teele, D. W., J. O. Klein, B. Roener, and The Greater Boston Otitis Media Study Group.** 1989. Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective cohort study. *J. Infect. Dis.* **160:**83–94.
19. **Vandamme, P., M. Heyndrickx, M. Vancanneyt, B. Hoste, P. De Vos, E. Falsen, K. Kersters, and K. H. Hinz.** 1996. *Bordetella trematum* sp. nov., isolated from wounds and ear infections in humans, and reassessment of *Alcaligenes denitrificans* Ruger and Tan 1983. *Int. J. Syst. Bacteriol.* **46:**849–858.

SUPPLEMENTAL READING

Bannatyne, R. M., C. Clausen, and L. R. McCarthy. 1979. *Cumitech 10, Laboratory Diagnosis of Upper Respiratory Tract Infections.* Coordinating ed., I. B. R. Duncan. American Society for Microbiology, Washington, DC.

Giebink, G. S. 1989. The microbiology of otitis media. *Pediatr. Infect. Dis. J.* **8**(Suppl. 1)**:**18–20.

Hendolin, P. H., L. Paulin, and J. Ylikoski. 2000. Clinically applicable multiplex PCR for four middle ear pathogens. *J. Clin. Microbiol.* **38:**125–132.

3.11.6 *Bordetella* Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

A resurgence of cases of pertussis (whooping cough) and outbreaks in North America in the last decade created a renewed interest in establishing a laboratory diagnosis of the disease. Compared to the period from 1990 to 1993, the period from 1994 to 1996 had average incidences of pertussis among age groups 5 to 9, 10 to 19, and ≥20 years that increased 40, 106, and 93%, respectively, in the United States (6). Clinical diagnosis may be elusive, since the typical cough with whoop occurs late in the illness (weeks 3 to 6) and symptoms are often nonspecific and virus-like, especially in older children and adults. In young children, the disease may be severe, with choking, apnea, or cyanosis, and with significant morbidity and mortality. Adults can serve as a reservoir for transmission of the disease to unvaccinated infants or may suffer from the disease themselves.

Treatment in the early stages of infection reduces the severity of illness and further spread of the disease. Thus, an accurate early laboratory diagnosis remains a key tool in control and prevention. Pertussis is caused primarily by *Bordetella pertussis* and occasionally by *B. parapertussis*. Other species of *Bordetella*, such as *B. holmesii*, *B. hinzii*, and *B. bronchiseptica*, can cause respiratory infections in humans but not specifically "whooping cough."

Bordetella spp. are small, faintly staining, gram-negative coccobacilli. They are nonmotile obligate aerobes that do *not* utilize carbohydrates, and they grow slowly in vitro; some can be recovered from the respiratory tracts of animals. *B. pertussis*, in particular, is very fastidious and must be cultured initially on special media. *B. parapertussis* is less fastidious. *B. parapertussis*, *B. bronchiseptica*, *B. holmesii*, and *B. hinzii* grow on blood agar within 2 to 3 days.

Laboratory confirmation of pertussis is subject to many constraints, and the primary method used, culture, is considered no more than 50% sensitive. Unfortunately, prior to 1996, culture was the only acceptable laboratory criterion for confirming a case of pertussis. Diagnosis by culture is insensitive because of issues related to specimen collection and transport, the fastidious nature of the organism, and interference from other bacterial microbiota. In June 1996, the Council for State and Territorial Epidemiologists revised the laboratory criteria to include positive PCR assays for *B. pertussis* as official laboratory confirmation of pertussis (1). Therefore, PCR has become the test of choice for the diagnosis of pertussis (3, 13, 20). For PCR testing protocol, see procedure 12.2.3, part 11. Reagents (polyclonal and monoclonal) are available for direct fluorescent-antibody (DFA) testing, but a positive DFA result without culture or PCR confirmation is *not* considered suitable confirmation of pertussis in any state in the United States. Culture still has a role in isolation of *B. pertussis* and related species, especially for antimicrobial susceptibility testing (AST) in cases of treatment failure (8). This procedure describes the processing and culturing of specimens for *Bordetella* spp. Refer to Appendix 3.11.6–1 for the DFA procedure and procedure 12.2.3.11, part 11, for PCR.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

◩ **NOTE:** Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

A. Specimen collection

◩ **NOTE:** Cough plates and nasal swabs, previously thought to be ideal specimens, are no longer recommended for culture of *Bordetella*.

1. Timing

a. Collect as soon as possible after symptoms develop.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

b. Collect specimens up to 4 weeks after onset, provided that antimicrobial therapy has not been started.

◩ **NOTE:** The organism is rarely found by culture after the fourth week of illness, and the percentage of positive culture results decreases with time. Positive results have been reported using PCR with specimens collected as late as 60 days after onset of symptoms.

2. Collection of nasopharyngeal specimen

◩ **NOTE:** *B. pertussis* exhibits a tropism and binds specifically to ciliated respiratory epithelial cells. Since the nasopharynx is lined with these cells, it is a far superior site for detection of the bacterium.

a. Nasopharyngeal swabs (refer to Fig. 3.11.6–1A)

◩ **NOTE:** Nasopharyngeal swabs *cannot* be used for PCR since both the alginate component and the aluminum shaft inhibit PCR-based assays (19).

(1) Use a *calcium alginate or Dacron fiber tip swab* on a fine flexible wire. Bend the wire so that it mimics the curve of the nasal airway and gently pass the swab through the nostril to the posterior nasopharynx. Do not force the swab; resistance will be felt when the posterior nasopharynx is reached.

(2) Rotate the swab and leave it in place for up to 30 s or until the patient coughs. Withdraw as quickly as possible.

(3) *Repeat procedure through the second nostril.*

(4) Submit both swabs for culture and DFA testing.

b. Nasal wash: syringe method (refer to Fig. 3.11.6–1B)

(1) Use a 3- to 5-ml syringe with a 2-in. 18-gauge tubing attachment. Fill the syringe with saline.

(2) Instruct the patient not to swallow during the procedure.

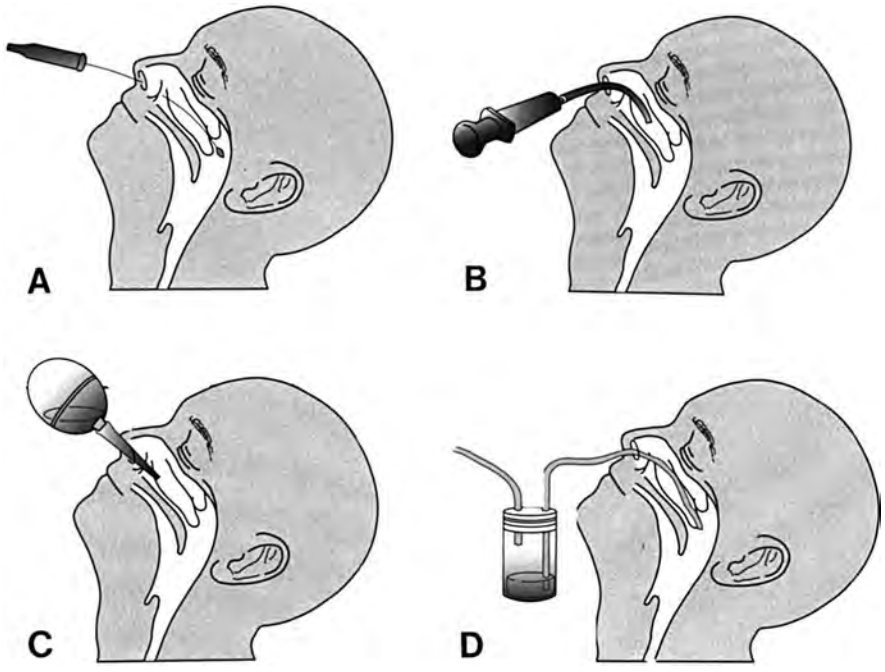


Figure 3.11.6–1 Collection of nasal pharyngeal swab(s) (A), nasal wash specimen(s) by syringe method (B), nasal wash specimen(s) by bulb method (C), and nasal aspirate specimen(s), assisted by vacuum (D). Diagrams courtesy of BD Diagnostic Systems, Sparks, MD, with permission.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

(3) With the patient's head hyperextended (approximately 70° angle), quickly instill approximately 5 ml of sterile 0.85% NaCl into one nostril.
(4) Immediately aspirate the saline solution back into the syringe, *or*
(5) Tilt the head forward and allow the fluid to run out of the nares into a sterile container, *or*
(6) Aspirate the fluid by inserting a rubber bulb syringe into each nostril.
(7) Place the specimen in a sterile container.

c. Nasal wash: bulb method (refer to Fig. 3.11.6–1C)
(1) Suction 3 to 5 ml of sterile 0.85% NaCl into a 1- to 2-oz tapered rubber bulb.
(2) Instruct the patient not to swallow during the procedure.
(3) With the patient's head hyperextended (approximately 70° angle), insert the bulb into one nostril until the nostril is occluded.
(4) Quickly instill the sterile saline into the nostril with one squeeze of the bulb.
(5) Immediately release the bulb to collect the nasal wash specimen.
(6) Empty the bulb contents into a sterile container and transport.

d. Nasal aspirate: vacuum assisted (refer to Fig. 3.11.6–1D)
(1) Connect a mucus trap (i.e., Luken's tube) to a suction pump and catheter, turn on suction, and adjust to suggested suction pressure (*see* chart below).
(2) Insert the end of the catheter though the external nares to the posterior pharynx.
(3) Apply suction while slowly withdrawing the catheter, allowing the catheter to remain in the nasopharynx no longer than 10 s.
(4) After aspiration, flush material out of the catheter with a small volume (1 to 1.5 ml) of sterile saline.

Patient age	Catheter size (French)[a]	Suction pressure (mm Hg)
Premature infant	6	80–100
Infant	8	80–100
Toddler	10	100–120
School age	12	100–120
Adolescent/adult	14	120–150

[a] To determine length of catheter tubing, measure distance from tip of nose to external opening of ear.

◩ **NOTE:** Nasopharyngeal aspiration or wash yields sufficient material for numerous diagnostic procedures and gives a 11% higher yield on culture than nasopharyngeal swabs (7). Aspirate specimens are easily divided and saved, are suitable for all testing methodologies, and can be frozen for long periods (2 years at −70°C).

◩ **NOTE:** Nasopharyngeal specimen collection directions are taken in part with permission from BD Diagnostic Systems, Sparks, MD.

B. Specimen transport

1. Inoculate plates at bedside.

◩ **NOTE:** The best culture results are achieved when specimens are plated directly onto culture media at the bedside; unfortunately, for many this is not possible or practical to establish as a routine. Bedside plating results in a higher positive culture rate that that obtained with nasopharyngeal aspirates taken at the same time and cultured the same day in the laboratory.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

2. Alternatively, submit in transport tube; choose a transport medium based on the length of time the specimen will be in transit.
- **a.** Transport tubes
  - (1) 0.5 to 1% casein hydrolysate (Casamino Acid; Remel, Inc.)
  - (2) Amies transport medium
  - (3) Half-strength charcoal blood agar stab, also called Regan-Lowe transport medium (15) (prepared media available from Hardy Diagnostics)
    - (a) Weigh 25.5 g of CM119 charcoal agar (Oxoid, Inc., Ogdenburg, NY) into 900 ml of water, autoclave, and cool.
    - (b) Add 100 ml of sterile defibrinated horse blood (available from most medium vendors) and 40 mg of cephalexin.
    - (c) Dispense into screw-cap vials to fill half full, seal, and store for up to 2 months at 4°C. Label containers with the expiration date.
- **b.** If delay is brief (≤2 h), use 0.5 to 1% casein hydrolysate (Casamino Acid) solution (Remel, Inc.) as a transport medium. With nasopharyngeal aspirates, add the solution to the specimen container and mix well.
- **c.** If specimens will be plated within 24 h of collection, use Amies medium *with* charcoal.
- **d.** If delay is prolonged (>24 h but ≤3 days), use Regan-Lowe transport medium.
  - (1) Incubate at 35°C for 2 days.
  - (2) Then hold at *room temperature* during transport.
  - (3) For this choice, transit time should not exceed 3 days.
- **e.** If transit time will exceed 3 days, use Regan-Lowe transport medium.
  - (1) Incubate at 35°C for 2 days.
  - (2) Then hold at 4°C during transport.
  - (3) Organisms can remain viable for as long as 8 days with this choice.

C. Rejection criteria

1. Do not perform culture if transport conditions are not followed.

2. Throat specimens, nares swabs, and sputum are unacceptable specimens.

3. Do not perform cultures from specimens collected on rayon or cotton swabs, as they contain fatty acids that inhibit growth.

III. MATERIALS

A. Primary tests

See Appendix 3.11.6–1 for DFA reagents.

B. Media

1. Regan-Lowe agar media containing Oxoid CM119 charcoal agar (51 g/liter), 10% defibrinated horse blood, and 0.04 g of cephalexin per liter (BD Diagnostic Systems; Hardy Diagnostics; Remel, Inc.). Some formulas use cyclodextrin in place of charcoal.
- **a.** Store commercially prepared plates at 4°C for 4 to 8 weeks in sealed container.
- **b.** Prepare in-house from Regan-Lowe base dehydrated medium (CM119).
  - (1) Dehydrated base has a 4-year shelf life.
  - (2) Dehydrated media can be purchased with or without 40 mg of cephalexin per liter.
  - (3) Add 10% horse blood to autoclaved and cooled medium prior to use.
  - (4) Plates are good for 4 to 8 weeks from date of preparation (10).

◩ **NOTE:** Bordet-Gengou agar has been supplanted by Regan-Lowe agar, which has a longer shelf life and greater ability to isolate the organism and is easier to prepare (10). Some *Bordetella* spp. are inhibited by cephalexin, and some investigators recommend using media with and without antimicrobial agents (9). Unfortunately, medium without antimicrobial agents becomes overgrown by contaminants very quickly, and this practice does not appear to increase the positivity rate. As a result, most laboratories do not follow this recommendation.

III. MATERIALS *(continued)*

2. BAP
3. MAC—used to verify growth requirements

C. Identification methods

1. Gram stain (procedure 3.2.1)
2. Catalase test (procedure 3.17.10)
3. Oxidase test (procedure 3.17.39)
4. Rapid urea test (procedure 3.17.48)
5. Nitrate medium (procedure 3.17.35)
6. Mueller-Hinton agar for pigment
7. Serologic reagents
   a. *B. pertussis* agglutination antiserum (BD Diagnostic Systems no. 210590, formerly Difco 2310-50-0) (*B. parapertussis* agglutination reagent is not available)
   b. DFA antisera (*see* Appendix 3.11.6–1).

D. Other supplies

1. Incubator at 35°C
2. Fluorescent microscope

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. See Appendix 3.11.6–1 for QC of DFA reagents.

B. Test each lot of serologic agglutination reagents prior to use and, additionally, at least every 6 months with a positively (*B. pertussis* ATCC 9797) and negatively (*Escherichia coli* ATCC 25922) reacting control.

C. For biochemical tests, see each biochemical procedure for QC requirements.

D. Perform QC on each lot of Regan-Lowe agar. Incubate aerobically overnight at 35°C.

1. Verify that media meet expiration date and QC parameters per current Clinical and Laboratory Standards Institute (formerly NCCLS) M22 document. See procedures 14.2 and 3.3.1 for further details.
2. For controls, it is better to freeze aliquots of clinical specimens that have not been cultivated through several passages.
   a. Freeze in sheep blood, skim milk, or 15% glycerol at −70°C (*see* item V in procedure 14.2).
   b. Use these isolates or ATCC strains for control of media, by following inoculum preparation described in procedure 14.2.

Test organism	Result
Bordetella pertussis ATCC 9797 or in-house strain	Growth
Bordetella parapertussis ATCC 15311	Growth
Staphylococcus aureus ATCC 25923	Partial to complete inhibition

V. PROCEDURE

Observe standard precautions.

A. See Appendix 3.11.6–1 for DFA tests.

◩ **NOTE:** Always perform culture with DFA because of the insensitivity of DFA and the subjectivity of interpretation of DFA tests.

B. Commercial multiplex PCR assays have recently become available for the detection of *B. pertussis*. The ProPertussis (Prodesse, Inc.) assay detects *B. pertussis*. The Pneumoplex (Prodesse, Inc.) PCR assay simultaneously detects *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* as well as *Legionella pneumophila, Legionella micdadei,* and *B. pertussis* (12). PCR detection of *B. pertussis* has been shown to be able to distinguish this organism from other clinically important *Bordetella* spp. and has a much higher sensitivity than other detection methods (16).

C. Culture inoculation

1. Firmly roll swab over one-third of the Regan-Lowe agar surface, and streak carefully for isolation in four quadrants to minimize overcrowding by breakthrough microorganisms that may release inhibitory substances to suppress growth of *Bordetella.*
2. Inoculate a BAP and incubate with the Regan-Lowe agar for comparison of growth.

V. PROCEDURE *(continued)*

D. Incubation

1. Place plates in a plastic bag or moist chamber with a sterile moistened filter paper to avoid drying. *B. pertussis* is very susceptible to drying.

2. Place at 35°C in an aerobic atmosphere (*without 5% CO_2*).

◩ **NOTE:** An incubation temperature of 37°C will not support the growth of many strains of *B. pertussis;* maintaining 35°C is critical.

3. Incubate plates for 5 to 12 days.

◩ **NOTE:** While most colonies of *B. pertussis* and *B. parapertussis* are detected in 5 to 7 days, maximum recovery can take as long as 12 days. Katzko et al. (11) showed that 16% of *B. pertussis* organisms and 50% of *B. parapertussis* organisms were recovered only after extended incubation.

E. Culture examination

1. Observe BAP at 24 h and daily thereafter for a total of 72 h.

a. *B. pertussis* does not grow on BAP, but other species do (Table 3.11.6–1).

b. *B. parapertussis* colonies are smooth and opaque and usually hemolytic.

c. *B. bronchiseptica* colonies are gray-white, somewhat flat, and dull and may have hazy hemolysis.

2. Observe Regan-Lowe plates at 48 h and daily thereafter with the use of a magnifying lens.

a. *B. pertussis* colonies appear at a minimum of 72 h as small, convex, gray, smooth, and very shiny, like drops of mercury. They may vary in size and run together.

b. *B. parapertussis* colonies appear similar to those of *B. pertussis* but are grayer and less domed, and they grow more rapidly.

c. *Legionella* may grow on Regan-Lowe plates and will appear as ground-glass colonies.

3. Gram stain colonies suggestive of *Bordetella,* especially those growing on Regan-Lowe plates and not on BAP.

a. Apply the counterstain (carbol fuchsin or basic fuchsin preferred) for 1 to 2 min to enhance staining intensity.

b. Observe for gram-negative coccobacilli or short rods consistent with *Bordetella* spp.

4. Perform catalase and oxidase tests. *B. pertussis* is positive for both. *B. parapertussis* is catalase positive and oxidase negative.

Table 3.11.6–1 Biochemical differentiation of *Bordetella* species of importance in respiratory cultures[a]

Test	*B. pertussis*	*B. parapertussis*	*B. holmesii* (CDC NO-2)	*B. trematum*	*B. bronchiseptica*[b]	*B. avium*[c]	*B. hinzii*[c]
Motility	−	−	−	+	+	+	+
Urease	−	+	−	−	+	−	−
Oxidase	+	−	−	−	+	+	+
Catalase	+	+	V (W)	+	+	+	+
Growth on MAC	−	+ (D)	+ (D)	+	+	+	+
Soluble pigment (on peptone agar, e.g., MH)	NA	Brown	Brown	−	−	W, amber	−
Nitrate/with gas[d]	NA	−/−	−/−	V	+/−	−/−	−/−
Growth on BAP (hemolysis)	− (NA)	+ (beta)	+ (V green)	+ (−)	+ (rare beta)	+ (V beta)	+ (−)

[a] Table extrapolated from references 4, 17, 18, and 21. NA, not applicable; W, weak; V, variable; D, delayed.

[b] *B. bronchiseptica* grows on salmonella-shigella agar and is tartrate negative, unlike other closely related organisms (*Oligella ureolytica* and *Ralstonia paucula* [IVc-2]). Also see Table 3.18.2–12 for other tests to separate these genera. *Brucella* spp. are similar to *B. bronchiseptica,* but the former are not motile and generally do not grow on MAC.

[c] *B. avium* is malonate negative, and *B. hinzii* is malonate positive; *B. avium* is not a human pathogen (4).

[d] The nitrate reaction is first; if the gas reaction is known, it is listed second, preceded by a slash.

V. PROCEDURE *(continued)*

5. Immediately confirm suspicious colonies.
   - **a.** Perform DFA for *B. pertussis* and *B. parapertussis* (Appendix 3.11.6–1).
   - **b.** For *B. pertussis,* perform slide agglutination test.
     (1) Add a drop of a turbid saline suspension (no. 3 McFarland standard) from a colony suspension to a drop of the working dilution (1:10) of the *B. pertussis* antiserum on a slide.
     (2) Also add a drop of suspension without antiserum for comparison.
     (3) Observe for agglutination under $\times 30$ magnification.
     (4) A 4+ rapid and complete reaction (large clumps and clear background) is considered a positive result.
     (5) No agglutination should be visible in the saline control. If agglutination in the control does occur, the culture is rough and not suitable for serologic techniques.
   - **c.** Additional testing is not necessary unless results are equivocal.
6. If DFA or agglutination is negative, confirm species with biochemical tests.
   - **a.** Perform rapid urea test.
     (1) Both *B. parapertussis* and *B. bronchiseptica* are urea positive.
     (2) *B. pertussis* is negative.
     ■ Brucella *spp. are oxidase, catalase, and urea positive. Work in a biological safety cabinet until this genus has been eliminated from consideration.* Brucella *spp. grow on blood agar, generally not on MAC, and are* not *motile.*
   - **b.** If isolate grows on BAP, inoculate Mueller-Hinton agar for pigment production.
   - **c.** See additional tests for identification of *Bordetella* spp. in Table 3.11.6–1 or submit to reference laboratory for confirmation.
7. Do not perform AST routinely. Seal positive culture plates and hold for at least 7 days at 4°C, should AST be needed for determination of erythromycin resistance.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Negative results

1. If the culture is negative, report "No *Bordetella pertussis* or *Bordetella parapertussis* isolated."
2. Report the number of days the culture was incubated.

B. Positive results

1. If the culture is positive, report the appropriate species identified, such as "*Bordetella pertussis* isolated."
2. Report the presence of *B. pertussis* or *B. parapertussis* as soon as preliminary tests are completed.
   ◩ **NOTE:** The report of *Bordetella* is of critical value because patients with pertussis are placed in isolation rooms.
   Call positive results to the attending physician(s) or caregiver and to infection control for inpatients. Document notification.
3. Pertussis is a reportable disease in most states; however, a positive DFA result without culture or PCR confirmation (procedure 12.2.3, part 11) is *not* considered suitable confirmation of pertussis in any state in the United States.

VII. INTERPRETATION

A. The isolation of *B. pertussis* is always significant. Patients who are symptomatic by definition have pertussis; those who do not have symptoms are carriers and represent a public health risk. Both should be treated.

B. Virtually all *B. pertussis* organisms are susceptible to erythromycin (the drug of choice to treat colonization), negating the need for AST (5). However, erythromycin resistance has been recently reported, and AST may be indicated in

VII. INTERPRETATION *(continued)*

cases of treatment failure. Testing is nonstandard, since it has to be performed on Regan-Lowe media. Both disk diffusion and the Etest method have been used (8).

C. *B. bronchiseptica* has been isolated from lower respiratory tract specimens, usually after 48 h of incubation. Rule out this pathogen, using Table 3.11.6–1, when examining such specimens.

VIII. LIMITATIONS

A. Methods that employ PCR are more rapid and sensitive than culture or DFA techniques.

B. *Legionella* spp. cross-react with *B. pertussis* antiserum (14).

C. Some *B. pertussis* organisms can grow on buffered charcoal-yeast extract medium with α-ketoglutarate, used for recovery of *Legionella*.

D. False-positive results can be caused by misinterpretation of the confirmatory tests.

E. The polyclonal antibody reagents used for DFA are capable of cross-reacting with several non-*B. pertussis* bacteria, resulting in false-positive results (2).

F. The monoclonal DFA reagent may produce a positive result with some strains of *B. bronchiseptica* (package insert, Altachem Pharma Inc.)

G. Treatment with antimicrobial agents can affect both DFA and culture, resulting in false negatives.

REFERENCES

1. **Centers for Disease Control and Prevention.** 1997. Case definitions for infectious conditions under public health surveillance. *MMWR Morb. Mortal. Wkly. Rep.* **46**(RR-10)**:**1–57.
2. **Ewanowich, C. A., L. W. L. Chui, M. G. Paranchych, M. S. Peppler, R. G. Marusyk, and W. L. Albritton.** 1993. Major outbreak of pertussis in northern Alberta, Canada: analysis of discrepant direct fluorescent-antibody and culture results by using polymerase chain reaction methodology. *J. Clin. Microbiol.* **31:**1715–1725.
3. **Farrell, D. J., G. Daggard, and T. K. S. Mukkur.** 1999. Nested duplex PCR to detect *Bordetella pertussis* and *Bordetella parapertussis* and its application in diagnosis of pertussis in nonmetropolitan southeast Queensland, Australia. *J. Clin. Microbiol.* **37:**606–610.
4. **Funke, G., T. Hess, A. von Graevenitz, and P. Vandamme.** 1996. Characteristics of *Bordetella hinzii* strains isolated from a cystic fibrosis patient over a 3-year period. *J. Clin. Microbiol.* **34:**966–969.
5. **Gordon, K. A., J. Fusco, D. J. Biedenbach, M. A. Pfaller, and R. N. Jones.** 2001. Antimicrobial susceptibility testing of clinical isolates of *Bordetella pertussis* from northern California: report from the SENTRY antimicrobial surveillance program. *Antimicrob. Agents Chemother.* **45:**3599–3600.
6. **Guris, D., P. M. Strebel, B. Bardenheier, M. Brennan, R. Tachdjian, E. Finch, M. Wharton, and J. R. Livengood.** 1999. Changing epidemiology of pertussis in the United States: increasing reported incidence among adolescents and adults, 1990–1996. *Clin. Infect. Dis.* **28:**1230–1237.
7. **Hallander, H. O., E. Reizenstein, B. Renemar, G. Rasmuson, L. Mardin, and P. Olin.** 1993. Comparison of nasopharyngeal aspirates with swabs for culture of *Bordetella pertussis. J. Clin. Microbiol.* **31:**50–52.
8. **Hill, B. C., C. N. Baker, and F. C. Tenover.** 2000. A simplified method for testing *Bordetella pertussis* for resistance to erythromycin and other antimicrobial agents. *J. Clin. Microbiol.* **38:**1151–1155.
9. **Hoppe, J. E., and M. Sehlagenhauf.** 1989. Comparison of three kinds of blood and two incubation atmospheres for cultivation of *Bordetella pertussis. J. Clin. Microbiol.* **27:**2115–2117.
10. **Hoppe, J. E., and R. Vogl.** 1986. Comparison of three media for culture of *Bordetella pertussis. Eur. J. Clin. Microbiol. Infect. Dis.* **5:**361–363.
11. **Katzko, G., M. Hofmeister, and D. Church.** 1996. Extended incubation of culture plates improves recovery of *Bordetella* spp. *J. Clin. Microbiol.* **34:**1563–1564.
12. **Khanna, M., J. Fan, K. Pehler-Harrington, C. Waters, P. Douglass, J. Stallock, S. Kehl, and K. J. Henrickson.** 2005. The Pneumoplex assays, a multiplex PCR-enzyme hybridization assay that allows simultaneous detection of five organisms, *Mycoplasma pneumoniae, Chlamydia pneumoniae, Legionella pneumophila, Legionella micdadei,* and *Bordetella pertussis,* and its real-time counterpart. *J. Clin. Microbiol.* **43:**565–571.
13. **Meade, B. D., and A. Bollen.** 1994. Recommendations for the use of the polymerase chain reaction in the diagnosis of *Bordetella pertussis* infections. *J. Med. Microbiol.* **41:**51–55.
14. **Ng, V. L., L. Weir, M. K. York, and W. K. Hadley.** 1992. *Bordetella pertussis* versus non-*L. pneumophila Legionella* spp.: a continuing diagnostic challenge. *J. Clin. Microbiol.* **30:**3300–3301.

REFERENCES *(continued)*

15. **Regan, J., and F. Lowe.** 1977. Enrichment medium for isolation of *Bordetella*. *J. Clin. Microbiol.* **6:**303–309.
16. **Templeton, K. E., S. A. Scheltinga, A. van der Zee, B. M. Diederen, A. M. van Kruijssen, H. Goossens, E. Kuijper, and E. C. Claas.** 2003. Evaluation of real-time PCR for detection of and discrimination between *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella holmesii* for clinical diagnosis. *J. Clin Microbiol.* **41:**4121–4126.
17. **Vandamme, P., M. Heyndrickx, M. Vancanneyt, B. Hoste, P. De Vos, E. Falsen, K. Kersters, and K. H. Hinz.** 1996. *Bordetella trematum* sp. nov., isolated from wounds and ear infections in humans, and reassessment of *Alcaligenes denitrificans* Ruger and Tan 1983. *Int. J. Syst. Bacteriol.* **46:**849–858.
18. **Vandamme, P., J. Hommez, M. Vancanneyt, M. Monsieurs, B. Hoste, B. Cookson, C. H. Wirsing von Konig, K. Kersters, and P. J. Blackall.** 1995. *Bordetella hinzii* sp. nov., isolated from poultry and humans. *Int. J. Syst. Bacteriol.* **45:**37–45.
19. **Wadowsky, R. M., S. Laus, T. Libert, S. States, and G. D. Ehrlich.** 1994. Inhibition of PCR-based assay for *Bordetella pertussis* by using calcium alginate fiber and aluminum shaft components of a nasopharyngeal swab. *J. Clin. Microbiol.* **32:**1054–1057.
20. **Wadowsky, R. M., R. H. Michaels, T. Libert, L. A. Kingsley, and G. D. Ehrlich.** 1996. Multiplex PCR-based assay for detection of *Bordetella pertussis* in nasopharyngeal swab specimens. *J. Clin. Microbiol.* **34:**2645–2649.
21. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria,* 2nd ed. Williams & Wilkins, Baltimore, MD.

SUPPLEMENTAL READING

American Academy of Pediatrics. 2003. Pertussis, p. 472–486. *In* L. K. Pickering (ed.), *2000 Red Book: Report of the Committee on Infectious Diseases,* 26th ed. American Academy of Pediatrics, Elk Grove Village, IL.

Friedman, R. L. 1988. Pertussis: the disease and new diagnostic methods. *Clin. Microbiol. Rev.* **1:**365–376.

Jackson, L. A., J. D. Cherry, S. P. Wang, and J. T. Grayston. 2000. Frequency of serological evidence of *Bordetella* infections and mixed infections with other respiratory pathogens in university students with cough illnesses. *Clin. Infect. Dis.* **31:**3–6.

Müller, F. C., J. E. Hoppe, and C. H. Wirsing von König. 1997. Laboratory diagnosis of pertussis: state of the art in 1997. *J. Clin. Microbiol.* **35:**2435–2443.

APPENDIX 3.11.6–1

Detection of *Bordetella pertussis* by Direct Fluorescent Antibody

I. PRINCIPLE

The DFA test performed on nasopharyngeal specimens is an important adjunct to culture for early presumptive diagnosis of pertussis. DFA tests are rapid but are too insensitive and subjective to be done in lieu of culture. Success in interpreting DFA examinations of clinical material is directly proportional to the experience of the reader. A positive DFA result without culture or PCR confirmation is *not* considered suitable confirmation of pertussis in any state in the United States.

Reagents (polyclonal and monoclonal) are available for testing, but the polyclonal reagent has variable sensitivity and low specificity. The monoclonal reagent contains fluorescein-conjugated anti-*B. pertussis* monoclonal antibody and rhodamine-conjugated anti-*B. parapertussis* monoclonal antibody. Limited information is available at this time concerning the sensitivity and specificity of the monoclonal reagent (2).

DFA is performed directly on the patient specimen but can also be used to confirm an isolate grown in culture as *B. pertussis* or *B. parapertussis.*

II. SPECIMENS

A. Nasopharyngeal specimen (*see* procedure 3.11.6 for collection)

B. Smears prepared from presumptive *B. pertussis* or *B. parapertussis* cultures, to confirm identification

III. MATERIALS

A. DFA reagents

1. Polyclonal reagents: BD Diagnostic Systems (formerly Difco reagents)
   a. Bacto chicken anti-*B. pertussis* fluorescein isothiocyanate (FITC) conjugate (formerly Difco no. 2359-56; BD Diagnostic Systems catalog no. 223591)
   b. Bacto chicken anti-*B. parapertussis* FITC conjugate (formerly Difco no. 2378-56; BD Diagnostic Systems catalog no. 223781)
   c. Reagent preparation
      (1) Bring lyophilized reagents to room temperature.

APPENDIX 3.11.6–1 *(continued)*

(2) To rehydrate, add 5 ml of sterile distilled or deionized water to each vial and rotate gently to completely dissolve contents.
(3) Determine the working dilution of each FITC conjugate immediately after rehydration, since it varies with each lot of reagent, the fluorescent microscope and filters used, and the age of the bulb.
(a) Prepare twofold dilutions of each FITC conjugate ranging from 1:5 to 1:80 in phosphate-buffered saline (PBS) buffer and test using stock strains.
(b) Choose the working dilution as one dilution less than the highest dilution yielding a 4+ fluorescence. For example, if the last dilution yielding a 4+ fluorescence is 1:40, then 1:20 (one less dilution) is the working dilution for that reagent.
(4) Aliquot the working dilution of each conjugate and store at −20°C until the expiration date of original vial.
(5) Label with reagent name, lot number, dilution, and preparation and expiration date.

2. Monoclonal reagents
a. Accu-Mab Plus *B. pertussis*/*parapertussis* DFA (Altachem Pharma Inc., Edmonton, Alberta, Canada)
(1) Catalogue no. BP002-01 (one-vial, 40-test kit)
(2) Catalog no. BP002-04 (four-vial, 160-test kit).
b. Supplied ready for use in PBS, pH 7.2, in an amber glass serum vial
c. Store at 2 to 8°C.

B. Supplies
1. Wash buffer: PBS, 0.01 M phosphate, 0.15 M sodium chloride (pH 7.2 to 7.4)
a. Stock solution
(1) Dissolve 2.56 g of $NaH_2PO_4 \cdot H_2O$ (or 2.89 g of $NaH_2PO_4 \cdot 2H_2O$) and 11.94 g of Na_2HPO_4 in 500 ml of distilled H_2O.
(2) Add 97.66 g of NaCl, and bring to a total volume of 1,000 ml.
(3) Sterilize by autoclaving or filtration and adjust pH to 7.2.
(4) Store at 4°C for up to 6 months.
b. Working solution
(1) Dilute stock 1:10 in sterile distilled H_2O.
(2) Store at 4°C for up to 2 weeks.
2. McFarland standard no. 3 (Appendix 3.16–1)
3. 0.5 to 1% Casamino Acids (catalog no. 060462; Remel, Inc.)
4. 95% Ethanol
5. Staining tray and moisture chamber
6. FA mounting fluid, pH 7.2; (9 parts reagent-grade glycerin to 1 part PBS)
7. 25-μl pipette for monoclonal reagents
8. Miscellaneous: microscope slides, immersion oil, wooden applicator sticks, staining jar, coverslips

C. Fluorescent microscope with excitation and emission filters for FITC conjugate (and rhodamine conjugate for monoclonal reagent). Check with your microscope supplier for the proper combination of barrier and excitation filters needed for use with conjugates.

IV. QUALITY CONTROL

A. Keep both the reagent and stained slides in reduced light to minimize photobleaching of the fluorescent dyes.
B. Always use aseptic technique when opening reagents for use.
1. Perform a visual inspection of the vial for turbidity and/or mold growth prior to each use.
2. Do not use reagents if any signs of contamination (turbidity or growth) are present.

C. Prior to use of new lots and each time a fluorescent antigen test is performed, test a positively and negatively reacting control with *each antiserum.*
1. Positive controls must fluoresce brilliant apple green with the fluorescein filters and bright orange-red with the rhodamine filters, as indicated in the chart below.
2. For positive controls, coccobacilli must be brightly fluorescent at the edges and have dark centers.

APPENDIX 3.11.6–1 *(continued)*

3. A positive control should show a 4+ reaction and the negative control should not exceed a 1+ reaction according to the chart below. See grading system in item VI below.
4. If a positive control is less than 3+, or if a negative control exceeds 1+, the test should be repeated and patient specimen slides should not be read and/or reported.

Test organism	Polyclonal reagent (apple green)		Monoclonal reagent	
	B. pertussis conjugate	*B. parapertussis* conjugate	Fluorescein filter (apple green)	Rhodamine filter (orange-red)
B. pertussis ATCC 9797	Positive, 3–4+	Negative or 1+	Positive, 3–4+	Negative or 1+
B. parapertussis ATCC 15311	Negative or 1+	Positive, 3–4+	Negative or 1+	Positive, 3–4+
*Bordetella (*not *B. pertussis, B. parapertussis,* or *B. bronchiseptica)*—optional			Negative or 1+	Negative or 1+

D. Preparation of positive and negative control slides
1. Store stock strains of QC organisms frozen at −70°C indefinitely (*see* item V in procedure 14.2) or on sealed plates at 4°C for 1 month.
2. Pick appropriate colonies from Regan-Lowe agar and emulsify in 2 ml of sterile distilled or deionized water (use PBS wash buffer for monoclonal reagents) until a cell density equal to a no. 3 McFarland standard is achieved. (An ideal smear will contain 10 to 100 organisms/oil immersion field.)
3. From the cell suspension smear a drop or large loopful of specimen on two areas of each clean glass microscope slide. Each area should be 10 to 15 mm in diameter. Do this for each control organism used. Only one area per slide is needed for monoclonal reagent.
4. Allow the smears to air dry and then fix the slide by a 1-min immersion in 95% ethanol. Remove slides, and allow them to air dry (or for monoclonal reagents, flood with 95% ethanol and allow the ethanol to evaporate at room temperature).
5. Place in a slide storage box, and keep at −20°C for up to 1 year. However, condensation and frost buildup can cause problems over time. Fixed control slides may also be kept in a desiccator in the dark at room temperature for up to 1 year.
6. Each time the test is performed, remove one *B. pertussis* and one *B. parapertussis* control slide from the freezer and allow them to come to room temperature.
7. Test each control slide in the same manner as each patient or culture specimen is tested.

◩ **NOTE:** *B. pertussis* ATCC 12742 and *B. parapertussis* ATCC 15237 are both mutant strains lacking lipopolysaccharide structures that are required for the monoclonal reagent to attach. They must *not* be used as positive controls in this procedure.

V. PROCEDURE

A. Preparation of slides

Observe standard precautions.

1. Specimens
   a. If swab is received, place in a tube containing 0.5 ml of sterile 0.1% casein hydrolysate, and vortex for 20 s.
      ◩ **NOTE:** This step is very important, as smears made directly from the specimen swabs often have so much background fluorescence from nasal secretions that interpretation becomes difficult if not impossible.
   b. For aspirate specimens, vortex the aspirate for 20 s and then use the aspirate directly.

APPENDIX 3.11.6–1 *(continued)*

2. Cultures
   a. Pick appropriate colonies from Regan-Lowe agar and emulsify in 2 ml of sterile distilled or deionized water (*use PBS wash buffer for monoclonal reagents*) until a cell density equal to a no. 3 McFarland standard is achieved.
   b. An ideal smear will contain 10 to 100 organisms/microscopic field.

B. DFA procedure (polyclonal reagents)
1. Process patient specimen, culture, and control slides in the same manner.
2. Smear a drop or large loopfull of emulsified specimen or culture in two separate areas on a glass slide. Each area should be 10 to 15 mm in diameter.
3. Draw a line with a wax pencil to separate sides or, preferably, use Teflon-coated slides with wells (Precision Laboratory Products, Middleton, WI, http://www.precisionslides.com).
4. Fix slides.
   a. Heat fix gently using either a flame or hot plate until warm to the touch *or*
   b. Immerse for 1 min in 95% ethanol.
5. Add several drops (1 drop = 35 μl) of the appropriate FA working conjugate to the fixed smears.
   a. Use FITC-conjugated *B. pertussis* antiserum on one half of each slide and FITC-conjugated *B. parapertussis* antiserum on the other half.
   b. Keep the same antiserum location on each slide (i.e., *B. pertussis* antiserum on left side of each slide).
6. Spread the conjugate over the entire area using a wooden applicator stick.
7. Place the slide(s) in a staining tray or moisture chamber.
8. Incubate at room temperature for 30 min.
9. Remove excess conjugate.
10. Place slide(s) in a staining jar containing PBS wash buffer for 10 min; change buffer two times during wash step.
11. Rinse for 2 min in distilled water.
12. Remove the slide(s), allow to drain, and air dry or blot with bibulous paper.
13. Add a small drop of mounting fluid (pH 7.2) to the center of each stained area, and mount each with a coverslip.
14. Examine slides using a fluorescent microscope with filters for fluorescein conjugate.
15. Examine control slides with 100× objective before proceeding to specimen slides.
16. Screen specimen slides with 40× objective and confirm with 100× objective.

C. DFA procedure (monoclonal reagents)
1. Process patient specimen, culture, and control slides in the same manner.
2. Smear a drop or large loopful of emulsified specimen or culture on a glass slide approximately 10 to 15 mm in diameter.
3. Fix slides immediately.
   a. Flood the smear with 95% ethanol and allow the ethanol to evaporate at room temperature.
   b. In addition, if specimen was transported or mixed in casein hydrolysate, dip the slide several times in PBS wash buffer to remove casein and allow slide(s) to dry.
4. Add 25 μl of the DFA reagent to each fixed smear.
5. Spread the reagent over the entire area using a wooden applicator stick.
6. Place the slide(s) in a moisture chamber for 30 min at room temperature. Do not allow the reagent to dry on the slide(s) or results will not be accurate.
7. Place slide(s) in a staining jar containing PBS wash buffer for 5 min.
8. Place positive control slides in a separate staining jar.
9. Repeat wash with fresh wash buffer for an additional 5 min. (If background fluorescence is found to be too high, increase wash steps to 10 min each.)
10. Dip slide(s) for 1 min in distilled water.
11. Blot excess water from slide(s) with lint-free tissue or bibulous paper and allow to air dry.
12. Add a small drop of mounting fluid (pH 7.2) to the center of each stained area, and mount each with a coverslip.

APPENDIX 3.11.6–1 *(continued)*

13. Examine slides with fluorescent microscope with filters for both fluorescein and rhodamine.
    a. Since fluorescein fades more rapidly than rhodamine after light exposure, examine each slide with fluorescein filters before proceeding to rhodamine filters.
    b. Examine control slides with 100× objective before proceeding to specimen slides.
    c. Screen specimen slides with 40× objective and confirm with 100× objective.

VI. INTERPRETATION

A. Score fluorescence from 1+ to 4+.
    1. Score immediately when each field is first viewed since the dyes, especially fluorescein, may fade rapidly after exposure to the excitation beam of the microscope.
    2. Use the following grading for controls and specimens with fluorescein conjugate and filters.
        a. 4+: brilliant apple green fluorescence with clear-cut cell outline and sharply defined center
        b. 3+: bright apple green peripheral fluorescence with clear-cut cell outline and sharply defined center.
        c. 2+: definite but dull apple green peripheral fluorescence with cell outline less well defined
        d. 1+: very dim, barely visible apple green fluorescence with cell outline indistinguishable from center
    3. Use the following grading for controls and specimens with rhodamine conjugate and filters.
        a. 4+: brilliant orange-red fluorescence with clear-cut cell outline and sharply defined center
        b. 3+: bright orange-red peripheral fluorescence with clear-cut cell outline and sharply defined center
        c. 2+: definite but dull orange-red peripheral fluorescence with cell outline less well defined
        d. 1+: very dim, barely visible orange-red fluorescence with cell outline indistinguishable from center

B. A positive cell must appear as a small oval-shaped or coccobacillary cell with an intense (3+ to 4+) fluorescence surrounding a *dark cell center*.
    1. Ignore occasional nonspecifically staining diplococci, or diphtheroid-like rods.
    2. Consider typical morphology as well as fluorescence in the interpretation.

C. For a positive result
    1. Polyclonal reagents
        a. Slide positive for *B. pertussis* must show *four or more cells* with the characteristic morphology and 3 to 4+ apple green fluorescence on the area stained with *B. pertussis* antiserum, with no or 1+ fluorescence on the area stained with *B. parapertussis* antiserum.
        b. Slide positive for *B. parapertussis* must show *four or more cells* with the characteristic morphology and 3 to 4+ apple green fluorescence on the area stained with *B. parapertussis* antiserum, with no or 1+ fluorescence on the area stained with *B. pertussis* antiserum.
    2. Monoclonal reagents
        a. Slide positive for *B. pertussis* must show one cell with the characteristic morphology and 3 to 4+ yellow-green fluorescence with fluorescein filters and no or 1+ fluorescence with rhodamine filters.
        b. Slide positive for *B. parapertussis* must show one cell with the characteristic morphology and 3 to 4+ orange-red fluorescence with rhodamine filters and no or 1+ fluorescence with fluorescein filters.
        c. Depending on your microscope filters, positive *B. parapertussis* cells may give a faint orange fluorescence (maximum, 1+) when viewed using the fluorescein filter. This faint orange fluorescence should not be confused with the expected bright green fluorescence of positive *B. pertussis* cells.

APPENDIX 3.11.6–1 *(continued)*

d. Depending on your microscope filters, positive *B. pertussis* cells may give a pale yellow fluorescence (maximum, 1 +) when viewed using the rhodamine filter. This pale yellow fluorescence should not be confused with the expected bright orange-red fluorescence of positive *B. parapertussis* cells.

◩ **NOTE:** The manufacturer claims that the monoclonal reagents are so specific that a slide should be considered positive for *B. pertussis* or *B. parapertussis* if a single cell with the characteristic morphology and appropriate fluorescence is observed. The single evaluation of the monoclonal reagent published to date used the stringent criterion of a minimum of four fluorescing organisms with typical morphology to qualify as a positive, but it was felt that the manufacturer's recommendation of a single cell was valid (2).

VII. REPORTING RESULTS

A. Negative results
   1. Report the DFA test result as "Negative for *Bordetella pertussis* and *Bordetella parapertussis* by direct fluorescent-antibody testing."
   2. If an isolate from a culture does not fluoresce with reagents, report "No *Bordetella pertussis* or *Bordetella parapertussis* isolated."

B. Positive results
   1. For positive DFA test, report "Positive for *Bordetella pertussis* or *Bordetella parapertussis* by DFA."
   2. For positive DFA test from culture, report "Positive for *Bordetella pertussis* or *Bordetella parapertussis*" depending on which conjugate gave a positive result.

VIII. LIMITATIONS

A. Methods that employ PCR are more rapid and sensitive than culture or DFA techniques.
B. Polyclonal reagent for DFA has variable sensitivity and low specificity.
C. False-positive results can be caused by misinterpretation of the confirmatory tests.
D. The polyclonal antibody reagents used for DFA are capable of cross-reacting with several non-*B. pertussis* bacteria, resulting in false-positive results (1).
E. The monoclonal DFA reagent may produce a positive result with some strains of *B. bronchiseptica* (package insert, Altachem Pharma Inc.)
F. *Legionella* spp. cross-react with *B. pertussis* antiserum (3).
G. Treatment with antimicrobial agents can affect both DFA and culture, resulting in false negatives.

References

1. **Ewanowich, C. A., L. W. L. Chui, M. G. Paranchych, M. S. Peppler, R. G. Marusyk, and W. L. Albritton.** 1993. Major outbreak of pertussis in northern Alberta, Canada: analysis of discrepant direct fluorescent-antibody and culture results by using polymerase chain reaction methodology. *J. Clin. Microbiol.* **31:**1715–1725.
2. **McNichol, P., S. M. Giercke, M. Gray, D. Martin, B. Brodeur, M. S. Peppler, T. Williams, and G. Hammond.** 1995. Evaluation and validation of a monoclonal immunofluorescent reagent for direct detection of *Bordetella pertussis. J. Clin. Microbiol.* **33:**2868–2871.
3. **Ng, V., L. Weir, M. K. York, and W. K. Hadley.** 1992. *Bordetella pertussis* versus non-*L. pneumophila Legionella spp.:* a continuing diagnostic challenge. *J. Clin. Microbiol.* **30:**3300–3301.

Supplemental Reading

Gilchrist, M. J. R. 1990. Laboratory diagnosis of pertussis. *Clin. Microbiol. Newsl.* **12:**49–53.

Halperin, S. A., R. Bortolussi, and A. J. Wort. 1989. Evaluation of culture, immunofluorescence, and serology for the diagnosis of pertussis. *J. Clin. Microbiol.* **27:**752–757.

Müller, F. C., J. E. Hoppe, and C. H. Wirsing von König. 1997. Laboratory diagnosis of pertussis: state of the art in 1997. *J. Clin. Microbiol.* **35:**2435–2443.

Streubel, P. M., S. L. Cochi, K. M. Farizo, B. J. Payne, S. D. Hanauer, and A. L. Baughman. 1993. Pertussis in Missouri: evaluation of nasopharyngeal culture, direct fluorescent antibody testing, and clinical case definitions in the diagnosis of pertussis. *Clin. Infect. Dis.* **16:**276–285.

Young, S. A., G. L. Anderson, and P. D. Mitchell. 1987. Laboratory observations during an outbreak of pertussis. *Clin. Microbiol. Newsl.* **9:**176–179.

3.11.7 *Corynebacterium diphtheriae* Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Diphtheria is an acute, toxin-mediated, communicable infectious disease primarily of the upper respiratory tract (throat, larynx) and occasionally of the skin. It is caused by toxigenic lysogenized strains of *Corynebacterium diphtheriae,* of which there are four biotypes: mitis, intermedius, belfani, and gravis. Diphtheria exacts a high mortality rate, approximating 10%. A less severe diphtheria-like disease (pharyngitis and atypical symptoms, such as endocarditis, septic arthritis, and other forms of systemic disease) caused by nontoxigenic strains has been reported. The classic pseudomembrane of the pharynx may be lacking in mild cases, which usually mimic streptococcal pharyngitis.

Although the number of cases reported in the United States has significantly declined, studies of diphtheria immunity levels among adults in the United States have shown that from 20 to 90% do not possess adequate immunity against the disease (12). In 1996, isolation of toxigenic *C. diphtheriae* from the blood of an American Indian woman living in the Northern Plains region of the United States prompted public health officials to conduct diphtheria surveillance in the patient's community, resulting in the recovery of 10 *C. diphtheriae* isolates (3, 11, 12). There is currently no geographic concentration of cases in the United States. Antimicrobial agents have little or no effect on the clinical outcome, and laboratory confirmation of diphtheria often requires several days, yet diagnosis and antimicrobial treatment are essential in limiting further spread (6, 13).

This procedure presents proper specimen collection and transport and culture for *C. diphtheriae.* Some laboratories may not be able to store the culture medium because of few requests and may elect to submit specimens to a reference laboratory for culture. It is a CAP requirement that the laboratory have available a mechanism to provide culture for *C. diphtheriae,* when requested.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

◩ **NOTE:** Refer to procedure 3.3.1 for additional details on specimen collection. The following can be copied for preparation of a specimen collection instruction manual for physicians and other caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

A. Specimen collection

1. Contact the clinical microbiology laboratory prior to specimen collection for diphtheria, because special techniques and/or media are required for the isolation of these agents.
2. In case of respiratory diphtheria, obtain material for culture on a swab (either a cotton- or a polyester-tipped swab) from the inflamed areas in the nasopharynx (5).
   - **a.** If membranes are present and can be removed, swab from beneath the membrane.
   - **b.** Collect from several areas to increase sensitivity.
   - **c.** Collect nasopharygeal swabs from suspected carriers.
   - **d.** For details, see Specimen Collection, Transport, and Handling for culture of beta-hemolytic streptococci, procedure 3.11.8.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

3. If cutaneous diphtheria is suspected, collect skin (aspirate/swab), throat (swab), and nasopharynx (swab) specimens (13, 14).

B. Specimen transport

1. Use any routine swab collection systems, such as Amies or Stuart's medium, because the organism is not fastidious (2).

   ◩ **NOTE:** BD Culturette EZ is preferred because the polyurethane adsorbs and releases >95% of the organisms, unlike other swabs that absorb the organisms, making release inefficient.
2. If inoculation of media is anticipated to exceed 24 h, use silica gel transport packages or sterile, desiccated silica gel crystals in tubes (6). However, it is best to avoid delays in culture inoculation to provide speed and accuracy in the diagnosis.
3. Follow the guidelines for the packaging and shipping of infectious substances as outlined in the respective Department of Transportation (DOT) or International Air Transport Association (IATA) regulations for shipping of specimens and/or cultures suspected of containing *C. diphtheriae* (*see* procedure 15.6).
4. Include patient demographics on the culture request form (age, sex, and name), including vaccination and travel history. Record collection time and source of specimen.

C. Rejection criteria

1. Specimens not representative of the anatomic site from which *C. diphtheriae* is commonly found
2. Specimens not accompanied by appropriate information (patient name, age, and vaccination history; source of specimen)
3. Specimens submitted in unapproved packaging and shipping containers
4. Specimen transport time exceeds 24 h and specimen is not contained in silica gel transport medium.

III. MATERIALS

A. Media

1. BAP inoculated for the detection of hemolytic streptococci and *C. diphtheriae* present in large numbers. Addition of a 50-μg disk of fosfomycin (BD Diagnostic Systems) to the initial inoculum will inhibit most contaminating oral microbiota and allow coryneforms to grow (6).
2. Selective media (*see* Appendix 3.11.7–1 for details)
   a. Cystine tellurite blood agar (CTBA) (Remel, Inc.) or serum tellurite agar with lamb serum (BD Diagnostic Systems)

      ◩ **NOTE:** This medium inhibits oronasal microbiota and turns colonies of corynebacteria, staphylococci, and yeasts black.
   b. Tinsdale (TIN) agar base medium (Remel, Inc.)

      ◩ **NOTE:** This medium contains cysteine and forms a halo around colonies that produce cystinase.
3. Slants (optional)
   a. Loeffler agar slant (LAS) (Remel, Inc.) or Loeffler blood serum with beef serum (BD Diagnostic Systems) *or*
   b. Pai agar slant (formula in Appendix 3.11.7–1)
4. Todd-Hewitt broth with 3% sterile rabbit blood (optional). Defibrinated rabbit blood is available from most medium vendors and from Hemostat (Los Angeles, CA, [800] 572-6888) or Quad-5 (Ryegate, MT, [406] 568-2911).

B. Reagents

1. Gram stain reagents (*see* procedure 3.2.1)
2. Alkaline Loeffler methylene blue stain (LMBS) (optional)
   a. Dissolve 0.3 g of methylene blue in 30 ml of 95% ethanol.
   b. Then add 100 ml of 0.01% potassium hydroxide.
3. 3% Hydrogen peroxide for catalase (*see* procedure 3.17.10)

III. MATERIALS *(continued)*

4. Conventional biochemical media: see procedures 3.17 and 3.18.1.
- **a.** Sugar fermentation media (procedure 3.17.9)
  - (1) Preferably cysteine Trypticase agar for determining oxidation (found at surface of the tube) and fermentation (acid or alkali production in entire tube) for acid production from glucose, maltose, sucrose, mannitol, and xylose, *or*
  - (2) Rapid sugar fermentation tubes (Remel, Inc.)
- **b.** Nitrate broth (procedure 3.17.35)
- **c.** Motility media (procedure 3.17.31)
- **d.** Urea hydrolysis (procedure 3.17.48)
- **e.** Esculin hydrolysis (procedure 3.17.5)
- **f.** A beta-hemolysin-producing strain (e.g., *Staphylococcus aureus* ATCC 25923) for CAMP reaction (procedure 3.17.8)

5. Commercial identification systems: RapID CB Plus system (Remel, Inc.), API (RAPID) Coryne system (bioMérieux), Biolog GP plate (Biolog, Hayward, CA), BBL CRYSTAL GP system (BD Diagnostic Systems)

◩ **NOTE:** Publications related to the performance of these systems are limited to the first three systems (7–10). It is recommended that the use of any of the above-listed commercial identification systems be supplemented with macroscopic morphology and Gram stain characteristics. Any isolate that is unidentifiable and deemed to be clinically significant should be sent to a creditable reference laboratory with expertise in identification of corynebacteria, especially for identification of toxin-producing *C. diphtheriae.*

C. Other supplies
- **1.** Incubator at 35°C
- **2.** Microscope

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Verify that media meet expiration date and QC parameters per Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22-A3 (5). See procedures 14.2 and 3.3.1 for further procedures.

B. QC checks need not be performed in-house on commercially purchased BAP. However, each new lot number and each new shipment of CTBA, LAS, Pai, and TIN must be tested with stock strains of *C. diphtheriae* (preferably biotype intermedius) for characteristic growth (13). Incubate aerobically overnight at 35°C.

Test organism	Result
Corynebacterium diphtheriae bv. intermedius ATCC 9675 or ATCC 51696	CTBA: growth, gray to black colonies, some with dark centers LAS: growth Pai: growth TIN: black colony with halo
Streptococcus pyogenes ATCC 19615	TIN: black, pinpoint without halos
Staphylococcus aureus ATCC 25923	CTBA: inhibition (partial to complete)

C. Test each lot of reagents and kits used for identification with organisms known to produce reliable positive and negative reactions.

D. Test methylene blue stain with a characteristic *Corynebacterium* and another gram-positive microorganism with each use of the test.

V. PROCEDURE

◩ **NOTE:** Refer to Fig. 3.11.7–1 for a flowchart of steps in the isolation, detection, and identification of *C. diphtheriae*.

Observe standard precautions.

A. Inoculation

◩ **NOTE:** Use of a biosafety cabinet will avoid contamination of the culture or specimen as well as protect laboratory processing personnel.

1. If the swab appears desiccated, was collected several days prior to receipt, or is received in silica gel, place it into Todd-Hewitt broth supplemented with 3% sterile rabbit blood, incubate it overnight, and then inoculate isolation media.

2. Inoculate the specimen to CTBA and BAP; streak for isolation. Incubate media at 35°C aerobically.

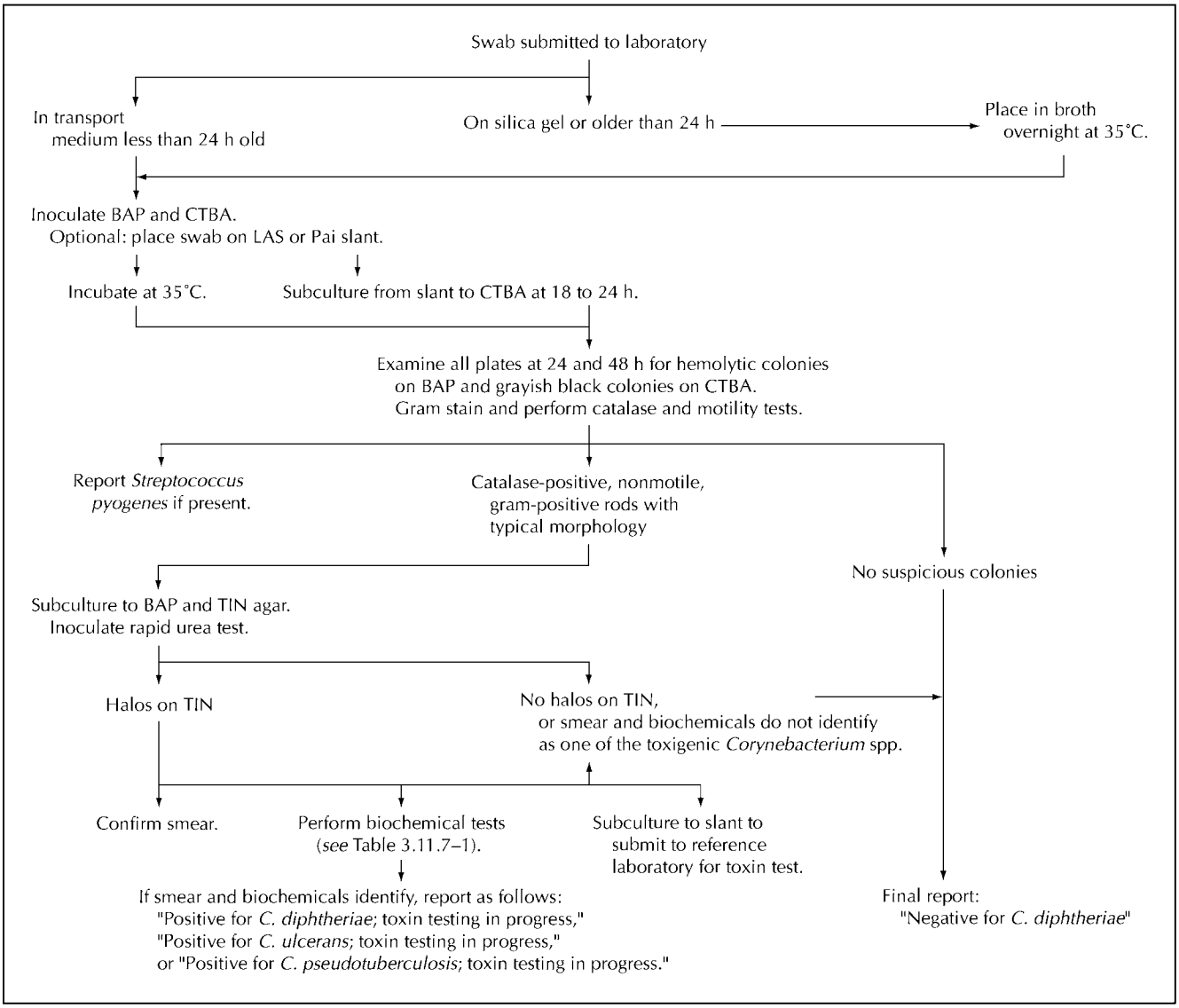

Swab submitted to laboratory
In transport medium less than 24 h old
On silica gel or older than 24 h
Place in broth overnight at 35°C.
Inoculate BAP and CTBA.
Optional: place swab on LAS or Pai slant.
Incubate at 35°C.
Subculture from slant to CTBA at 18 to 24 h.
Examine all plates at 24 and 48 h for hemolytic colonies on BAP and grayish black colonies on CTBA.
Gram stain and perform catalase and motility tests.
Report *Streptococcus pyogenes* if present.
Catalase-positive, nonmotile, gram-positive rods with typical morphology
No suspicious colonies
Subculture to BAP and TIN agar.
Inoculate rapid urea test.
Halos on TIN
No halos on TIN, or smear and biochemicals do not identify as one of the toxigenic *Corynebacterium* spp.
Confirm smear.
Perform biochemical tests (*see* Table 3.11.7–1).
Subculture to slant to submit to reference laboratory for toxin test.
If smear and biochemicals identify, report as follows:
"Positive for *C. diphtheriae*; toxin testing in progress,"
"Positive for *C. ulcerans*; toxin testing in progress,"
or "Positive for *C. pseudotuberculosis*; toxin testing in progress."
Final report:
"Negative for *C. diphtheriae*"

Figure 3.11.7–1 Flowchart for evaluation of culture for *C. diphtheriae*.

V. PROCEDURE *(continued)*

3. Optionally, inoculate an LAS *or* a Pai agar slant, and leave the swab on the slant during incubation.
   a. Incubate media at 35°C aerobically.
   b. After 18 to 24 h of incubation, subculture the agar slant to a second plate of CTBA.

B. Culture evaluation
   1. Examine all agar plates at 24 and 48 h for colonies typical of *C. diphtheriae* (*see* descriptions below). Pursue any beta-hemolytic colonies on BAP and dark colonies on CTBA.
   2. Subculture colonies that are catalase positive and exhibit typical morphology on Gram stain (Fig. 3.11.7–2) to the following.
      a. BAP to provide growth for identification procedures
      b. Modified TIN plate. Stab the agar in several places for halos.
   3. Incubate plates aerobically at 35°C and examine at 24 and 48 h. Do *not* incubate in CO_2.

C. Morphology of *C. diphtheriae*
   1. Cellular morphology (Fig. 3.11.7–2 and reference 1)
      a. Gram stain: pleomorphic gram-positive rods that occur in angular arrangements (commonly referred to as palisades or Chinese letters); possibly coccobacillary forms, most notably in older cultures

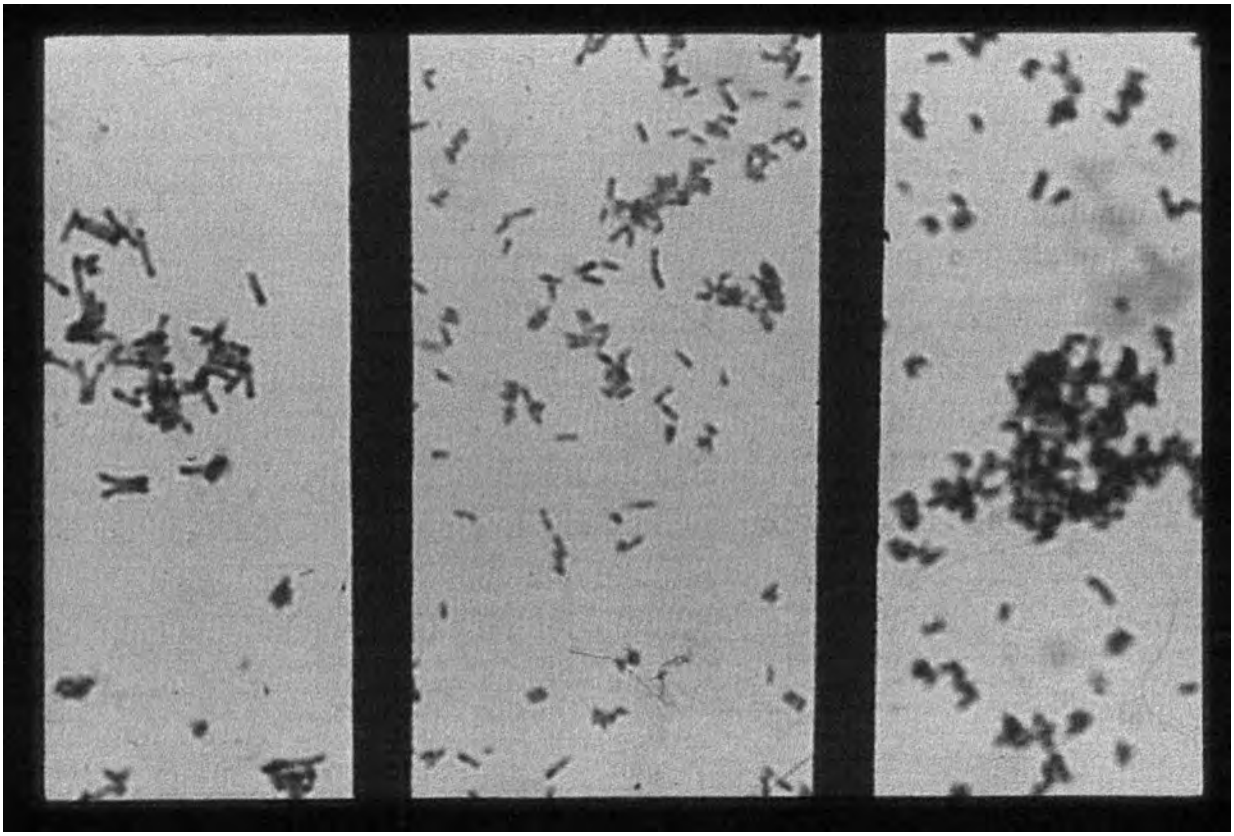


Figure 3.11.7–2 Microscopic morphology of three types of *C. diphtheriae* grown on Loeffler media and stained with Loeffler methylene blue: from right to left they are biotypes gravis, mitis, and intermedius. Photos reproduced from Clinical Microbiology, ASM Committee on Educational Materials, 1985.

V. PROCEDURE *(continued)*

b. LMBS: pleomorphic beaded rods whose ends may be swollen, producing a club shape; angular arrangements; reddish purple metachromatic granules or bars apparent

2. Colonial morphology

a. CTBA: grayish black (gunmetal gray) and 1 to 3 mm in size, with a garlic-like odor

(1) Biotype intermedius: smallest, flat

(2) Biotypes mitis and gravis: larger, convex, smooth or wrinkled

b. BAP (Fig. 3.11.7–3 and reference 1)

(1) Biotype intermedius: smaller, flat, creamy, transparent, nonhemolytic

(2) Biotypes mitis and gravis: larger, convex, with weak beta-hemolysis

(3) *Streptococcus pyogenes:* gram-positive cocci in chains; production of beta-hemolysis

c. TIN: brownish black zone (halo) around the colony darkens with age.

D. Identification procedures

1. Examine TIN at 24 and 48 h for brownish black halos.

2. Make a suspension of suspected colony on any of the agars in Todd-Hewitt broth. Use this suspension to do the following.

a. Prepare stains

(1) Examine LMBS or Gram stain black colonies to confirm characteristic cellular morphology. *Staphylococcus* spp. may produce a characteristic halo on TIN.

(2) Perform wet mount motility to eliminate *Bacillus* spp.

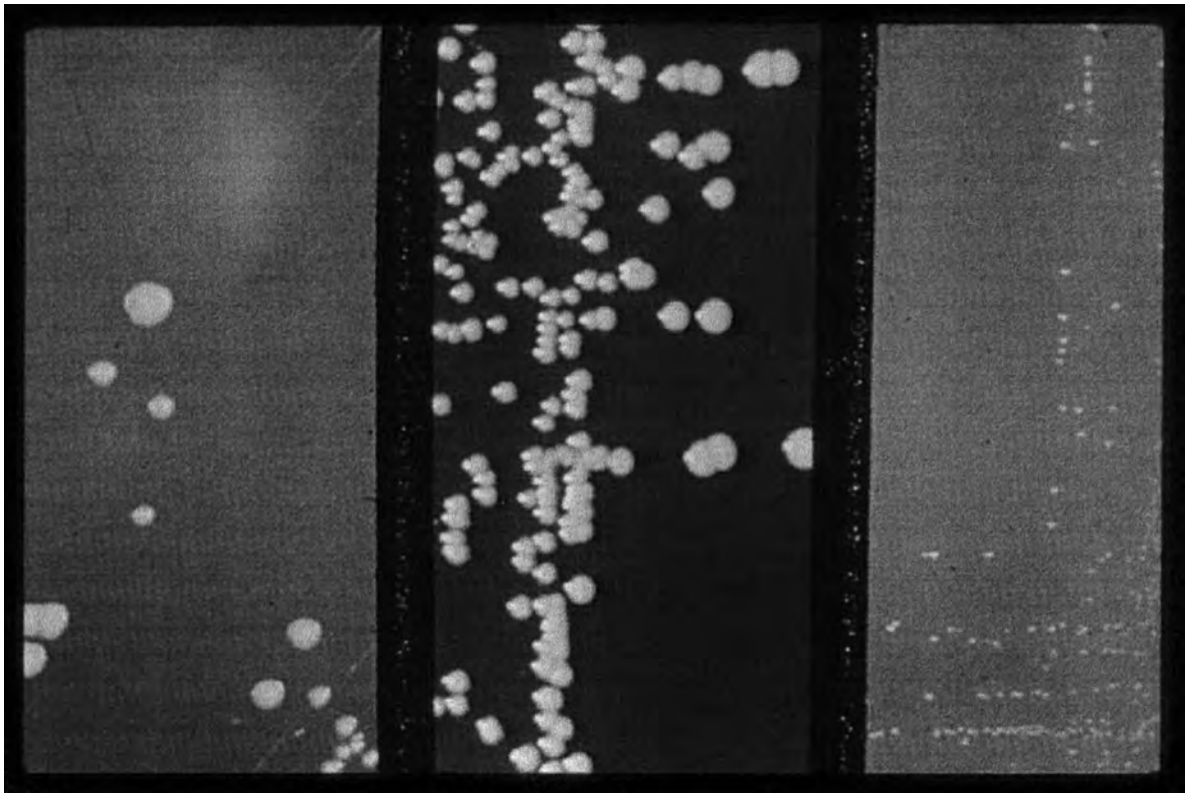


Figure 3.11.7–3 Colonial morphology of *C. diphtheriae* grown on BAP for 48 h; from right to left they are biotypes gravis, mitis, and intermedius. Photos reproduced from Clinical Microbiology, ASM Committee on Educational Materials, 1985.

V. PROCEDURE *(continued)*

b. Inoculate CAMP test.

c. Inoculate biochemical tests.

(1) Conventional

(a) Urea

i. For agar slant, overlay urea slant with sterile mineral oil.

ii. Or use rapid disk test (preferred) (procedure 3.17.48)

a. *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* produce a characteristic halo on TIN and are urea positive (Table 3.11.7–1).

◩ **NOTE:** *C. ulcerans* has been known to cause diphtheria-like disease and can elaborate diphtheria toxin. *C. pseudotuberculosis* can also produce the toxin.

b. *C. diphtheriae* is urea negative.

(b) Nitrate

(c) Sugar fermentation

i. Conventional media may be supplemented with a drop or two of sterile rabbit serum to enhance growth (*see* procedure 3.17.9). Incubate aerobically at 35°C and examine daily for 3 days, *or*

ii. Inoculate 1 drop of each rapid sugar identification medium heavily with a loopful of colony, making an even suspension. Incubate for 1 h in a 37°C water bath. Interpret as for conventional tests.

(2) As an alternative to the above-mentioned biochemical tests, inoculate a commercial identification test kit and read according to the manufacturer's instructions.

E. If identification procedures indicate *C. diphtheriae, C. ulcerans,* or *C. pseudotuberculosis,* ship (per DOT or IATA packaging and shipping guidelines) LAS or Pai or other slant to a public health laboratory for toxigenicity testing. The isolate must be toxigenic to confirm a clinical diagnosis of diphtheria (13). Contact the local public health laboratory to determine where it wishes the isolate to be sent. The CDC Pertussis and Diphtheria Laboratory performs toxin testing 7 days a week.

F. Antimicrobial susceptibility testing

In the absence of published CLSI guidelines for susceptibility testing of corynebacteria (4), it is recommended that if testing is requested, tests be performed on Mueller-Hinton agar with sheep blood using the Etest method (procedure 5.8). Report the MIC without an interpretive breakpoint, unless it is obviously resistant.

Table 3.11.7–1 Key biochemical reactions to identify toxic *Corynebacterium* species[a]

Species	Halos on TIN	Motility	Nitrate	Urea	Hemolysis	Can have diphtheria toxin	Reverse CAMP
C. diphtheriae	+	−	+[b]	−	V	+	−
C. ulcerans	+	−	−	+	+	+	+
C. pseudotuberculosis	+	−	V	+	+	+	+
C. pseudodiphtheriticum	Unknown	−	+	+	−	−	−

[a] Table extrapolated from text and tables in reference 6. +, positive; −, negative; V, variable. *C. ulcerans* is trehalose and glycogen positive; *C. pseudotuberculosis* is not. Many commercial kits do an excellent job of identification of these species. *C. diphtheriae, C. ulcerans,* and *C. pseudotuberculosis* are pyrazinamidase negative; *C. pseudodiphtheriticum* and many common corynebacteria are positive. For laboratories that must screen numerous isolates for *C. diphtheriae,* tablets to rapidly test for pyrazinamidase activity are available from Key Scientific.

[b] *C. diphtheriae* biotype belfani is nitrate reductase negative.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. If the culture is negative, report "No *Corynebacterium diphtheriae* isolated after 3 days."

B. Positive culture results

1. If biochemical results are consistent with *C. diphtheriae,* report "Presumptive for *Corynebacterium diphtheriae;* sent to public health laboratory for confirmation and toxigenicity testing." Do not report any enumeration.

2. Notify physician and infection control of positive results. Diphtheria is a reportable disease in most states.

3. Document notification.

VII. INTERPRETATION

A. Pleomorphic gram-positive rods that usually grow well on BAP and are catalase positive, nonmotile, CAMP negative, and urea negative and form halos on TIN are presumptively *C. diphtheriae.*

B. Confirm such results with further biochemical kit or sugar tests.

C. Phenotypic identification of *C. diphtheriae* does not mean that the organism is a toxin producer. Toxin genes are carried on plasmids which can be lost. Toxin production must be demonstrated.

VIII. LIMITATIONS

A. Smears made directly from clinical specimens are not reliable for diagnostic purposes, since metachromatic granules are found in other bacteria as well (13). Methylene blue stain (Loeffler) smears from media incubated for a short time are no longer recommended, for the same reason.

B. *C. diphtheriae* must be a toxigenic strain to be diagnostic of diphtheria.

C. TIN is not suitable as a primary plating medium, since it may not support the growth of some strains of *C. diphtheriae.*

D. Do not read TIN too soon, since several organisms may give some slight browning on this medium in 18 h.

E. Incubation at 5 to 10% CO_2 retards the development of halos on TIN.

F. Black colonies on CTBA can be due to other organisms capable of reducing tellurite to tellurium. Other *Corynebacterium* spp., staphylococci, and some streptococci possess this ability.

G. *C. diphtheriae* rapidly loses viability in saline. Use broth for suspensions of the organism or to moisten specimen swabs.

H. Sterile rabbit serum will enhance growth of *C. diphtheriae,* in particular biotype intermedius, and is especially advised in biochemical media.

I. Toxigenicity testing (modified Elek test) requires skill and experience in the preparation of reagents and interpretation of results and is therefore best left to public health laboratories or other reference laboratories that perform these procedures with some regularity.

REFERENCES

1. **Buxton, R.** 2007. *Examination of Gram Stains of Bacterial Skin Infections.* http://microbelibrary.org.
2. **CDC Pertussis and Diphtheria Laboratory.** *Instructions for Specimen Collection and Transport for Laboratory Testing of* C. diphtheriae. Centers for Disease Control and Prevention, Atlanta, GA. http://cdc.gov/vaccines/vpd-vac/diphtheria/dat/downloads/dipcollection.rtf.
3. **Centers for Disease Control and Prevention.** 1997. Toxigenic *Corynebacterium diphtheriae*—Northern Plains Indian community. August–October 1996. *MMWR Morb. Mortal. Wkly. Rep.* **46:**506–510.

REFERENCES *(continued)*

4. **Clinical and Laboratory Standards Institute.** 2008. *Performance Standards for Antimicrobial Susceptibility Testing.* Eighteenth informational supplement. Approved standard M100-S18. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
6. **Funke, G., and K. Bernard.** 2007. Coryneform gram-positive rods, p. 485–514. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. C. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
7. **Funke, G., K. Peters, and M. Aravena-Roman.** 1998. Evaluation of the RapID CB Plus system for identification of coryneform bacteria and *Listeria* spp. *J. Clin. Microbiol.* **36:**2439–2442.
8. **Funke, G., F. N. R. Renaud, J. Freney, and P. Riegel.** 1997. Multicenter evaluation of the updated and extended API (RAPID) Coryne database 2.0. *J. Clin. Microbiol.* **35:**3122–3126.
9. **Hudspeth, M. K., S. H. Gerardo, D. M. Citron, and E. J. C. Goldstein.** 1998. Evaluation of the RapID CB Plus system for identification of coryneform species and other gram-positive rods. *J. Clin. Microbiol.* **36:**543–547.
10. **Lindenmann, K., A. von Graevenitz, and G. Funke.** 1995. Evaluation of the Biolog system for the identification of asporogenous, aerobic gram-positive rods. *Med. Microbiol. Lett.* **4:**287–296.
11. **Marston, C. K., F. Jamieson, F. Cahoon, G. Lesiak, A. Golaz, M. Reeves, and T. Popovic.** 2001. Persistence of a distinct *Corynebacterium diphtheriae* clonal group within two communities in the United States and Canada where diphtheria is endemic. *J. Clin. Microbiol.* **39:**1586–1590.
12. **Popovic, T., C. Kim, J. Reiss, M. Reeves, H. Nakao, and A. Golaz.** 1999. Use of molecular subtyping to document long-term persistence of *Corynebacterium diphtheriae* in South Dakota. *J. Clin. Microbiol.* **37:**1092–1099.
13. **Sottnek, F. O., and J. M. Miller.** 1980. *Isolation and Identification of* Corynebacterium diphtheriae. Centers for Disease Control, Atlanta, GA.
14. **Waites, K. B., M. A. Saubolle, D. F. Talkington, S. A. Moser, and V. Baselski.** 2005. *Cumitech 10A, Laboratory Diagnosis of Upper Respiratory Tract Infections.* Coordinating ed., S. E. Sharp. ASM Press, Washington, DC.

SUPPLEMENTAL READING AND WEBSITES

Clarridge, J. E., T. Popovic, and T. J. Inzana. 1998. Diphtheria and other corynebacterial and coryneform infections, p. 347–371. *In* W. J. Hausler and M. Sussman (ed.), *Topley and Wilson's Microbiology and Microbial Infections,* vol. 3. Oxford University Press, New York, NY.

Efstratiou, A., and P. A. C. Maple. 1994. *Manual for the Laboratory Diagnosis of Diphtheria.* Copenhagen: Expanded Programme on Immunization in the European Region of World Health Organization, ICP/EPI 038(C).

Efstratiou, A., K. H. Engler, I. K. Mazurova, T. Glushkevich, J. Vuopio-Varkila, and T. Popovic. 2000. Current approaches to the laboratory diagnosis of diphtheria. *J. Infect. Dis.* **181:**S138–S145.

http://www.bu.edu/cohis/infxns/bacteria/cory.htm

http://www.cdc.gov

http://www.phls.co.uk/facts/Immunisation/Diphtheria/diptintro.htm

APPENDIX 3.11.7–1

Media for Detection of *Corynebacterium diphtheriae*

Refer to manufacturers' package inserts for additional information.

A. TIN agar: a peptone agar base enriched with bovine serum, cysteine hydrochloride, potassium tellurite, and sodium thiosulfate
   1. The TIN kit (Remel) includes one tube of TIN agar base along with one bottle of desiccated TIN enrichment containing tellurite and serum. One tube and one bottle make one plate. Prepare a sufficient number of plates for QC testing and patient specimens.
   2. Melt the agar in the bottle by boiling.
   3. Rehydrate the enrichment by adding 3.3 ml of sterile distilled water aseptically to the bottle. Allow to sit until completely hydrated.
   4. Cool the base to 47 to 50°C. Add the enrichment and pour one plate. Plate is good for 5 days after pouring. Perform QC.

B. CTBA is a heart infusion agar supplemented with 5% rabbit blood, tellurite, and L-cystine; shelf life is 1 month. Deeps with a longer shelf life are available.

C. Loeffler blood serum medium contains eggs and beef serum, rather than agar, to coagulate to produce a solid medium. It is best purchased commercially.

APPENDIX 3.11.7–1 *(continued)*

D. Pai agar slant
   1. 666 ml of fresh eggs, 333 ml of distilled water, and 80 ml of glycerin
   2. Filter eggs and water through gauze and add glycerin.
   3. Tube in 3-ml amounts, slant, and autoclave for 1 h at 110°C.
   4. Tightly cap; shelf life is 1 year (1).

Reference

1. **Sottnek, F. O., and J. M. Miller.** 1980. *Isolation and Identification of* Corynebacterium diphtheriae. Centers for Disease Control, Atlanta, GA.

3.11.8 Group A Streptococcus: Culture and Nonculture Tests

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Group A beta-hemolytic streptococcus (GABHS) is an important pathogen that causes pharyngitis, cellulitis, and bacteremia. Serious sequelae, including scarlet fever, acute glomerulonephritis, toxic shock syndrome, and acute rheumatic fever, can result from infections with this microorganism.

The primary reason this microorganism is an effective pathogen is the variety of virulence factors it can produce. Numbering among them are somatic constituents like M protein and capsule, enzymes like streptolysin O, DNase B, and streptokinase, and toxins like erythrogenic toxins A to C.

GABHS accounts for 30% of pharyngitis cases in children aged 5 to 15 years but only 10% of adult cases. Other bacterial causes of pharyngitis include group C and G beta-hemolytic streptococci, *Neisseria gonorrhoeae*, *Corynebacterium diphtheriae*, and *Arcanobacterium haemolyticum*. However, most cases of pharyngitis have a viral etiology. In children, the most worrisome complications of GABHS infection are acute rheumatic fever, acute glomerulonephritis, and peritonsillar abscess. Antimicrobial therapy alleviates pharyngeal symptoms and prevents some of the sequelae of infection.

Since Breese et al. (5) first described throat culturing for GABHS, numerous methods of detection have been published. It is important to select diagnostic tests that are highly sensitive for use in children, since low numbers of GABHS in specimens may still indicate infection. That is because collecting high-quality pharyngeal specimens from uncooperative children can be difficult. For this reason, the American Academy of Pediatrics and the Infectious Diseases Society of America (IDSA) recommend that negative nonculture tests (rapid antigen detection and the Gen-Probe Group A Streptococcus Direct Test) be confirmed by culture (1, 4), even though clinical studies have documented that the probe tests have a sensitivity similar to that of culture (5, 17).

Guidelines were published recently for testing adults with pharyngitis who lacked the complications of heart disease, chronic lung disease, or a history of acute rheumatic fever (13). They state that these patients should be screened for presence of the Centor criteria (9): history of fever, tonsillar exudates, absence of cough, and tender anterior cervical lymphadenopathy (lymphadenitis). Patients with two or more criteria should have rapid GABHS antigen detection performed, with antimicrobial therapy prescribed only for patients with positive results. Alternatively, antimicrobial therapy can be administered to patients who meet three or four of the criteria without a need for diagnostic testing.

The IDSA does not support diagnosing GABHS pharyngitis on clinical grounds alone, which it feels could result in the unnecessary use of antimicrobial agents in uninfected patients. It recommends instead that adult patients suspected of being infected have their diagnosis confirmed by at least a positive antigen detection test prior to antimicrobial therapy (4).

Both sets of guidelines discourage pharyngeal cultures during the routine evaluation of pharyngitis in adults or for confirming negative antigen test results when test sensitivities exceed 80%. However, the IDSA guidelines leave the decision to the physician as to whether cultures should be performed instead of or in addition to antigen detection assays. Additional indications for pharyngeal cultures in adults are (i) investigations of outbreaks of GABHS disease, (ii) monitoring the appearance and spread of antimicrobial resistance, and (iii) examination for pathogens other than GABHS.

GABHS are important causes of bacteremia and skin and soft tissue infections as well. Recently appreciated is the role of GABHS as an etiologic agent of perianal dermatitis in children following swallowing or direct inoculation of infectious respiratory secretions (2, 20). Two other highly publicized GABHS diseases in recent years have been necrotizing fasciitis ("flesh-eating" bacterial disease) and streptococcal toxic shock syndrome.

This procedure describes laboratory options for detection and identification of GABHS by direct methods and by culture. The emphasis is on pharyngeal specimens, since they are often submitted specifically for culture of this organism.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

◩ **NOTE:** Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

Observe standard precautions.

A. Specimen collection

1. Pharynx (7)

a. Place gentle pressure on the tongue with a tongue depressor.

b. Extend one or two sterile swabs (one for the antigen test and one for culture if necessary) between the tonsillar pillars and behind the uvula, avoiding the tongue, buccal mucosa, and uvula.

c. Sweep the swabs back and forth across the posterior pharynx, tonsillar areas, and any inflamed or exudative areas.

d. Do not obtain specimens if the epiglottis is inflamed, since sampling may trigger life-threatening respiratory obstruction.

2. Perianal dermatitis (cellulitis)

a. Cleanse the sampling area with saline, followed by two applications of 70% alcohol.

b. Collect an aspirate from the leading edge of the inflamed area.

c. Order an aerobic (routine) wound culture (*see* procedure 3.13.1)

3. Blood culture (*see* procedure 3.4.1)

4. Wound culture (*see* procedure 3.13.1)

B. Specimen transport

1. Submit swabs for GABHS culture to the laboratory as follows.

a. Dry in a paper envelope *or*

b. Moistened or immersed in transport medium

2. Label specimens with the patient's demographic information, name of person requesting the test, date of specimen collection, the specimen collection site, and the test to be performed.

C. Rejection criteria

1. Pharyngeal cultures for GABHS are not recommended for confirmation of negative antigen detection results for adults when the antigen test sensitivity exceeds 80%.

2. Do not perform Gram stains of pharyngeal specimens for diagnosis of GABHS pharyngitis, since the results are not helpful.

III. MATERIALS

A. Primary tests: antigen detection tests for GABHS

◩ **NOTE:** Many kits for detection of GABHS antigen are commercially available (14). Some of these products are Clinical Laboratory Improvement Amendments (CLIA) waived and are readily performed in physician offices. For an up-to-date list of CLIA-waived tests, please refer to the website http://www.cms.hhs.gov/clia/waivetbl.pdf. Almost all of the assays are based on nitrous acid extraction of group A streptococcal antigen from specimens followed by particle agglutination, staphylococcal coagglutination, EIA, immunochromatography, or optical immunoassay techniques for detection of antigen. Kits vary in their sensitivities in detection of GABHS, and reports of the same kit can differ depending on the culture media and conditions used for the comparison (15).

B. The Gen-Probe Strep A Direct Test is a DNA hybridization probe that is complementary to the rRNA target of the group A streptococcus.

C. Culture

1. Inoculate one of the following.

a. BAP

b. NNA: BAP with 30 µg of neomycin per ml and 15 µg of nalidixic acid per ml (BD Diagnostic Systems, Sparks, MD)

III. MATERIALS *(continued)*

c. Selective streptococcal agar: BAP with neomycin and polymyxin B (Remel, Inc.; Hardy Diagnostics) or colistin and oxolinic acid (Remel, Inc.).

d. Selective group A streptococcus agar containing trimethoprim-sulfamethoxazole with or without colistin or crystal violet (available from most vendors)

◩ **NOTE:** Media with trimethoprim-sulfamethoxazole can be used for growth of GABHS and group B beta-hemolytic streptococci but not for group C, F, or G beta-hemolytic streptococci. Contact the vendor for information on the antimicrobial agents in their selective media, as they vary from vendor to vendor.

2. Identification methods

a. Gram stain (procedure 3.2.1)

b. Catalase test (procedure 3.17.10)

c. One or more of the following

(1) Pyrrolidonyl-β-naphthylamide (PYR) (procedure 3.17.41)

(2) AccuProbe STREP A DNA probe assay for RNA (Gen-Probe Inc.)

(3) Latex particle agglutination or staphylococcal protein A coagglutination tests for streptococcal grouping

(a) Directigen (BD Diagnostic Systems)

(b) Meritec Strep (Meridian Diagnostics)

(c) Oxoid Strep Grouping (Oxoid)

(d) PathoDx (Diagnostic Products Corp., Los Angeles, CA)

(e) Prolex (Pro-Lab Diagnostics, Austin, TX)

(f) Slidex Strep (bioMérieux Vitek, Hazelwood, MO)

(g) Streptex (Abbott Diagnostics, Abbott Park, IL)

3. Other supplies needed

a. Incubator maintained at 35°C with provision for ambient, 5 to 10% CO_2, or anaerobic incubation

b. Disposable cards for latex particle agglutination or staphylococcal protein A coagglutination identification tests

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Antigen detection tests

1. Test a positive and negative control antigen with each new lot and at the start of each work shift.

◩ **NOTE:** Use of some commercial kits, having an internal patient control, does not necessitate other daily QC. Consult the package insert.

2. Determine whether the antigen detection test is at least 80% sensitive compared to culture as a QA check.

B. Immunological identification tests

1. Test each new lot of antiserum with a corresponding positively and negatively reacting control organism prior to use and every 6 months thereafter.

2. Test each lot of extraction reagent with a known culture of GABHS to verify its ability to extract the antigen.

C. Culture media

1. Verify that culture media meet expiration date and QC parameters per Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22-A3 (10). See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

IV. QUALITY CONTROL *(continued)*

2. Perform growth checks on each new lot of selective blood agar that contains antimicrobial agents (*see* below).

Test organism	Result
Proteus mirabilis ATCC 12453	Partial to complete inhibition
Streptococcus pyogenes ATCC 19615	Growth of beta-hemolytic colonies

◩ **NOTE:** CLSI QC standards (10) propose the elimination of user QC for commercially prepared group A selective agars. Consult with local regulatory agencies and CLSI documents prior to discontinuation of user QC.

V. PROCEDURE

Observe standard precautions.

A. GABHS antigen detection tests

1. Perform antigen detection tests on pharyngeal specimens to provide rapid results.
- **a.** Follow each step of the manufacturer's instructions carefully.
- **b.** Include control steps, as indicated by the manufacturer.

2. Confirm negative results from pediatric patients with culture, because current tests are unable to detect low numbers of GABHS in poorly collected specimens.

◩ **NOTE:** Confirmation of negative test results for adult patients is generally unnecessary, unless the sensitivity of the test is less than 80%.

3. Do not confirm positive results for any patients, except during validation testing, to determine the test specificity, or to recover isolates for antimicrobial susceptibility testing (AST) (19).

B. Gen-Probe Group A Strep Direct Test

Perform according to the manufacturer's instructions. Only the swab types outlined in the manufacturer's product insert should be used for collection (Gen-Probe).

C. Culture

1. Inoculation of culture media
- **a.** Choose either BAP and/or one of the selective sheep blood agars

  ◩ **NOTE:** Inoculating two swabs with both nonselective and selective media adds to the expense but will increase the sensitivity of culture (21).
- **b.** Firmly roll a pharyngeal swab over one-sixth (no more) of the agar surface, and streak carefully for isolation in four quadrants to minimize overgrowth by other microorganisms. Carefully stab the agar several times with the same loop both in an area that has been streaked and in an area that has not been streaked, so as not to leave gaps, in order to improve detection of beta-hemolysis when plates are incubated aerobically (Fig. 3.11.8–1).

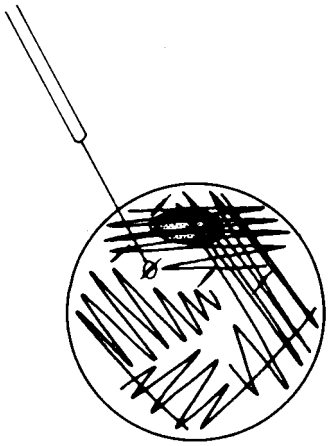


Figure 3.11.8–1 Method of streaking plate for throat culture with stabs in agar.

2. Incubate culture plates at 35 to 37°C for 48 h under one of the following conditions.
- **a.** Aerobic with ambient air for BAP with agar stabs
- **b.** Aerobic with 5 to 10% CO_2 for selective streptococcal agar with agar stabs *(This method is not acceptable for BAP without antimicrobials.)*
- **c.** Anaerobic for either BAP or selective agars (3)

◩ **NOTE:** Each of the methods described above has good sensitivity; in comparative studies no one method was consistently better for detection of GABHS (19, 21). However, aerobic incubation in CO_2 is helpful for isolating pathogens other than GABHS, such as group C and G streptococci. Incubation for 48 h consistently increases the yield of positive cultures by 5 to 46% depending on the study (19). Prolonged incubation is most important

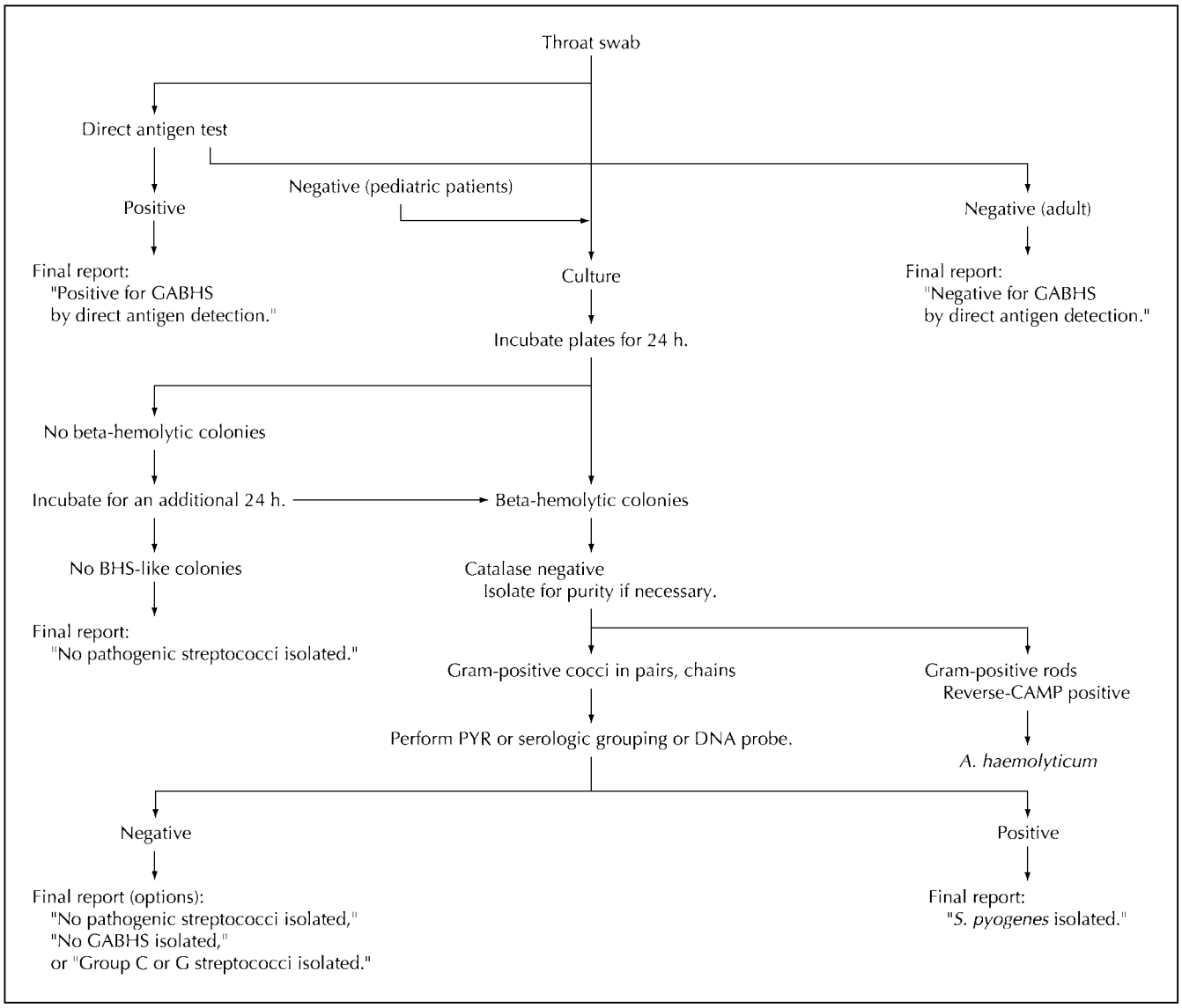


Figure 3.11.8–2 Algorithm for laboratory diagnosis of streptococcal pharyngitis.

V. PROCEDURE *(continued)*

if selective media, which may somewhat inhibit the growth of GABHS, or if anaerobic incubation is used.

3. Examination of culture media (Fig. 3.11.8–2)
- **a.** Observe culture media after 24 (and 48) h for small translucent or transparent colonies that are dome shaped, have an entire edge, and are surrounded by a relatively wide zone of complete (beta-) hemolysis.
- **b.** Perform a catalase test on one or two colonies. Catalase-positive colonies are not streptococci.
- **c.** Perform a Gram stain on catalase-negative colonies. Organisms other than gram-positive cocci in pairs or chains are not streptococci.

4. Confirmatory tests
- **a.** Confirm beta-hemolytic, catalase-negative, gram-positive cocci in pairs and chains as *Streptococcus pyogenes* by *one* of the following.
  - (1) Positive PYR test (12). For specimens from nonpharyngeal sites, confirm with a negative bile-esculin or esculin test (procedure 3.17.5).

V. PROCEDURE *(continued)*

(2) Positive result for group A streptococcal antigen with an immunological grouping test
(3) Positive DNA probe result

◩ **NOTE:** The presence of a zone of growth inhibition around a bacitracin (Taxo A) disk to identify streptococci as GABHS is not reliable (22).

b. If a catalase-negative, beta-hemolytic isolate is PYR negative, test it with immunological grouping reagents to identify it as group B, C, F, or G. Do not report GABHS that are PYR negative as *S. pyogenes* because they belong to the *Streptococcus anginosus* group of normal respiratory microbiota.

c. If a colony Gram stain reveals gram-positive bacilli, perform the CAMP test (procedure 3.17.8). *A. haemolyticum* is catalase negative and reverse-CAMP test positive and forms slowly growing beta-hemolytic colonies on nonselective or selective sheep blood agar.

5. Do not perform AST as a routine, since all GABHS continue to exhibit penicillin susceptibility. Save positive culture plates (preferably for 7 days) in case AST is needed for determination of erythromycin or clindamycin resistance. See section 5 and reference 11 for methods.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Negative results from pharyngeal specimens

1. Report the antigen detection result as "Negative for group A streptococci by antigen detection assay."

2. Report the culture result as "No pathogenic streptococci isolated" if there are no beta-hemolytic colonies or if beta-hemolytic colonies are identified as group A (PYR negative), B, D, or F.

B. Positive results from pharyngeal specimens

1. Report the antigen detection result as "Positive for group A streptococcus by direct antigen test."

2. Report the culture results with a comment that indicates the specific pathogens for which the specimen was analyzed. For negative cultures include a comment such as "Negative for group A streptococcus" if there are no beta-hemolytic colonies.

3. If *A. haemolyticum* is identified, report the culture as positive for this microorganism.

4. If group C or G beta-hemolytic streptococci are identified, report or do not report according to the preferences of the ordering physician.

VII. INTERPRETATION

A. A positive pharyngeal culture for GABHS indicates the presence of *S. pyogenes* but does not distinguish between infection and colonization (16, 18).

B. All *S. pyogenes* organisms are susceptible to penicillin (the antimicrobial agent of choice for treating infection), negating the need for susceptibility testing. If testing is performed, penicillin resistance must be confirmed by a reference laboratory (11). For penicillin-allergic patients, erythromycin is the therapeutic agent of choice. Recurrent tonsillitis may require surgery and treatment with clindamycin (8).

C. GABHS can be resistant to erythromycin and clindamycin; in some geographic areas the incidence is high (22), and testing may be indicated when penicillin therapy is contraindicated.

VIII. LIMITATIONS

A. Falsely negative pharyngeal cultures can result from overgrowth of cultures by normal oral microorganisms or from the lack of beta-hemolysis in cultures incubated aerobically.

B. Falsely positive pharyngeal culture reports can result from misinterpretation of identification tests. PYR-positive colonies may be enterococci, which are rarely beta-hemolytic and uncommonly found in the pharynx. Isolates from rectal swabs should be identified by immunological methods or the combination of PYR and esculin hydrolysis tests.

C. PYR-negative GABHS are not *S. pyogenes* but members of the *S. anginosus* group, which is not a cause of pharyngitis.

D. Group C and G beta-hemolytic streptococci can cause pharyngitis and fever, but they do not place patients at risk of acute rheumatic fever.

REFERENCES

1. **American Academy of Pediatrics.** 2003. Group A streptococcal infections, p. 573–584. *In* L. K. Pickering (ed.), *2003 Red Book: Report of the Committee on Infectious Diseases*, 26th ed. American Academy of Pediatrics, Elk Grove Village, IL.
2. **Amren, D. P., A. S. Anderson, and L. W. Wannamaker.** 1966. Perianal cellulitis associated with group A streptococci. *Am. J. Dis. Child.* **112:**546–548.
3. **Belli, D. C., R. Auckenthaler, L. Paunier, and P. E. Ferrier.** 1984. Throat cultures for group A beta-hemolytic *Streptococcus.* Importance of anaerobic incubation. *Am. J. Dis. Child.* **138:**274–276.
4. **Bisno, A. L., M. A. Gerber, J. M. Gwaltney, Jr., E. L. Kaplan, and R. H. Schwartz.** 2002. Practice guidelines for the diagnosis and management of group A streptococcal pharyngitis. Infectious Diseases Society of America. *Clin. Infect. Dis.* **35:**113–125.
5. **Bourbeau, P. P.** 2003. Role of the microbiology laboratory in diagnosis and management of pharyngitis. *J. Clin. Microbiol.* **41:**3467–3472.
6. **Breese, B. B., F. A. Disney, and W. Talpey.** 1966. The nature of a small pediatric group practice. II. The incidence of beta hemolytic streptococcal illness in a private pediatric practice. *Pediatrics* **38:**277–285
7. **Brien, J. H., and J. W. Bass.** 1985. Streptococcal pharyngitis: optimal site for throat culture. *J. Pediatr*. **106:**781–783.
8. **Capper, R., and R. J. Canter.** 2001. Is there agreement among general practitioners, paediatricians and otolaryngologists about the management of children with recurrent tonsillitis? *Clin. Otolaryngol.* **26:**371–378.
9. **Centor, R. M., J. M. Witherspoon, H. P. Dalton, C. E. Brody, and K. Link.** 1981. The diagnosis of strep throat in adults in the emergency room. *Med. Decis. Making* **1:**239–246.
10. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
11. **Clinical and Laboratory Standards Institute.** 2008. *Performance Standards for Antimicrobial Susceptibility Testing.* Eighteenth informational supplement. Approved standard M100-S18. Clinical and Laboratory Standards Institute, Wayne, PA.
12. **Clinical and Laboratory Standards Institute.** 2008. *Abbreviated Identification of Bacteria and Yeast*, 2nd ed. Approved guideline M35-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
13. **Cooper, R. J., J. R. Hoffman, J. G. Bartlett, R. E. Besser, R. Gonzales, J. M. Hickner, and M. A. Sande.** 2001. Principles of appropriate antibiotic use for acute pharyngitis in adults: background. *Ann. Intern. Med.* **134:**509–517.
14. **Evangelista, A. T., A. L. Truant, and P. P. Bourbeau.** 2002. Rapid systems and instruments for the identification of bacteria, p. 22–49. *In* A. L. Truant (ed.), *Manual of Commercial Methods in Microbiology.* ASM Press, Washington, DC.
15. **Graham, L., Jr., F. A. Meier, R. M. Centor, B. K. Garner, and H. P. Dalton.** 1986. Effect of medium and cultivation conditions on comparisons between latex agglutination and culture detection of group A streptococci. *J. Clin. Microbiol.* **24:**644–646.
16. **Gunnarsson, R. K., S. E. Holm, and M. Soderstrom.** 1997. The prevalence of beta–haemolytic streptococci in throat specimens from healthy children and adults. Implications for the clinical value of throat cultures. *Scand. J. Prim. Health Care* **15:**149–155.
17. **Heiter, B. J., and P. P. Bourbeau.** 1993. Comparison of the Gen-Probe Group A Streptococcus Direct Test with culture and a rapid streptococcal antigen detection assay for diagnosis of streptococcal pharyngitis. *J. Clin. Microbiol* **31:**2070–2073.
18. **Kaplan, E. L., F. H. Top, Jr., B. A. Dudding, and L. W. Wannamaker.** 1971. Diagnosis of streptococcal pharyngitis: differentiation of active infection from the carrier state in the symptomatic child. *J. Infect. Dis.* **123:**490–501.

REFERENCES *(continued)*

19. **Kellogg, J. A.** 1990. Suitability of throat culture procedures for detection of group A streptococci and as reference standards for evaluation of streptococcal antigen detection kits. *J. Clin. Microbiol.* **28:**165–169.
20. **Kokx, N. P., J. A. Comstock, and R. R. Facklam.** 1987. Streptococcal perianal disease in children. *Pediatrics* **80:**659–663.
21. **Kurtz, B., M. Kurtz, M. Roe, and J. Todd.** 2000. Importance of inoculum size and sampling effect in rapid antigen detection for diagnosis of *Streptococcus pyogenes* pharyngitis. *J. Clin. Microbiol.* **38:**279–281.
22. **York, M. K., L. Gibbs, F. Perdreau-Remington, and G. F. Brooks.** 1999. Characterization of antimicrobial resistance in *Streptococcus pyogenes* isolates from the San Francisco Bay Area of Northern California. *J. Clin. Microbiol.* **37:**1727–1731.

SUPPLEMENTAL READING

Bisno, A. L., G. S. Peter, and E. L. Kaplan. 2002. Diagnosis of strep throat in adults: are clinical criteria really good enough? *Clin. Infect. Dis.* **35:**126–129.

Waites, K. B., M. A. Saubolle, D. F. Talkington, S. A. Moser, and V. Baselski. 2005. *Cumitech 10A, Laboratory Diagnosis of Upper Respiratory Tract Infections.* Coordinating ed., S. E. Sharp. ASM Press, Washington, DC.

3.11.9 Nasal Sinus Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Acute rhinosinusitis, an infection of one or both of the paranasal sinuses, is among the most common health problems (8). It is manifest by an inflammatory response of the mucous membranes of the nasal cavity, seen as edema and hypersecretion of mucus following a common upper respiratory viral infection (7, 10). The disease is mild but results in considerable medical cost to relieve facial pain and swelling, nasal discharge, fatigue, and symptoms related to the inflammatory process (10). Therapies to alleviate the symptoms of congestion and improve nasal drainage are recommended early in the viral infection and can prevent progression to bacterial infection (9). Antimicrobial agents are important, if the disease progresses beyond 7 to 10 days, to reduce time to recovery, aid in symptom resolution, and prevent complications from progression of the disease (3, 9). Antimicrobial therapy is usually targeted to the most common agents, *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis,* and *Streptococcus pyogenes* (2, 3). Some authors include anaerobes and *Staphylococcus aureus* as possible agents of disease, but these bacteria are less often associated with acute inflammation (12). In 40% of cases, no agent is cultured, which has suggested that *Mycoplasma pneumoniae* or *Chlamydia pneumoniae* is involved (7). Sinus infections can become chronic, although the etiology of this presentation has been controversial. For chronic infections, particularly in diabetic patients and in tropical countries, fungi can invade the sinuses and cause progressive, life-threatening infections. Anaerobes have also been implicated, and therapy usually includes agents, such as metronidazole or clindamycin, which are active against anaerobes (1).

This procedure deals only with invasively collected specimens for diagnosis of acute sinusitis. Refer to other procedures in this handbook for culture for viruses, fungi, and mycobacteria and for nasal carriage of contagious pathogens or *Bordetella pertussis* (*see* Table 3.11.1–1). Collection of specimens from patients with sinusitis is performed by otolaryngologists who perform nasal endoscopy or sinus puncture and aspiration.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

NOTE: Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

Observe standard precautions.

A. Specimen collection is performed by an otolaryngologist.

1. Rigid endoscopy (12)

a. Provide patient with an intranasal decongestant and then a topical anesthetic.

b. Identify the middle meatus adjacent to the maxillary sinus ostium ipsilateral to the side to be aspirated.

c. Collect drainage from the middle meatus with a small swab on a wire.

2. Maxillary sinus puncture and aspiration

a. Clean the anterior nares with antiseptic solution.

b. Apply topical anesthetic.

c. Puncture the maxillary antrum and aspirate secretions with a needle and syringe.

d. If no material is aspirated, irrigate with 2 ml of nonbacteriostatic saline.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

B. Specimen transport
- **1.** Submission to the laboratory
  - **a.** Submit swabs in tube of transport medium or in BD Culturette EZ.
  - **b.** Aspirates should be transferred to a sterile container for submission to the laboratory. If an anaerobic culture is required, submit a portion of the sample in a sterile tube containing prereduced anaerobically sterilized (PRAS) media. Syringes with the needle attached should never be sent to the laboratory because of the sharps and biohazard risk to staff. Syringes with a Luer-Lok are also not ideal for culture since the specimen may become congealed in transport in the device and become contaminated during handling.
- **2.** Label specimens with demographic information, date and time of collection, and site of collection.
- **3.** List the diagnosis and whether it is chronic or acute.
- **4.** Order anaerobic culture if appropriate to the diagnosis.

C. Rejection criteria
- **1.** Reject requests for routine bacterial culture of nasal washes and nasal aspirates not collected by otolaryngologists as inappropriate and lacking specificity to diagnose either sinusitis or lower respiratory disease (6, 11). These specimens are acceptable for *B. pertussis* and for viral culture.
- **2.** Nasal swabs not collected by otolaryngologists are acceptable only for specific culture (e.g., *B. pertussis* or *Corynebacterium diphtheriae*) or staphylococcal carriage, which should be indicated along with the culture request. They are unacceptable to diagnose acute sinusitis (6, 11). They may, however, be acceptable for diagnosis of fungal invasion of the sinuses (*see* section 8).

III. MATERIALS

A. Primary media
- **1.** BAP
- **2.** CHOC
- **3.** Anaerobic plate media (on request). See section 4.

B. Identification methods
- **1.** Gram stain (procedure 3.2.1)
- **2.** Refer to procedure 3.3.2 for identification of the common agents of sinusitis.

C. Other supplies
- **1.** Incubator at 35°C with 5% CO_2 or a CO_2-generating system
- **2.** Inoculating sticks or loops
- **3.** Petri dishes and filter paper

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that media meet expiration date and QC parameters per Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22-A3 (5). See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

B. Test each lot of CHOC per procedure 3.3.1.

V. PROCEDURE

Observe standard precautions.

A. Inoculation
- **1.** Inoculate specimen to BAP and CHOC.
- **2.** Firmly roll swab over one-sixth (no more) of the agar surface, or deposit 3 or 4 drops of fluid onto agar. Streak carefully for isolation in four quadrants to minimize overgrowth by other microorganisms.

B. Incubation
- **1.** Incubate plate at 35 to 37°C in 5% CO_2 for a minimum of 48 h.
- **2.** For special requests in cases of chronic infections, extend incubation to 4 days.

V. PROCEDURE *(continued)*

C. Gram stain

1. Perform a gram stain from the swab or fluid (procedure 3.2.1)

2. Note the presence of inflammatory cells and bacteria.

D. Culture examination

1. Observe for growth of *S. pneumoniae, H. influenzae, M. catarrhalis,* and *S. pyogenes.*

a. Refer to Table 3.3.2–5 for rapid identification tests.

b. *S. pneumoniae*

(1) Confirm bile solubility with optochin disk, if questionable or resistant.

(2) Perform antimicrobial susceptibility testing (AST), using standardized methods (4), per laboratory protocol and physician policy.

c. Perform beta-lactamase test on *H. influenzae.*

d. Since greater than 90% of *M. catarrhalis* organisms are beta-lactamase positive, testing is not helpful for treatment.

2. Observe plates at 24 and 48 h for growth of enteric gram-negative rods and *S. aureus.*

a. These organisms are usually considered contaminants in sinus specimens and are not identified unless they are the predominant species in the culture.

b. Perform AST only if the Gram stain suggests that they are involved in an inflammatory process and no other pathogens are isolated.

◩ **NOTE:** Normal skin microbiota (coagulase-negative staphylococci and corynebacteria) are not identified to the species level.

3. Identify any molds that are present. Yeasts need not be identified to the species level, as they have not been implicated in sinusitis.

4. Identify any other predominant organism that is not part of the normal respiratory microbiota.

5. If anaerobic cultures were ordered, refer to section 4 for identification; gram-positive anaerobes are more likely to be present in sinus infections than gram-negative rods.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Gram stain: report smear as indicated in procedure 3.2.1.

B. Negative culture results

1. Report preliminary and final reports as "No growth."

2. Indicate the number of days the culture was incubated.

C. Positive results

1. Report all pathogens and susceptibility tests performed, using preliminary reports as indicated in procedure 3.3.2 and CLSI guidelines (4).

2. Indicate presence of aerobic skin microbiota, without species identification.

3. If the culture is mixed, with no predominating pathogen, indicate the genera and do not report further, e.g., "Mixed microbiota present, consisting of three morphologies of gram-negative rods and skin bacteria."

◩ **NOTE:** Mixed microbiota without a predominant pathogen generally indicates that the specimen was not collected appropriately. Exception is made if the Gram stain demonstrates inflammation by the presence of PMNs.

VII. INTERPRETATION

A. A positive culture with *S. pneumoniae, H. influenzae, M. catarrhalis,* and *S. pyogenes* generally indicates infection with that organism.

B. A positive culture with a predominant gram-negative rod or *S. aureus* may or may not indicate infection with that agent.

VII. INTERPRETATION *(continued)*

C. A negative culture cannot rule out sinusitis. In fact, often a pathogen is not isolated.

D. Most physicians agree on the benefits of antimicrobial therapy for acute sinus infections. Generally a beta-lactam agent, such as amoxicillin, amoxicillin-clavulanate, or a cephalosporin, is used, negating the need for culture (3). However, for persistent chronic infection in the face of therapy, cultural evaluation and antimicrobial profiling are indicated.

VIII. LIMITATIONS

A. Inaccurate culture reporting can result from contamination of the specimen with normal oral microbiota.

B. False-negative cultures can be caused by delays in processing.

C. False-positive cultures can be caused by overinterpretation of the culture results.

REFERENCES

1. **Brook, I., P. Yocum, and K. Shah.** 2000. Aerobic and anaerobic bacteriology of concurrent chronic otitis media with effusion and chronic sinusitis in children. *Arch. Otolaryngol. Head Neck Surg.* **126:**174–176.
2. **Brook, I., W. M. Gooch III, S. G. Jenkins, M. E. Pichichero, S. A. Reiner, L. Sher, and T. Yamauchi.** 2000. Medical management of acute bacterial sinusitis. Recommendations of a clinical advisory committee on pediatric and adult sinusitis. *Ann. Otol. Rhinol. Laryngol. Suppl.* **182:**2–20.
3. **Chow, A. W., C. B. Hall, J. Klein, R. B. Kammer, R. D. Meyer, and J. S. Remington.** 1992. General guidelines for the evaluation of new anti-infective drugs for the treatment of respiratory tract infections. *Clin. Infect. Dis.* **15**(Suppl. 1)**:**S62–S88.
4. **Clinical and Laboratory Standards Institute.** 2008. *Performance Standards for Antimicrobial Susceptibility Testing.* Eighteenth informational supplement. Approved standard M100-S18. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
6. **Dagan, R., R. Melamed, M. Muallem, L. Piglansky, and P. Yagupsky.** 1996. Nasopharyngeal colonization in southern Israel with antibiotic-resistant pneumococci during the first 2 years of life: relation to serotypes likely to be included in pneumococcal conjugate vaccines. *J. Infect. Dis.* **174:**1352–1355.
7. **Gwaltney, J. M., Jr.** 2005. Sinusitis, p. 772–783. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), *Principles and Practice of Infectious Diseases,* 6th ed. Churchill Livingstone, New York, NY.
8. **Gwaltney, J. M., Jr.** 1996. Acute community-acquired sinusitis. *Clin. Infect. Dis.* **23:**1209–1225.
9. **Hadley, J. A.** 2001. The microbiology and management of acute and chronic rhinosinusitis. *Curr. Infect. Dis. Rep.* **3:**209–216.
10. **Lanza, D. C., and D. W. Kennedy.** 1997. Adult rhinosinusitis defined. *Otolaryngol. Head Neck Surg.* **117:**S1–S7.
11. **Robinson, D. A., K. M. Edwards, K. B. Waites, D. E. Briles, M. J. Crain, and S. K. Hollingshead.** 2001. Clones of *Streptococcus pneumoniae* isolated from nasopharyngeal carriage and invasive disease in young children in central Tennessee. *J. Infect. Dis.* **183:**1501–1507.
12. **Talbot, G. H., D. W. Kennedy, W. M. Scheld, and K. Granitol for the Endoscopy Study Group.** 2001. Rigid nasal endoscopy versus sinus puncture and aspiration for microbiologic documentation of acute bacterial maxillary sinusitis. *Clin. Infect. Dis.* **33:**1668–1675.

3.12 Urine Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Urinary tract infections (UTI) account for seven million visits to physicians' offices and over one million hospital admissions per year. Epidemiological studies by E. H. Kass (10) have shown that bacterial counts of $\geq 10^5$ CFU/ml for a pure culture of gram-negative bacilli were found to be associated with acute bacterial infections of the urinary tract. In females with dysuria and acute UTI, other investigators reported that $>10^2$ CFU/ml can be significant (8, 9, 17). For infants and catheterized patients, low counts also have been shown to be significant (6, 18, 19). Because of the importance of colony counts for infection, urine cultures are always performed with an accompanying colony count (2).

Urine is normally a sterile body fluid. However, it is easily contaminated with microbiota from the perineum, prostate, urethra, or vagina. The microbiologist must provide detailed instructions to ensure proper specimen collection, preservation, labeling, and transport of urine for culture.

The etiologic agents of urinary tract infection are generally limited to the patient's own intestinal microbiota, with *Escherichia coli*, *Enterococcus* spp., *Klebsiella-Enterobacter* spp., and *Proteus* spp. representing a majority of isolates from both hospitalized patients and outpatients. Refer to Table 3.12–1 for a list of usual microbiota of contaminated and infected urine.

Several terms may be encountered with the submission of specimens for culture. These are defined in Table 3.12–2. Since the significance of bacterial counts depends on the disease presentation, different criteria are often used to determine if the count of bacteria in the urine is significant and requires treatment. Because the laboratory rarely knows details other than patient age and sex and type of specimen, the significance of bacteria in the urine is usually based only on these criteria. Pyuria, in addition to bacteriuria, is also an important factor in establishing the presence of a UTI (*see* procedure 3.2.3 and Appendix 3.12–1).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

◩ **NOTE:** Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and other caregivers. It is the responsibility of the laboratory director or designee to educate physicians and other caregivers on proper specimen collection.

A. Specimen collection

1. Clean-voided midstream urine collection

a. Preparation

(1) Females

◩ **NOTE:** Collection of midstream urine specimens should be avoided during menses.

(a) While the labia are held apart with the aid of a pair of sponges, wash the vulva thoroughly from front to back with two successive cotton pledgets or sponges soaked in soap. Special attention should be paid to the urethral meatus (benzalkonium or hexachlorophene should not be used, as a single drop of residual can sterilize the urine before the specimen reaches the laboratory).

Table 3.12–1 Urinary microbiota[a]

Microbiota	Organism	Extent of workup if count is appropriate per Table 3.12–4
Urogenital	Viridans group streptococci, *Neisseria* spp., diphtheroids, *Lactobacillus* spp., anaerobes	Report as urogenital microbiota.
Skin	Diphtheroids, *Staphylococcus* spp.	Report as skin or with urogenital microbiota unless present in amounts >10-fold more than other microbiota. Then treat as uropathogen.
Uropathogens	Gram-negative bacilli	ID to species level and AST
	Staphylococcus	ID and AST of *S. aureus;* ID of *Staphylococcus saprophyticus* with novobiocin disk for females of childbearing age; AST generally not needed for *S. saprophyticus* or other coagulase-negative staphylococci.
	Yeasts	ID of *C. albicans* and *Candida glabrata;* ID of others to species level only on request
	Beta-hemolytic *Streptococcus*	ID, especially of group B in women in childbearing years
	Enterococcus spp.	Check for VRE on inpatients; ID to species level and AST only if VRE and on request
	G. vaginalis	ID only if number is 10 times greater than that of all other microbiota
	Aerococcus urinae	ID only if number is 10 times greater than that of all other microbiota (22) (*see* Table 3.18.1–4b for tests to identify)
	Corynebacterium (urease positive)	ID and AST, if number is 10 times greater than that of all other microbiota and ≥100,000 CFU.
Bacteremia	All pathogens	Full identification and AST

[a] Abbreviations: AST, antimicrobial susceptibility testing; ID, identification; VRE, vancomycin-resistant enterococcus.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

(b) Then with two additional sponges and sterile water or saline, rinse the vulva.

(2) Circumcised males: no preparation for midstream specimen.

(3) Uncircumcised males: the process is similar to that described above for females.

(a) Retract the foreskin, and wash the glans penis thoroughly with two successive cotton pledgets or sponges soaked in soap, paying special attention to the urethral meatus.

(b) Then rinse the glans with additional successive pledgets with sterile water or saline.

b. Have the patient collect voided urine directly into a disposable leakproof container, instructing the patient to not halt and restart the urinary stream for a "midstream" collection but preferably move the container into the path of the already voiding urine.

◩ **NOTE:** Never collect urine from a bedpan or urinal.

◩ **NOTE:** Most laboratories still recommend that patients follow the above instructions for cleansing the genital area before collection of a

Table 3.12–2 Definitions

Term	Definition
Bacteriuria	Presence of uropathogenic bacteria in the urine
Cystitis	Inflammation of the bladder
Cystostomy	Surgical procedure of inserting tube directly in the bladder through the suprapubic area to drain urine
Dysuria	Pain or burning on urination, a common complaint on presentation of UTI
Nephrostomy	Surgical procedure leaving tubing directly in the kidney
Prostatitis	Infection of the prostate gland; patient may present with fever or be asymptomatic
Pyelonephritis	Acute infection of the kidney and renal pelvis usually with fever, chills, and flank pain; chronic; may be without symptoms
Pyuria	Urine WBC count of 8–10/μl (or >5/high-power field in a conventional urinalysis), which correlates with WBC excretion rate of >400,000 WBC/h)
Urethritis	Inflammation of the urethra; may be caused by sexually transmitted diseases or uropathogens
Urosepsis	Pyelonephritis with accompanying bacteremia and septic shock
Urostomy	Surgical procedure leaving an external opening in the abdominal wall, usually made of intestine, for the egress of urine
Suprapubic aspirate	Specimen of urine collected with a syringe and needle inserted directly through the skin into the bladder
Straight catheter urine	Specimen collected by the insertion of a catheter through the urethra into the bladder
Uncomplicated UTI	Infection primarily in otherwise healthy women who have no structural or functional abnormalities of the urinary tract
Complicated UTI	Infection in a patient with structural and/or functional abnormalities of the urinary tract (e.g., spinal cord injury, neurogenic bladder, urinary tract obstruction, etc.)
Asymptomatic UTI	Infection with $>10^5$ bacteria/ml with no symptoms of infection

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

urine culture specimen. Limited studies have so far shown that this practice may not be warranted. One study found that cleansing by both asymptomatic and symptomatic women did not decrease contamination of specimens (11). Cleansing of the male genitalia did not improve the detection of bacteriuria (13). No difference in contamination rates was seen when a clean but nonsterile container was used for urine collection.

2. Catheter urine
   a. Using a needle and syringe, collect urine through the catheter port, after cleaning with alcohol. Alternatively, collect the sample directly into a Vacutainer tube without anticoagulant, using a Vacutainer holder and needle. *Do not send urine obtained from a catheter bag.*
   b. A straight catheter (in and out) is used by a physician or trained health care worker (HCW) to obtain urine directly from the bladder.
      (1) This procedure must be carried out with aseptic technique, to avoid the risk of introducing microorganisms into the bladder.
      (2) Discard the initial 15 to 30 ml of urine and submit the next flow of urine for culture.

3. Ileal conduit
   a. Remove the external device.
   b. Cleanse the stoma with 70% alcohol followed by iodine.
   c. Remove the iodine with alcohol.
   d. Insert a double catheter into the cleansed stoma, to a depth beyond the fascial level, and collect the urine.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

4. Urine collection by suprapubic needle aspiration directly into the bladder is performed by a physician or trained HCW. This method is the preferred method for infants, for patients for whom the interpretation of results of voided urine is difficult, and when anaerobic bacteria are suspected as the cause of infection.
   a. Bladder should be full and palpable before aspiration.
   b. Shave and disinfect the skin over the bladder.
   c. Make a small lance wound through the epidermis above the symphysis pubis.
   d. Aspirate using a needle and syringe. Place the urine specimen in a sterile container before submission to the laboratory. Syringes with the needle attached should not be accepted because of the sharps and biohazard risk to staff. Syringes with a Leur-Lok may leak during transport, resulting in contamination of the specimen.
5. Lower UTI in men is localized to the prostate by collection of sequential urine samples and expressed prostatic secretions (*see* procedure 3.9.1 and Appendix 3.9.1–2). Uropathogenic bacteria are the most common cause of acute and chronic prostatitis, but skin microbiota such as coagulase-negative *Staphylococcus* and *Corynebacterium* spp. may be found in low numbers in prostatic secretions from chronic prostatitis patients. *Neisseria gonorrhoeae* is found infrequently but has been reported as a cause of acute prostatitis.
6. Cystoscopy is a bilateral ureteral catheterization to determine the site of infection in the urinary tract. This procedure is usually performed in specially designated areas such as operating rooms or specialty clinics.
   a. Clean the urethral area (and vaginal vestibule in females) with soapy water, and rinse the area well with water.
   b. Insert a cystoscope (obturator in place) into the bladder.
   c. With sterile technique, collect approximately 5 to 10 ml of urine from open stopcock into a sterile container.
   d. Label this sample "CB," for catheterized bladder urine, and refrigerate it. Then irrigate the bladder. (Use sterile nonbacteriostatic 0.85% NaCl to irrigate the bladder.)
   e. After irrigation of the bladder and insertion of the ureteral catheters, collect irrigating fluid passing from the bladder through the ureteral catheters by holding the ends of both catheters over an opened sterile container.
   f. Label this sample "WB," for washed bladder urine, and refrigerate it.
   g. Pass the ureteral catheters to each midureter or renal pelvis without introducing additional irrigating fluid. Open both stopcocks of the cystoscope to empty the bladder.
   h. Discard the first 5 to 10 ml of urine from each ureteral catheter.
   i. Collect four consecutive paired cultures (5 to 10 ml each) directly into opened sterile containers.
   j. Label these specimens "LK-1," "RK-1," "LK-2," and "RK-2" (LK for left kidney and RK for right kidney). Submit all samples for culture.

B. Timing of specimen collection

1. Obtain early-morning specimens whenever possible. Allowing urine to remain in the bladder overnight or for at least 4 h will decrease the number of false-negative results.
2. Do not force fluids in order to have the patient void urine. Excessive fluid intake will dilute the urine and may decrease the colony count to $<10^5$ CFU/ml.

C. Specimen transport

1. Transport urine to the laboratory after collection or, if urine cannot be delivered to the laboratory within 2 h after collection, refrigerate for up to 24 h both during the holding period and during transport. *Do not freeze.*

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

2. If refrigeration is not possible and specimens are delayed in transport, collect in transport tubes with preservatives.
   a. Examples: 0.5 ml of freeze-dried boric acid-glycerol or boric acid-sodium formate (B-D urine tubes, Becton Dickinson, Rutherford, NJ).
   b. Place at least 3 ml of urine into the transport tube to avoid an inhibiting or diluting effect on the microorganisms.

D. Specimen labeling and request submission

1. Label the urine container with demographic information of the patient and the time of collection. Using the laboratory's protocol, ensure that the collection method is communicated to the laboratory.
2. Be alert to the fact that the person ordering the test may desire a Gram stain, may request only a colony count screen without preliminary identification, or may request omission of antimicrobial susceptibility testing (AST) in cases of uncomplicated UTI. Perform only those tests ordered.

   ◩ **NOTE:** Fungal cultures of urine usually are requests to detect the presence of yeasts. It is rarely necessary to inoculate these to selective fungal media with centrifugation and prolonged incubation. Notify the physician that yeast cultures are included as part of the routine urine culture and yeasts will be cultured and reported if found. However, when yeast cultures are requested, culture at least 0.01 ml per plate and hold cultures for 48 to 72 h to detect yeasts in low numbers.

E. Rejection criteria:

1. Request a repeat urine specimen when there is no evidence of refrigeration and the specimen is >2 h old.
2. Request a repeat specimen or obtain the information when the collection time and method of collection have not been provided.
3. Reject 24-h urine collections.
4. Reject dipslide specimens that have been improperly collected (e.g., urine left in the container, or paddle not fully inoculated on both sides or only partially inoculated).
5. Reject urine specimens obtained with the same collection method within 48 h of receipt of first specimen. Call this a duplicate specimen.
6. Neonates, infants, and young toddlers cannot cooperate to provide a voided sample. Although collection of urine by in-and-out straight catheterization is the recommended method to obtain an accurate urine culture sample, catheterization is not always possible, particularly in neonates. Collection of urine by pedibag may be the only way to obtain a urine sample in this age group. Although the laboratory should discourage submission of bagged specimens as the routine for urine culture, this type of sample should be analyzed if it is the only sample that can be obtained.
7. Reject Foley catheter tips as unacceptable for culture; they are unsuitable for the diagnosis of urinary infection (5).
8. Reject urine from the bag of a catheterized patient.
9. Reject specimens that arrive in leaky containers.
10. Except for suprapubic bladder aspirates, reject specimen requests for anaerobic culture.
11. If an improperly collected, transported, or handled specimen cannot be replaced, document in the final report that specimen quality may have been compromised and who was notified. Generally, voided urine from inpatients is easily recollected.
12. For rapid urine screens for voided urine specimens from outpatients to eliminate those that do not warrant culture, see Appendix 3.12–2 and reference 14.

III. MATERIALS

A. Media

1. BAP

2. MAC or EMB

3. Columbia colistin-nalidixic acid agar (CNA) or phenylethyl alcohol agar (PEA) (optional)

◩ **NOTE:** The advantage of adding CNA to the culture is that it allows detection of gram-positive microbiota when overgrown with gram-negative microbiota. In addition, the Columbia base allows growth of some bacteria that are inhibited on tryptic soy-based agars. The added expense may allow more accurate and often less time-consuming evaluation of cultures.

4. CHOC: use for surgically collected kidney urine or specimens collected by cystoscopy or after prostatic message. These can be labeled "EPS" or "VB3."

5. Other media: see Table 3.12–3 for commercial systems. These systems work best for specimens from outpatients or those likely to have only one pathogen. When mixed microbiota are expected (e.g., specimens from patients with indwelling catheters or from geriatric patients), these systems are contraindicated.

B. Stain reagents

See Gram stain procedure (procedure 3.2.1).

C. Supplies

1. Loop method

a. Use either platinum or sterile plastic disposable loops.

b. Sizes

(1) 0.001-ml (1-µl) loop to detect colony counts greater than 1,000 CFU/ml

(2) 0.01-ml (10-µl) loop to detect colony counts between 100 and 1,000 CFU/ml

(3) Disposable loops are color coded, according to volume delivery.

2. Pipettor method: sterile pipette tips and pipettor to deliver 10 or 1 µl.

3. Sterile bent glass or plastic disposable sterile rod or "spreader," if desired, to spread inoculum (Excel Scientific, Wrightwood, CA, [760] 249-6371).

4. For commercial systems in Table 3.12–3, follow manufacturers' instructions.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Unless calibrated pipettors are used for the inoculum and spreaders are used to spread the inoculum, colony counts are only approximations and can be off by as much as a hundredfold (1). Especially at higher counts, one colony does not represent 1 CFU, nor is this accuracy necessary for urine culturing. Choose the method from Fig. 3.12–1 that is most efficient, depending on the number of specimens received and the expertise of the staff. When the method of colony count has been decided, validate the method with the following protocol.

1. Make a 0.5 McFarland suspension of *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Enterococcus faecalis* ATCC 29212 in broth or saline.

2. Using a sterile pipette tip and calibrated pipettor, dilute each 1:100 by placing 0.05 ml (50 µl) of culture into 5 ml of saline. Vortex. This should represent 10^6 CFU/ml.

3. Prepare a 1:10 dilution from the 1:100 dilution by inoculating 0.5 ml of suspension to 4.5 ml of saline (equals 10^5 CFU/ml). Vortex and repeat to prepare two more 1:10 dilutions.

4. Label the dilutions "10^6," "10^5," "10^4," and "10^3," respectively, beginning with the 1:100 dilution (the labeling may have to be adjusted after the final counts are calculated from the growth).

5. Inoculate each dilution to a BAP by the chosen colony count method for use in the laboratory, using both the 0.01- and 0.001-ml loops or pipettes. Label the plates with organism name, the expected CFU per milliliter (10^6, 10^5, 10^4, and 10^3), and the dilution used (10^{-2} and 10^{-3} for 0.01 and 0.001 ml, respectively).

Table 3.12–3 Summary of bacteriological culture systems[a]

Culture system	Principle	Comment(s)
BBL CHROMagar Orientation Media (BD Diagnostic Systems)	Chromogenic agar that screens for urinary tract pathogens by production of different color changes on the media	Compares well to routine media (4)
Bullseye Urine Plate (HealthLink Diagnostics, Jacksonville, FL)	Five-chambered plate containing media for isolation and identification of common uropathogens and their AST patterns	Colonies counted; direct AST
CPS ID 3 (bioMérieux Vitek, Inc., Hazelwood, MO)	Enumeration and presumptive ID of common urinary pathogens	Identifies *E. coli*, *Proteus mirabilis*, *Enterococcus* spp., and indole-positive *Proteus;* presumptive ID of *Klebsiella pneumoniae*, *P. aeruginosa*, *S. aureus*, *S. agalactiae*, and *Candida* spp.
Diaslide Urine Culture Device (Healthcare Technologies, Cape Town, South Africa)	Transparent hinged plastic casing contains CLED and MAC for detection and presumptive identification of common UTI pathogens	Growth is compared to reference photographs for quantitation; morphology and color determine presumptive ID.
DIP N COUNT (Starplex Scientific, Etobicoke, Ontario, Canada)	Paddle contains CLED and MAC or EMB	Colonies are compared to a color density chart; color chart is used for ID.
URI-CHECK (Troy Biologicals, Inc., Troy, Mich.)	Dipslide culture for the enumeration and ID of uropathogens; dipslide contains CLED and MAC or EMB; similar to DIP N COUNT	Growth density is compared to chart.
Uricult (Orion Diagnostica, Espoo, Finland)	Dipslide containing CLED-polymyxin, MAC, and a β-glucuronidase substrate for enumeration of microorganisms and presumptive ID of *E. coli*	*E. coli* appears as brown colonies.
Wampole Bactercult (Inverness Medical, Princeton, NJ)	Sterile, disposable tube coated with culture medium; contains phenol red indicator for presumptive ID of common uropathogens	Colonies counted

[a] Updated from reference 2. Abbreviations: ID, identification; CLED, cystine lactose electrolyte-deficient agar.

IV. QUALITY CONTROL *(continued)*

6. Also inoculate 0.05 ml (50 μl) of each of the last three 1:10 dilutions to a BAP, using a sterile pipette tip and calibrated pipettor. Spread these plates with a spreader for accurate colony counting. Label these plates with the organism names, "10^5," "10^4," or "10^3," and the words "1:20 dil." Do this in duplicate.

   ◩ **NOTE:** Spreading with a loop will not give an accurate count.

7. Incubate plates for 18 h, and find the lowest dilution made with the 50-μl pipette (1:20 dilution) that has 30 to 300 colonies. Multiply the count by 20 to determine the CFU per milliliter in that dilution of the culture. Repeat with the duplicate plate of that dilution and determine the average count of the two plates. Use this accurate count to adjust the final counts for each of the four dilutions (10^6, 10^5, 10^4, and 10^3) based on this calculation.

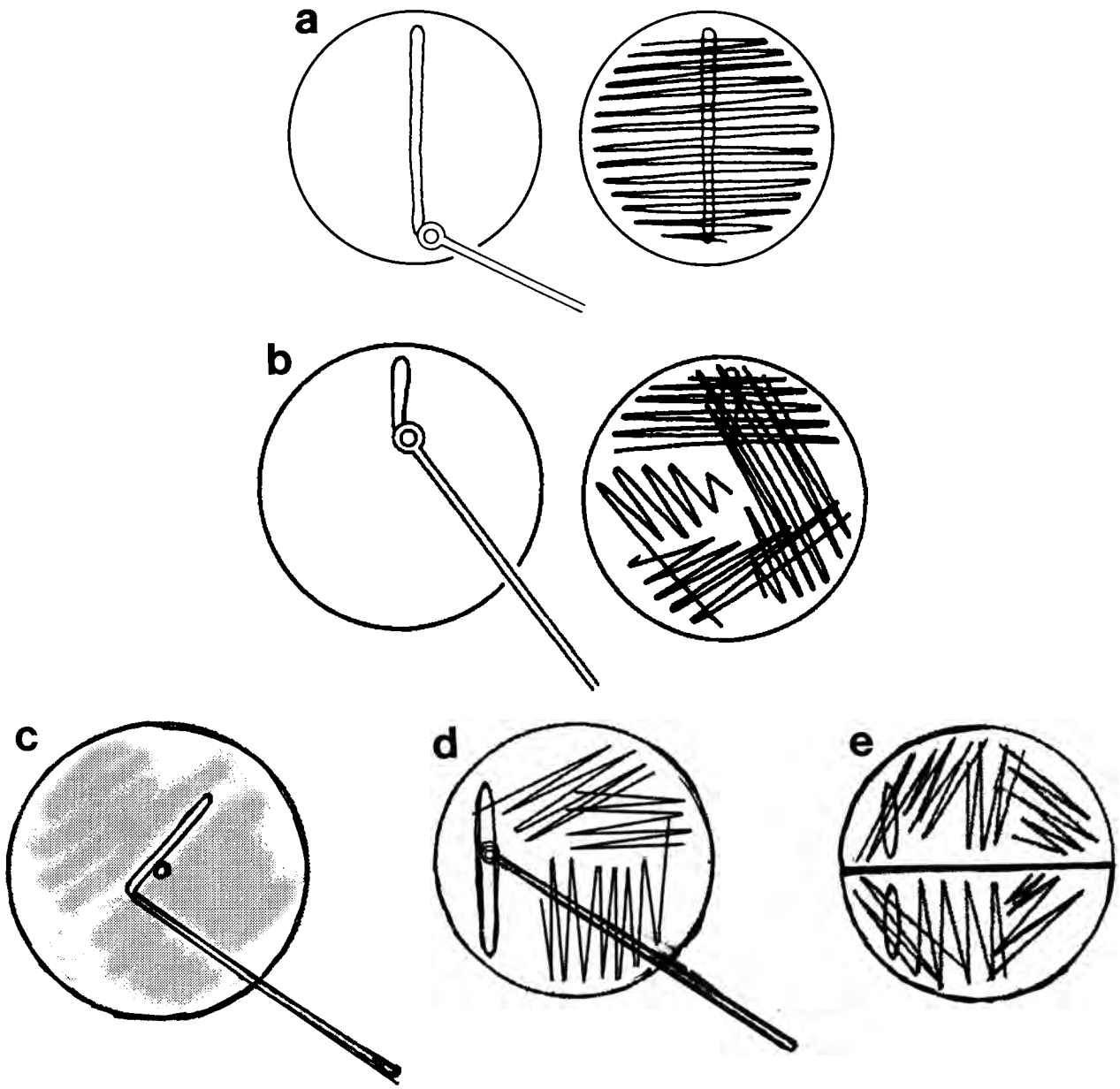


Figure 3.12–1 (a) Method of streaking urine for colony count using a 0.001-ml loop; (b) method of streaking urine for colony count using either a 0.01- or 0.001-ml loop; (c) spreader method of streaking urine for colony count after inoculation with either a 0.01- or 0.001-ml volume; (d) method of streaking 0.01 ml of urine using drip method for count and quadrant streaking for isolation; (e) MAC-CNA biplate with 0.01 ml of urine streaked in quadrants (EMB and PEA are other options).

IV. QUALITY CONTROL *(continued)*

Example: If the 1:20 dilution of the 10^4 plates has an average of 50 colonies, the true count is 50×20, or 10^3. All plates counts should then be reduced by a factor of 10 (e.g., the 10^4 is 10^3, the 10^3 is 10^2).

8. Relabel each of the plates inoculated by the urine count method, to match the actual counts determined by the calibrated method.

IV. QUALITY CONTROL
(continued)

9. Display plates for the laboratory staff to educate them on the appearance of each colony count. Photographs can be helpful. Prepare definitions of colony counts, using the appearance of each plate and enter as part of this procedure.
Example: "10^5 CFU/ml shows confluent growth in the initial drop of urine with the 0.01-ml inoculum but shows approximately 50 colonies with the 0.001-μl loop."

B. Inspect nondisposable calibrated loops regularly to confirm that they remain round and are free of bends, dents, corrosion, or incinerated material. Refer to Appendix 3.12–3 for procedures to QC microbiological loops.

C. For disposable loops, check for certificate of count validation from the manufacturer with each lot. Verify calibration for each of the first few lots when changing manufacturers, by following Appendix 3.12–3.

D. Calibrate pipettors at regular intervals, if used (*see* Appendix 3.12–4).

E. Verify that media meet expiration date and QC parameters per Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22-A3 (3). See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

V. PROCEDURE

Observe standard precautions.

A. Microscopic and other direct methods

◩ **NOTE:** The Gram stain is useful in rapidly determining the type and count of bacteria and cells in urine and should be performed on request (16, 21). It is particularly helpful if results can be made available while the patient is in the clinic. For inpatients, the urine Gram stain can help diagnose the cause of sepsis before blood cultures are positive.

1. Gram stain: refer to procedure 3.2.1.
- **a.** Place 10 μl of well-mixed, *uncentrifuged* urine onto a glass slide, and allow it to air dry without spreading. Fix with methanol.
- **b.** Determine the number of organisms per oil immersion field. One organism seen per ×1,000 correlates to a colony count of 10^5/ml of urine (2).
- **c.** The presence of many squamous epithelial cells and different microbial morphotypes suggests contamination.

2. Detection of pyuria

◩ **NOTE:** Pyuria with a WBC count of >10/μl (or >5 per high-power field [HPF] in a conventional urinalysis) has a specificity of 90% for predicting catheter-associated UTI with greater than 10^5 CFU/ml but a sensitivity of only 37% (20).
- **a.** Detect by either Gram stain or urinalysis (procedure 3.2.3), from examination of freshly collected uncentrifuged urine.
- **b.** Use enhanced urinalysis for pediatric patients less than 2 years old. Place uncentrifuged urine in a hemacytometer and count WBCs (7, 15, 16). This method is reported to have a sensitivity of 84% and specificity of 90% for infants (12).
- **c.** For commercial methods, see Appendix 3.12–1 and reference 15.

B. Culture methods

1. For voided urine specimens and those from indwelling catheters, culture at least 0.001 ml. For other urine specimens, culture 0.01 ml. See Table 3.12–4.

◩ **NOTE:** Only streak the blood plate for colony count. Other plates should be streaked in quadrants for isolation of colonies (Fig. 3.12–1b and e), rather than for colony count, to minimize delays in obtaining isolated colonies and false-negative culture results due to antimicrobial inhibition. If colony count cannot be performed due to overwhelming spreading *Proteus*, an estimate of the count can be made from the isolation plates.

V. PROCEDURE *(continued)*

2. Inoculation methods for colony count (*choose one method*)
- **a.** Calibrated-loop method
  - (1) Using the disposable or flamed and cooled calibrated loop, hold the loop vertically, and immerse it just below the surface of a well-mixed, uncentrifuged urine specimen.
  - (2) Deliver a loopful of well-mixed urine onto the blood agar plate and spread using one of the three methods below.
    - (a) Using the loop, make a straight line down the center of the plate and streak the urine by making a series of passes at 90° angles through the inoculum (method for 0.001 ml only). See Fig. 3.12–1a.
    - (b) Deliver the loopful onto one quadrant of plate by making a straight line or a V-shaped line. Streak urine onto the first quadrant, and proceed by streaking onto all four quadrants. See Fig. 3.12–1b.
    - (c) Use a sterile spreader or bent rod ("hockey stick") to spread the loopful of urine over the surface of the entire plate in three directions (Fig. 3.12–1c). An accurate colony count will not be obtained if the calibrated loop is used to inoculate the plates.
- **b.** Pipette inoculation methods
  - (1) With a sterile pipette tip and calibrated pipettor, aspirate 0.01 ml of well-mixed urine onto one side of plate and allow to drip down plate. Streak urine in quadrants on rest of plate with sterile loop. See Fig. 3.12–1d.
  - (2) Alternatively, drop onto plate and spread by one of the methods described above.
- **c.** For bacteriological culture systems, see Table 3.12–3. For each method, a measured amount of urine is placed on agar surfaces, and after incubation, colonies are counted. The sensitivities of these systems vary and are high when only one pathogen is likely.

3. Take an additional loopful or pipette full of well-mixed urine for selective agar plates to be inoculated and streak in quadrants for isolation as indicated in Fig. 3.12–1e and Fig. 3.3.1–1 and 3.3.1–2 in procedure 3.3.1.

4. Incubate in ambient air overnight at 35 to 37°C.
- **a.** Anaerobic cultures of the urine should be done on request on suprapubic aspirates, when a vesiculoenteric fistula is suspected, and when bacterial morphotypes are seen in the direct smear but fail to grow on aerobic culture.
- **b.** If convenient, incubate BAP and CNA in 5% CO_2 to enhance growth of gram-positive organisms.

5. Examination of culture media
- **a.** Examine cultures that have been incubated overnight but make final reading at 18 h unless the specimen fits the criteria below.
- **b.** Reincubate until culture has incubated for 48 h if one of the following is true.
  - (1) The specimen was collected by an invasive technique, such as suprapubic bladder aspiration or straight catheter method.
  - (2) Tiny or scant colonies are present that are barely discernible.
  - (3) Culture results do not correlate with Gram stain findings or clinical conditions (e.g., the patient has sterile pyuria or symptoms without a positive culture).
  - (4) The patient is immunocompromised, including patients who have transplanted organs.

Table 3.12–4 Protocol for workup of urine cultures

Type of urine	Inoculation	No. of isolates			Time to final report
		1 uropathogen[a]	2 uropathogens[a]	≥3 uropathogens	
Voided midstream; obtained in outpatient clinics and doctors' offices from patients <65 yr old	Streak 0.001 ml to BAP for count. Streak MAC (or EMB) in quadrants (CNA or PEA optional).	<10,000 CFU/ml, minimal ID[b] ≥10,000 CFU/ml (or ≥1,000 CFU of uropathogen/ml in females 14–30 yr old),[c] definitive ID[d] and AST	For each <100,000 CFU/ml, minimal ID For each that is ≥100,000 CFU/ml, definitive ID and AST	Report count plus "Multiple bacterial morphotypes present. Suggest appropriate recollection with timely delivery to the laboratory, if clinically indicated."	Day 1 or 2 (minimum incubation, 18 h)[e]
Indwelling catheter; voided from geriatric (≥65 yr old) and all inpatients	Streak 0.001 ml to BAP for count. Streak CNA (or PEA) and MAC (or EMB) in quadrants.	<10,000 CFU/ml, minimal ID ≥10,000 CFU/ml, definitive ID and AST	For each <100,000 CFU/ml, minimal ID For each that is ≥100,000 CFU/ml, definitive ID and AST	If voided urine, or if catheter collected and urinalysis WBCs or leukocyte esterase is available and normal, report as for voided outpatient urine. *or* Contact caregiver to determine if specimen was collected through catheter port and if patient is febrile or symptomatic. If so, follow protocol as for two uropathogens. *otherwise* Minimal ID of each uropathogen, with note: "Contact laboratory if definitive identifications are clinically indicated."	Day 2 or 3 (minimum incubation, 36 h)
Straight catheter; pediatric catheterized, suprapubic, kidney, cystoscopy yeast cultures	Streak 0.01 ml to BAP for count. Streak CNA (or PEA) and MAC (or EMB) in quadrants. Add CHOC for surgically collected or prostate specimens.	100 to 1,000 CFU/ml with normal urogenital or skin microbiota, minimal ID ≥1,000 CFU/ml or any pure culture of lower count of uropathogen, definitive ID and AST	For each <1,000 CFU/ml, minimal ID For each uropathogen that is ≥1,000 CFU/ml, definitive ID and AST	For each <10,000 CFU/ml, minimal ID For each that is ≥10,000 CFU/ml, definitive ID and AST *or* Contact physician to determine extent of workup.	Day 2 or 3 (minimum incubation, 48 h)

[a] Urogenital or skin microbiota that is at least 10 times less than the uropathogens is ignored for purposes of workup. If this microbiota is equal to the uropathogens, report "Multiple bacterial morphotypes present. Suggest appropriate recollection with timely delivery to the laboratory, if clinically indicated." For list of uropathogens and urogenital and skin microbiota, see Table 3.12–1.

[b] For minimal identification (ID), see Table 3.12–5.

[c] Report any amount of group B streptococci (*S. agalactiae*) in this age group. Check staphylococci with novobiocin disk for *Staphylococcus saprophyticus*, a uropathogen in this age group.

[d] For definitive ID, see Table 3.12–1.

[e] See text (item V.B.5.b) for exceptions.

V. PROCEDURE *(continued)*

(5) Yeast or fungal culture is requested or appropriate (e.g., neonatal intensive care unit cultures).

◩ **NOTE:** Incubation may need to be up to 72 h if CNA or EMB is not used. Many yeasts grow well on EMB.

c. For positive cultures, examine culture media for the quantity and morphological type of organisms present.
(1) With a 0.001-ml loop, one colony equals 1,000 CFU/ml.
(2) With a 0.01-ml loop, one colony equals 100 CFU/ml.
(3) When the colonies are too numerous to count
(a) The maximum readable using the 0.001-ml loop is $>10^5$ CFU/ml.
(b) The maximum readable on the 0.01-ml loop is $>10^4$ CFU/ml.
(4) Use the procedure in item IV to estimate counts between the maximum and minimum.

d. An accurate colony count may be difficult to establish for all morphotypes on dipslide paddles. In order to determine the clinical significance of potential uropathogens if growth is mixed, morphotypes should be subcultured to plated media to establish an accurate colony count.

6. Further workup of positive cultures
a. Determine the colony count of each morphotype in the culture separately by examining BAP.
b. Using Tables 3.12–1 and 3.12–4, determine the extent of workup of each organism.
c. For testing for minimal identification guidelines, refer to Table 3.12–5.
d. Do not identify normal urogenital microbiota to the genus or species level.
e. *Streptococcus agalactiae* should be reported from women in childbearing years and from known diabetics, regardless of the count.
f. Because *E. coli* represents 80% of the pathogens in voided urine cultures, use rapid identification methods to identify this species. See Table 3.3.2–5.
g. For definitive identifications, refer to Table 3.3.2–5 and procedures 3.18.1 and 3.18.2.
h. Perform AST as indicated in the tables and described in section 5.
i. Always identify to the species level if oxidase positive and indole positive, since such organisms are pathogens regardless of count (e.g., *Aeromonas* and *Vibrio* spp.).

7. Hold positive culture plates at room temperature for at least 2 to 3 days for possible further workup if requested by the patient's physician.

8. Hold urine specimens at refrigeration temperatures for 24 h to resolve any problems with the specimen or culture results.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report Gram stain results for bacteria and cells per Gram stain procedure (3.2.1).

B. Negative results
1. If no growth is observed on all media, report "Urine culture negative" or "No growth of uropathogens."
2. If 0.001 ml was cultured, report "No growth of $\geq 10^3$ CFU/ml at xx."

C. Positive results
1. If only urogenital or skin microbiota is observed, report as such.
Example: "10,000 CFU/ml normal urogenital microbiota."

Table 3.12–5 Reporting of isolates with minimal testing[a]

Report	Minimal testing
Presumptive *E. coli*	Grows typically on MAC or EMB and appears lactose positive or spreading on any medium or is indole positive and oxidase negative.
Presumptive *P. aeruginosa*	Appears lactose negative on MAC or EMB and is oxidase positive and indole negative or has odor of *Pseudomonas* or *Alcaligenes*. If desired, inoculation and incubation of KIA or TSI can be used to distinguish from enteric rods.
Gram-negative rod	Grows well on EMB or MAC but does not fit either of the above criteria for gram-negative rods.
Yeast	Morphology consistent with yeast in wet mount or "feet" present on plate.
S. aureus	Catalase-positive, gram-positive cocci and slide coagulase positive. *Caution: S. saprophyticus* can be positive in latex or coagglutination serologic coagulase tests.
Coagulase-negative staphylococci	Catalase-positive, gram-positive cocci and slide coagulase negative
Enterococcus	Catalase-negative, PYR-positive, nonhemolytic, and gram-positive cocci
Viridans group streptococci	Gram-positive cocci in pairs and chains, catalase negative, alpha-hemolytic, or PYR negative. Rule out *A. urinae* (differs by a Gram stain showing tetrads and clusters) prior to reporting, if numbers are significant (22).
Lactobacillus spp.	Gram-positive rods, catalase negative, alpha-hemolytic
Corynebacterium spp.	Gram-positive rods, catalase positive. Rule out uropathogenic *Corynebacterium* with rapid urease test (procedure 3.17.48), if numbers are significant.

[a] See Table 3.12–4 for indications. Abbreviations: KIA, Kligler's iron agar; TSI, triple sugar iron agar; PYR, pyrrolidonyl-β-naphthylamide.

VI. REPORTING RESULTS *(continued)*

2. As specified in Table 3.12–4, mixed cultures are reported with the count in CFU per milliliter, followed by "Multiple bacterial morphotypes present; possible contamination; suggest appropriate recollection, with timely delivery to the laboratory, if clinically indicated."

3. Otherwise, report the colony count of each pathogen separately, followed by the presumptive, minimal, or definitive identification and susceptibility test results, as indicated in Tables 3.12–4 and 3.12–5.

◩ **NOTE:** For general principles of reporting preliminary and final results, refer to procedure 3.3.2.

4. When antimicrobial inhibition is observed (i.e., no growth in the primary area of the plate but growth in the area where the inoculum is diluted), do not report the count but report "Colony count unreliable due to antimicrobial inhibition."

5. Notify physician of unusual positive findings (e.g., *Salmonella enterica* serovar Typhi or *Burkholderia pseudomallei*).

D. Document all testing in a hard copy or computerized work card.

VII. INTERPRETATION

A. The criterion of $\geq 10^5$ CFU/ml for significance can be applied to a majority of specimens submitted for culture. However, the following are true.

1. A mixed culture in an uncomplicated outpatient population likely indicates contamination.

VII. INTERPRETATION *(continued)*

2. Low levels ($<10^4$/ml) of organisms commonly found on the skin and external and internal genitalia are considered to be contaminants, but in selected circumstances, a count of *Enterobacteriaceae* of 10^2 CFU/ml or more, especially for *Salmonella,* can be considered significant.

3. Colony counts of $<10^5$ CFU/ml in voided specimens in the presence of dysuria and symptoms of UTI may be significant.

B. AST should not be performed directly from urine specimens. These methods are not Food and Drug Administration approved and are not standardized. Refer to *Cumitech 2B* for further discussion (2).

VIII. LIMITATIONS

A. Women with uncomplicated UTI for which the microbial etiology is established (e.g., *E. coli* or *S. saprophyticus*) may be treated empirically with a single high dose or a short course (3 days) of antimicrobial agents. Urine culture for identification and AST of the causative organisms should be done in cases where there is treatment failure and relapsing infection, suspected subclinical pyelonephritis, or recurrent infection.

B. In cases of sterile pyuria, the Gram stain is important. If organisms are seen but not cultured and findings persist, an anaerobic culture may be indicated.

C. False-negative results are due, in part, to interfering substances, diluted urine, low urine pH, and subjective interpretation of the criteria for further workup of the culture.

D. Acute urethral syndrome due to urethritis should be suspected in sexually active women who have dysuria and pyuria but sterile urine cultures. Genital testing for *N. gonorrhoeae*, *Chlamydia trachomatis,* and *Ureaplasma urealyticum* should be done. Dysuria also occurs in patients with vulvovaginitis, and vaginal swabs should be submitted for detection of bacterial vaginosis, *Candida* overgrowth, and *Trichomonas vaginalis* (*see* procedure 3.9.1).

E. For sterile pyuria, mycobacterial infection should be considered (*see* section 7). The specimen for culture should be the first voided urine of the day, three days in a row.

REFERENCES

1. **Albers, A. C., and R. D. Fletcher.** 1983. Accuracy of calibrated-loop transfer. *J. Clin. Microbiol.* **18:**40–42.
2. **Clarridge, J. E., J. R. Johnson, and M. T. Pezzlo.** 1998. *Cumitech 2B, Laboratory Diagnosis of Urinary Tract Infections.* Coordinating ed., A. L. Weissfeld. American Society for Microbiology, Washington, DC.
3. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Dejulius, K. L., K. Smith, S. Parshall, D. Warner, L. Foy, P. Shah, A. Miskov, S. Schindler, G. W. Procop, and G. Hall.** 2004. Use of BBL CHROMagar Orientation Media for the identification and enumeration of urinary tract pathogens: comparison to routine culture techniques. Presented at the 104th General Meeting of the American Society for Microbiology, New Orleans, LA.
5. **Gross, P. A., L. M. Harkauy, G. E. Barden, and M. Kerstein.** 1974. The fallacy of cultures of the tips of Foley catheters. *Surg. Gynecol. Obstet.* **139:**597–598.
6. **Heldrich, F. J., M. A. Barone, and E. Spiegler.** 2000. UTI: diagnosis and evaluation in symptomatic pediatric patients. *Clin. Pediatr.* (Philadelphia) **39:**461–472.
7. **Hoberman, A., E. R. Wald, L. Penchansky, E. A. Reynolds, and S. Young.** 1993. Enhanced urinalysis as a screening test for urinary tract infection. *Pediatrics* **91:**1196–1199.
8. **Hooton, T. M., and W. E. Stamm.** 1997. Diagnosis and treatment of uncomplicated urinary tract infection. *Infect. Dis. Clin. N. Am.* **11:**551–581.
9. **Johnson, J. R., and W. E. Stamm.** 1989. Urinary tract infections in women: diagnosis and treatment. *Ann. Intern. Med.* **111:**906–917.
10. **Kass, E. H.** 1956. Asymptomatic infections of the urinary tract. *Trans. Assoc. Am. Phys.* **69:**56–64.
11. **Lifshitz, E., and L. Kramer.** 2000. Outpatient urine culture: does collection technique matter? *Arch. Intern. Med.* **160:**2537–2540.
12. **Lin, D. S., F. Y. Huang, N. C. Chiu, H. A. Koa, H. Y. Hung, C. H. Hsu, W. S. Hsieh, and D. I. Yang.** 2000. Comparison of hemocytometer leukocyte counts and standard urinalyses for predicting urinary tract infections in febrile infants. *Pediatr. Infect. Dis. J.* **19:**223–227.

REFERENCES *(continued)*

13. **Lipsky, B. A., R. C. Ireton, S. D. Fihn, R. Hackett, and R. E. Berger.** 1987. Diagnosis of bacteriuria in men: specimen collection and culture interpretation. *J. Infect. Dis.* **155:**847–854.
14. **Pezzlo, M. T., D. Amsterdam, J. P. Anhalt, T. Lawrence, N. J. Stratton, E. A. Vetter, E. M. Peterson, and L. M. de la Maza.** 1992. Detection of bacteriuria and pyuria by URISCREEN, a rapid enzymatic screening test. *J. Clin. Microbiol.* **30:**680–684.
15. **Pfaller, M. A., B. Ringenberg, L. Rames, J. Hegeman, and F. Koontz.** 1987. The usefulness of screening tests for pyuria in combination with culture in the diagnosis of urinary tract infection. *Diagn. Microbiol. Infect. Dis.* **6:**207–215.
16. **Shaw, K. N., K. L. McGowan, M. H. Gorelick, and J. S. Schwartz.** 1998. Screening for urinary tract infection in infants in the emergency department: which test is best? *Pediatrics* **101:**1–5.
17. **Stamm, W. E., G. W. Counts, K. R. Running, S. Fihn, M. Turck, and K. K. Holmes.** 1982. Diagnosis of coliform infection in acute dysuric women. *N. Engl. J. Med.* **307:**463–468.
18. **Stark, R. P., and D. G. Maki.** 1984. Bacteriuria in the catheterized patient: what quantitative level of bacteriuria is relevant? *N. Engl. J. Med.* **311:**560–564.
19. **Tambyah, P. A., and D. G. Maki.** 2000. Catheter-associated urinary tract infection is rarely symptomatic: a prospective study of 1,497 catheterized patients. *Arch. Intern. Med.* **160:**678–682.
20. **Tambyah, P. A., and D. G. Maki.** 2000. The relationship between pyuria and infection in patients with indwelling urinary catheters: a prospective study of 761 patients. *Arch. Intern. Med.* **160:**673–677.
21. **Washington, J. A., II, C. M. White, M. Laganiere, and L. H. Smith.** 1981. Detection of significant bacteriuria by microscopic examination of urine. *Lab. Med.* **12:**294–296.
22. **Zhang, Q., C. Kwoh, S. Attorri, and J. E. Clarridge III.** 2000. *Aerococcus urinae* in urinary tract infections. *J. Clin. Microbiol.* **38:**1703–1705.

APPENDIX 3.12–1

Observe standard precautions.

Commercial Systems for Evaluation of Pyuria Prior to Culture

A. The KOVA Glasstic Slide 10 (Hycor Biomedical, Inc.) is an optically clear, plastic, disposable slide chamber with grids that holds up to 10 specimens and allows the user to perform quantitative microscopic counts of somatic and bacterial cells. Each of the 10 chambers will hold a standardized 6.6 μl of sample and has a depth of 0.1 mm.
   1. Add 6.6 μl of uncentrifuged urine to one grid of the KOVA Slide.
   2. Allow grid to fill by capillary action.
   3. Quantitate cells under ×400 magnification.
   4. A positive urine sample contains >10 WBCs/μl.

B. Yellow IRIS (International Remote Imaging Systems) is an automated urinalysis instrument for use on midstream urine specimens from immunocompetent adults. It incorporates a flow microscope, video camera, and digitalized picture image-processing computer.

C. Dipstick method for detection of bacteria and WBCs
   1. Specimen: fresh voided urine from immunocompetent patient
   2. Materials: Chemstrip LN (Bio-Dynamics, Division of Boehringer Mannheim Diagnostics, Indianapolis, IN)
   3. Procedure
      a. Dip the strip into the urine specimen for 1 s.
      b. Withdraw the strip over the specimen container rim to remove excess urine.
      c. Read results within 2 min.
      d. Compare the color intensity with the color guide provided by the manufacturer.
   4. Interpretation
      a. Nitrate reductase
         (1) Many, but not all, gram-negative bacilli reduce nitrate to nitrite.
         (2) Low urine pH (<6) may yield false-negative test results.
      b. WBC esterase in WBCs produces a positive reaction.
   5. Limitations
      a. Vitamin C, phenazopyridine, and high protein levels may interfere with test results.
      b. The leukocyte esterase is a sensitive test to determine pyuria, but the sensitivity of test results is higher when both parameters are used.
      c. Some non-glucose-fermenting gram-negative bacilli, e.g., *Acinetobacter*, are nitrate negative. All gram-positive microorganisms are nitrate negative. False-negative results will occur with infections with these organisms, which may be common in nosocomial UTI.
      d. Neutropenic immunocompromised patients, having very few if any WBCs in urine, have false-negative leukocyte esterase tests.

APPENDIX 3.12–2

Observe standard precautions.

Rapid Urine Screens

A. Accutest Uriscreen (Jant Pharmacal Corp., Encino, CA).
   1. Rapid screening test that combines the detection of both bacteriuria and pyuria in urine by detecting catalase activity
   2. Add 1.5 to 2.0 ml of well-mixed urine to a tube containing dehydrated reagent.
   3. Add 4 drops of 10% hydrogen peroxide.
   4. Gently mix the contents of the tube, avoiding formation of bubbles.
   5. Observe the tube for the formation of foam above the surface of the liquid.
   6. A positive reaction is indicated by the release of catalase and occurs within 2 min.
   7. Visual results are obtained within 2 min.

B. The semiautomated Coral UTI Screen and the Wave 180 Automated luminometer system (Coral Biotechnology, San Diego, CA) constitute an automated screening system that will detect the presence of organisms in urine. The Coral UTI Screen uses a somatic cell release agent (SRA) to release and destroy the ATP present in somatic cells (bacterial ATP is protected within bacterial cells). Bacterial ATP is then liberated from cells and detected as follows. A reduced form of luciferin reacts with bacterial (or yeast) ATP in the presence of the enzyme luciferase to produce oxidized luciferin, AMP, and light. The light produced is measured by a photomultiplier tube detector luminometer, and the signal is converted to relative light units (RLU). The RLU produced is proportional to the amount of bacterial ATP present in the urine sample. A 25-μl aliquot of urine is tested in the Coral UTI Screen collected with or without preservative. After addition of the sample to the SRA tube and incubation for a minimum of 15 min, the test result is generated within seconds of loading the Wave 180 luminometer system. Two UTI-chec calibration standards supplied by the manufacturer are tested during each run as part of the QC procedure. The Wave 180 luminometer reports patient results as negative, positive, or OLV (sample counts greater than the response range), which are calculated as a percentage of the calibrator RLU for each sample. The Coral UTI Screen system has been shown to be an accurate, sensitive, and cost-effective method for screening of urine samples (2).

References

1. **Pezzlo, M. T., D. Amsterdam, J. P. Anhalt, T. Lawrence, N. J. Stratton, E. A. Vetter, E. M. Peterson, and L. M. de la Maza.** 1992. Detection of bacteriuria and pyuria by URISCREEN, a rapid enzymatic screening test. *J. Clin. Microbiol.* **30:**680–684.
2. **Semeniuk, H., J. Noonan, H. Gill, and D. Church.** 2002. Evaluation of the Coral UTI Screen™ system for rapid automated screening of significant bacteriuria in a regional centralized laboratory. *Diagn. Microbiol. Infect. Dis.* **44:**7–10.

APPENDIX 3.12–3

Use and Calibration of Microbiological Loops

I. PRINCIPLE

The volume of fluid picked up by a calibrated loop depends on the volume and shape of the container holding the liquid and the direction and depth the loop is immersed. Variability results from surface tension and wetted surface of the loop. Liquids in containers with small diameters (<1 cm) have high surface tensions, which result in less loop pickup since glass-liquid and plastic-liquid (adhesive) forces are greater than liquid-liquid (cohesive) forces. When the wire above the loop is wetted by deep immersion into the fluid, excess liquid drains down the wire and enlarges the volume transferred (1).

Quantitative loops are commonly used to set up quantitative cultures and check inocula for antimicrobial tests. Quantitative loops are less accurate than pipettors yet are an excellent way to set up a semiquantitative culture or dilution. Quantitative loops are used when ≤20% error is acceptable.

Loops are calibrated either by the dye method or by the drill bit method. Another method, the pour plate method, is rarely used because it is cumbersome.

II. TYPES OF LOOPS

A. Volume is either 1 or 10 μl.

B. Reusable loops are made of wire, usually platinum or nichrome.

C. Disposable loops are made of plastic.

APPENDIX 3.12–3 *(continued)*

III. MATERIALS

A. Drill bit method (drill bits can be purchased at a hardware store)
1. Twist drill bits (no. 53 and 54) for 0.001-ml loop
2. Twist drill bits (no. 21 and 22) for 0.01-ml loop
3. For disposable loops, some manufacturers supply a "go-no go" bar to calibrate their loops. Drill bits do not work for these loops.

B. Dye method
1. Distilled water, either type I or type II
2. Evans blue dye solution (EBD)
   a. Stock
      (1) Add 0.75 g of EBD to 100 ml of distilled water.
      (2) Filter solution through no. 40 Whatman filter paper.
      (3) Store at room temperature in a dark bottle for 6 months. Label bottle with contents, expiration date, and initials of preparer. Note lot number and expiration date in the work record.
   b. Working solutions
      (1) Prepare dilutions of the EBD stock solution in distilled water to equal to 1:500, 1:1,000, 1:2,000, and 1:4,000 by diluting 1 ml of stock dye in 49 ml of distilled water.
      (2) Dilute 1 ml of this 1:50 dilution in 9 ml of distilled water. This is a 1:500 dilution.
      (3) Perform twofold serial dilutions, starting with 5 ml of the 1:500 solution and 5 ml of distilled water, to prepare the remaining dilutions.
      (4) Store the four dilutions for up to 6 months, but prepare new dilutions if the reading of any one dilution differs by 3% from previous readings.
3. Supplies
   a. Square glass cuvettes with a 1-cm light path. Use the same cuvette for all measurements.
   b. Glassware: several clean 15-ml test tubes, several class A 1- and 10-ml pipettes
   c. Good-quality spectrophotometer with the following specifications: linear through 2.0 absorbance units at 350 nm; spectral band width, $\leq$2 nm (348 to 352 nm); precision (drift) of $\leq$0.001 Å/h; accuracy of wavelength selected, $\leq$1 nm

IV. QUALITY CONTROL

A. General considerations
1. Reusable loops
   a. Inspect calibrated loops regularly to confirm that they remain round and are free of bends, dents, corrosion, or incinerated material.
   b. Check the loops to ensure that the delivery volume is accurate on a monthly basis (2).
2. Check delivery volumes of disposable loops upon receipt of a new lot number. Satisfactory performance of multiple lots and manufacturer's certificate of in-house QC eliminate the need for routine calibration.

B. Methods for calibration of loops
1. Food and Drug Administration (FDA) drill bit method

   ◩ **NOTE:** The FDA uses the standard 0.001-ml platinum-rhodium loop in plate counts on milk. The drill bit method of QC is simple and does not require a spectrophotometer.
   a. Calibration of 0.001-ml loop (inside diameter of 1.45 $\pm$ 0.06 mm)
      (1) Obtain two twist drill bits (no. 53 and 54).
      (2) Carefully slip the 0.001-ml loop over the end of the no. 54 bit. If the loop is calibrated, it will fit over the bit.
      (3) Repeat the procedure with the no. 53 bit. If the loop is calibrated, it will not fit over the end.
      (4) If the loop fits over the no. 53 bit, it is inaccurate. Follow corrective action.
   b. Calibration of 0.01-ml loop
      (1) Obtain two twist drill bits (no. 21 and 22).
      (2) Repeat the procedure described above for the 0.001-ml loop.
      (3) A calibrated 0.01-ml loop will fit over the end of a no. 22 drill bit and will not fit over a no. 21 bit.

APPENDIX 3.12–3 *(continued)*

c. For disposable loops, the drill bits will not work but the manufacturer may supply a calibration bar that is similar to the drill bits.

2. Colorimetric dye calibration procedure
   a. Set spectrophotometer to absorbance mode, using a wavelength of 600 nm.
   b. Zero spectrophotometer with distilled water.
   c. Measure and record the absorbance of each dye dilution (1:500, 1:1,000, 1:2,000, 1:4,000).
   d. Plot the optical density (vertical axis) against each of the concentrations of the dilutions (horizontal axis) on worksheet 1 (*see* p. 3.12.28). Draw a single line that most closely fits all four points to construct the calibration curve.
   e. Using the 0.001-ml loop, transfer 10 loopfuls of the EBD stock dye solution to 10 ml of distilled water. After thorough mixing, measure and record the absorbance of this solution. The absorbance should correspond to that of the 1:1,000 dilution on the calibration curve.
   f. Prepare and evaluate three additional test solutions for a total of four readings. Record the readings on the worksheet and determine the average. Then calculate the percent inaccuracy by following the instructions on the worksheet.
   g. If the average reading is more than $\pm 20\%$ of the 1:1,000 stock solution dilution, the loop is inaccurate. Follow with corrective action.
   h. To calibrate the 0.01-ml loop, transfer 10 loopfuls of the EBD stock solution to 100 ml of distilled water using the 0.01-ml loop. After thorough mixing, measure and record the absorbance of this solution. Prepare and evaluate three additional test solutions for a total of four readings. Then calculate the percent inaccuracy by following the instructions on the worksheet. The final reading should be the same as that of the 0.001 loop, i.e., $\pm 20\%$ of the 1:1,000 stock solution dilution.

C. Corrective action
   1. If delivery of loop does not fall within the specified tolerance limit, the loop can be discarded and replaced or repaired and retested.
   2. Repair by dipping in sand to remove deposits, and clean by wetting with alcohol and igniting.
   3. Loops can be returned to their calibrated diameter by using standard, circular gauges (Remel, Inc.). Reconditioned loops must be calibrated before being put back in service.
   4. For inaccurate disposable loops, contact manufacturer for replacement.

V. PROCEDURE FOR LOOP USE
   A. For reusable loops, flame and cool before the first and after each transfer. Use a new, unwetted disposable loop for each transfer.
   B. Fluid should be in a widemouthed container (diameter, >1 cm).
   C. Swirl the specimen to mix the bacterial suspension evenly.
   D. Hold the loop vertically, and immerse it to just below the surface of the liquid. Avoid any bubbles on the meniscus of the liquid.
   E. Check to ensure that no bubbles are within the loop.
   F. Move loop straight up and down only.
   G. Transfer contents of loop to the plate by pressing liquid to the plate until the liquid is no longer visible in the loop. Spread with loop or, for greater accuracy, spread with a bent rod.

VI. LIMITATIONS

Quantitative loops, both disposable and reusable, will be grossly inaccurate if bubbles are present in the film of fluid transferred. Avoid bubbles by not shaking liquid. Inspect loopful carefully for bubbles. Remove bubbles by flaming (reusable loop), replacing (disposable loop), or redipping (either loop).

References

1. **Albers, A. C., and R. D. Fletcher.** 1983. Accuracy of calibrated-loop transfer. *J. Clin. Microbiol.* **18:**40–42.
2. **Clarridge, J. E., M. T. Pezzlo, and K. L. Vosti.** 1987. *Cumitech 2A, Laboratory Diagnosis of Urinary Tract lnfections.* Coordinating ed., A. S. Weissfeld. American Society for Microbiology, Washington, DC.

APPENDIX 3.12–4

Use and Calibration of Pipettors

I. PRINCIPLE AND DESCRIPTION

A. Principle

Pipettors aspirate and expel by an air or positive-displacement mechanism. The former mechanism uses air to push out the liquid, and the latter uses a plunger. Positive-displacement pipettors are generally thought to be more accurate for smaller volumes and, in theory, do not need the pipettor tip replaced between samples. Air displacement pipettors are more commonly used in microbiology.

B. Description

The term "pipettor" describes dispensing, diluting, and pipetting equipment. The pipettor may be manual or automated. There are repeater, adjustable-volume, and multichannel models.

Manual pipettors are lightweight cylindrical tools consisting of a handheld mechanical handle for volume adjustment and calibration and a delivery shaft that fits to disposable tips. Pipettors are approximately 10 in. long and deliver microliter to milliliter volumes. On automated pipettors, the mechanical handle is replaced with instrumentation.

C. Pipetting technique

1. Forward pipetting
   a. Refer to Fig. 3.12–A1.
   b. When prerinsing by forward pipetting, follow procedure for forward pipetting but dispense aliquot back into original container or discard. Proceed with deliveries for calibration.
   c. Top and bottom stop positions do not include eject stroke position for ejecting pipetter tips.
2. Reverse pipetting
   a. Refer to Fig. 3.12–A2.
   b. When prerinsing by reverse pipetting, follow procedure for reverse pipetting. Dispense liquid back into original vessel or discard. Proceed with deliveries for calibration.
   c. Top and bottom stop positions do not include eject stroke position for ejecting pipettor tips.

D. Accuracy and precision

1. Definition of accuracy

   "Accuracy" is the closeness of agreement between the stated volume of the pipetter and the mean volume obtained during repeated, controlled deliveries. Accuracy is numerically expressed as inaccuracy, given as a percentage. Inaccuracy can be thought of as the difference between the expected or theoretical result and the calculated result.
2. Definition of precision

   "Precision" is the agreement between replicate measurements. Precision is numerically expressed as imprecision, given as the coefficient of variation. Imprecision can be thought of as the range of values in which 95% of the replicate measurements fall.
3. Use of inaccuracy and imprecision as they relate to calibration of liquid delivery instruments
   a. Proper calibration of pipettors requires calculation of both inaccuracy and imprecision.
   b. Inaccuracy and imprecision must be calculated for each volume of an adjustable pipettor and each channel of a multichannel pipettor unless the manufacturer's procedure instructs otherwise.

E. Uses for liquid delivery systems in microbiology

Pipettors are used to dilute sera, set up quantitative cultures, prepare inocula for antimicrobial tests, add ingredients to media and reagents, and add exact amounts of reagents or specimen during a test procedure. Pipettors are used because of their excellent accuracy and precision and because they expeditiously dispense small volumes repeatedly.

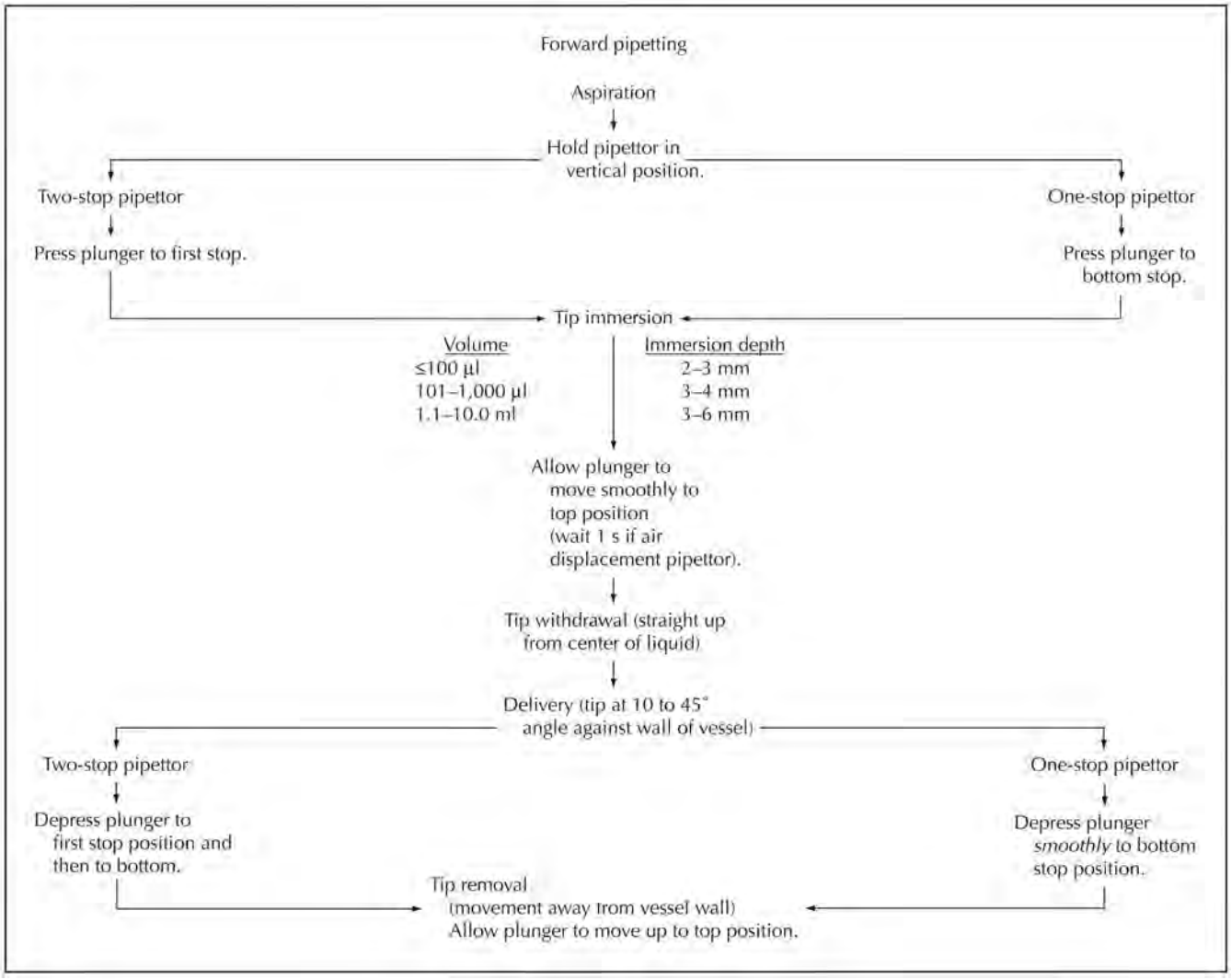

Forward pipetting
Aspiration
Hold pipettor in vertical position.
Two-stop pipettor
Press plunger to first stop.
One-stop pipettor
Press plunger to bottom stop.
Tip immersion
Volume
≤100 µl
101–1,000 µl
1.1–10.0 ml
Immersion depth
2–3 mm
3–4 mm
3–6 mm
Allow plunger to move smoothly to top position (wait 1 s if air displacement pipettor).
Tip withdrawal (straight up from center of liquid)
Delivery (tip at 10 to 45° angle against wall of vessel)
Two-stop pipettor
Depress plunger to first stop position and then to bottom.
One-stop pipettor
Depress plunger *smoothly* to bottom stop position.
Tip removal (movement away from vessel wall)
Allow plunger to move up to top position.

Figure 3.12–A1 Flowchart for forward pipetting (adapted from reference 8 with permission).

APPENDIX 3.12–4 *(continued)*

F. Calibration factors

1. Importance of calibration
   Volumetric measurement using pipettors is a potential source of error in the clinical laboratory. A small error in pipetting can cause a large error in the final result.
2. Requirement for pipettor calibration
   Laboratory inspecting agencies such as the CAP and the JCAHO require periodic testing of pipetting equipment to ensure accurate delivery.
3. Proposed standard for pipettor calibration
   Guidelines for calibration of volumetric pipettors are found in many sources (1–10).
4. Calibration methods available (Table 3.12–A1)
   Numerous methods for calibration are available. The gravimetric, spectrophotometric, and colorimetric methods are the most convenient and commonly used. Radioisotopic, enzymatic, and acid-base titration methods are less commonly recommended and are not discussed further. Commercially available pipettor calibration kits designed for in-house or send-out measurement and calculation are available (*see* Table 3.12–A2).

Reverse pipetting (for two-stop pipettors only)
Aspiration
Same as in forward pipetting
except press plunger to bottom
stop position for two-stop pipettors
Pause 1 s.
Tip immersion
Allow plunger to return to top position.
Tip withdrawal
(straight up from center of liquid)
Delivery
Tip at 10 to 45° angle against wall of vessel.
Depress plunger smoothly to
first stop position; wait 1 s.
Remove tip from liquid by moving away from vessel wall.
Some fluid will remain in pipettors tip.
Reuse of tip
Maintain plunger in
intermediate position.
Next immersion
Change tip.
Allow plunger to
return to top stop position.
New tip

Figure 3.12–A2 Flowchart for reverse pipetting (adapted from reference 8 with permission).

APPENDIX 3.12–4 *(continued)*

II. SPECIAL PRECAUTIONS AND ENVIRONMENTAL CONCERNS

A. Special precautions for pipettor calibration

1. Changing pipettor tips during calibration procedure
   Use the same pipettor tip for all deliveries during the calibration procedure, whether the pipettor is used for repetitive dispensing of several aliquots of the same liquid (e.g., buffers and reagents) or for transferring single aliquots of different liquids (e.g., serum). *Note:* During actual pipetting for routine use, a different tip must be used for each different liquid.
2. Prerinsing pipettor tips
   "Prerinsing" is the precoating of the inside of the tip with the liquid being dispensed. Prerinse by aspirating an aliquot of the liquid into the tip and then dispensing it back into the original container or discarding it. Prerinsing improves uniformity and precision by providing identical contact surfaces for all aliquots.
   a. If the pipettor is normally used for repetitive dispensing of several aliquots of the same liquid, prerinse pipettor tip *at the beginning,* before dispensing the first aliquot.

APPENDIX 3.12–4 *(continued)*

Table 3.12–A1 Calibration methods for volume-dispensing instruments

Method	Basis of system	Limitation
Gravimetric	1 ml of water = 1 g (adjusted for temp and pressure)	Vol dispensed must be >0.002 ml
Spectrophotometric	Absorbances of potassium dichromate used to create calibration curve	Vol dispensed must be >0.01 ml

Table 3.12–A2 Commercial pipettor calibration systems and related products

Supplier	Product(s)
Brinkman Instruments, Inc.	Autoclavable pipettors
Calibrite Corp.	Pipette calibration service
Laboratory Equipment Support Service, Inc.	Audit systems, reference service for pipettor calibration
Medical Laboratory Automation, Inc.	Automatic pipettors, pipette calibration kits
SMI Scientific Manufacturing Industries, Inc.	Positive-displacement pipettors
Streck Laboratories	VC-100, automated acid-base titration system

b. If the pipettor is normally used for transferring single aliquots of different liquids, prerinsing may not be necessary.

B. Environmental concerns for pipettor calibration

1. Temperature control
   a. Temperatures of pipettors to be calibrated, room air, test liquid (water), and other equipment should be identical (± 0.5°C).
   b. Temperature should be as close as possible to temperature at which pipettor is used.
   c. Keep temperature stable throughout procedure.
2. Miscellaneous environmental concerns
   a. Maintain relative humidity at 45 to 75%. This reduces evaporation and limits buildup of static electricity.
   b. Prepare aqueous test liquids from CLSI (NCCLS) type I or II water (*see* procedure 14.4), which prevents impurities from affecting water density.
   c. Use water with no visible bubbles. Air bubbles alter measured volume.
   d. Complete weighing steps quickly. Use a lid on the weighing vessel to decrease evaporation. These precautions obviate an evaporation factor in the calculations.

III. CALIBRATION OF PIPETTORS

A. Frequency of calibration

Microbiology laboratory guidelines for frequency of pipettor calibration (Table 3.12–A3)

In general, pipettors used in microbiology and serology laboratories are not used for critical-volume delivery and may not require a rigorous calibration schedule. Guidelines for testing frequency do not exist for pipettors that are used less frequently or that are not used for critical delivery.

1. Determine frequency of calibration for each pipettor on the basis of its use. Pipettors used daily require more frequent calibration than those used weekly or monthly.
2. Pipettors used for critical-volume delivery need more frequent testing than those used to deliver approximate volumes.
3. Carefully label pipettors for approximate delivery so that they are not used for more exact purposes.

APPENDIX 3.12–4 *(continued)*

Table 3.12–A3 Suggested calibration schedule for microbiology laboratory pipettors

Pipettor use	Calibration schedule[a]			
	New or after maintenance	Monthly	Quarterly	Annually
Critical-vol delivery required[b]				
Weekly or monthly use	A, P		A	P
Daily use	A, P	A		P
Critical-vol delivery not required[c]				
Weekly or monthly use	A, P			A, P
Daily use	A, P		A	P

[a] A, test for accuracy; P, test for precision.

[b] Critical-volume delivery may be required for reagent delivery in some assays.

[c] Critical-volume delivery generally not required for serum dilutions (including Venereal Disease Research Laboratory and rapid plasma reagin tests), quantitative culture, inocula for antimicrobial tests, adding ingredients to media and reagents, adding reagents to test procedure, or adding specimen to slide for fluorescent staining.

B. Calibration procedures: gravimetric method

1. Materials and supplies
   a. Reagent
      Distilled water: CLSI (NCCLS) type I or II (*see* procedure 14.4)
   b. Equipment
      (1) Analytic balance: balance capable of measuring to 0.001 mg (1.0 µg)
      (2) Thermometer: NBS calibrated to 0.1°C
      (3) Weighing vessel: nonporous glass, plastic, or metal weighing container
      Open surface area should be as small as possible to control evaporation. Use a loose-fitting lid to prevent evaporation of the small volumes of distilled water that will be weighed. Handle weighing vessel with forceps or washed gloves.
2. Procedure
   a. Record temperature (rounded to the closest 0.5°C) before test procedure.
   b. Tare the weighing vessel.
   c. Prerinse the pipettor tip, if necessary, with distilled water.
   d. Deliver one aliquot of distilled water into weighing vessel by using standard operating procedure for that pipettor (i.e., forward or reverse pipetting). The emphasis throughout is on smoothness, uniform timing, and uniform motion.
   e. Replace lid of weighing vessel.
   f. Record results of weighings on worksheet 2 (*see* p. 3.12.29).
   g. Deliver additional aliquots (4 for inaccuracy determination or 10 for imprecision determination) with uniform timing. Record weight of each aliquot. After all weighings are completed, calculate the net weight of each aliquot.
3. Calculations
   a. Use worksheet 2 (*see* p. 3.12.29).
   b. Calculate the mean weight ($\bar{W}$) by using the individual weights (W_i) and the number of weighings (n).

$$\bar{W} = \frac{\Sigma W_i}{n}$$

   c. Calculate the mean volume ($\bar{V}$) by using the mean weight ($\bar{W}$) and factor Z, which controls for water density variation resulting from temperature, atmospheric pressure, and relative humidity changes.

$$\bar{V} = (\bar{W})(Z)$$

Factor Z is calculated by using Table 3.12–A4. A standard Z value, using an average room temperature and barometric pressure, can be used in calculations

APPENDIX 3.12–4 *(continued)*

Table 3.12–A4 *Z* value for distilled water as a function of temperature and pressure[a]

Temp (°C)	*Z* at air pressure (mm Hg)[b] of:					
	600	640	680	720	760	800
15	1.0018	1.0018	1.0019	1.0019	1.0020	1.0020
15.5	1.0018	1.0019	1.0019	1.0020	1.0020	1.0021
16	1.0019	1.0020	1.0020	1.0021	1.0021	1.0022
16.5	1.0020	1.0020	1.0021	1.0022	1.0022	1.0023
17	1.0021	1.0021	1.0022	1.0022	1.0023	1.0023
17.5	1.0022	1.0022	1.0023	1.0023	1.0024	1.0024
18	1.0022	1.0023	1.0024	1.0024	1.0025	1.0025
18.5	1.0023	1.0024	1.0025	1.0025	1.0026	1.0026
19	1.0024	1.0025	1.0025	1.0026	1.0027	1.0027
19.5	1.0025	1.0026	1.0026	1.0027	1.0028	1.0028
20	1.0026	1.0027	1.0027	1.0028	1.0029	1.0029
20.5	1.0027	1.0028	1.0028	1.0029	1.0030	1.0030
21	1.0028	1.0029	1.0030	1.0030	1.0031	1.0031
21.5	1.0030	1.0030	1.0031	1.0031	1.0032	1.0032
22	1.0031	1.0031	1.0032	1.0032	1.0033	1.0033
22.5	1.0032	1.0032	1.0033	1.0033	1.0034	1.0035
23	1.0033	1.0033	1.0034	1.0035	1.0035	1.0036
23.5	1.0034	1.0035	1.0035	1.0036	1.0036	1.0037
24	1.0035	1.0036	1.0036	1.0037	1.0038	1.0038
24.5	1.0037	1.0037	1.0038	1.0038	1.0039	1.0039
25	1.0038	1.0038	1.0039	1.0039	1.0040	1.0041
25.5	1.0039	1.0040	1.0040	1.0041	1.0041	1.0042
26	1.0040	1.0041	1.0042	1.0042	1.0043	1.0043
26.5	1.0042	1.0042	1.0043	1.0043	1.0044	1.0045
27	1.0043	1.0044	1.0044	1.0045	1.0045	1.0046
27.5	1.0044	1.0045	1.0046	1.0046	1.0047	1.0047
28	1.0046	1.0046	1.0047	1.0048	1.0048	1.0049
28.5	1.0047	1.0048	1.0048	1.0049	1.0050	1.0050
29	1.0049	1.0049	1.0050	1.0050	1.0051	1.0052
29.5	1.0050	1.0051	1.0051	1.0052	1.0052	1.0053
30	1.0052	1.0052	1.0053	1.0053	1.0054	1.0055

[a] Adapted from reference 8 with permission.

[b] 1 mm Hg = 133.322 Pa.

to calibrate pipettors that are not used to deliver critical volumes. Calculate the actual *Z* value (by using current temperature and atmospheric pressure) when calibrating pipettors used to deliver critical volumes.

d. Calculate inaccuracy ($\bar{E}$%) by using mean volume ($\bar{V}$) and nominal volume (V_0). Use four weighings generally.

$$\bar{E}\% = \frac{\bar{V} - V_0}{V_0} \times 100$$

e. Calculate imprecision (CV%) by using individual weights (W_i), mean weight ($\bar{W}$), and number of weighings (n). Use 10 weighings generally.

$$\text{CV}\% = 100 \times \frac{\sqrt{\frac{\Sigma(W_i - \bar{W})^2}{n - 1}}}{\bar{W}}$$

APPENDIX 3.12–4 *(continued)*

C. Calibration procedures: spectrophotometric method
1. Materials
a. Reagents
(1) Potassium dichromate: $K_2Cr_2O_7$, analytical reagent grade
(2) Perchloric acid diluent: $HClO_4$, analytical reagent grade (0.001 N)
(3) Distilled water: CLSI (NCCLS) type I or II (*see* procedure 14.4)
(4) Stock solutions
Stock solutions are stable for 1 year if protected from evaporation.
(a) Stock solution I
Weigh approximately 10 g of $K_2Cr_2O_7$, and record weight to nearest 0.001 g. Transfer chemical to clean, 1,000-ml volumetric flask. Dilute to 1,000 ml with 0.001 N $HClO_4$.
(b) Stock solution II
Using a volumetric pipettor, transfer 100 ml of stock solution I to another clean, 1,000-ml volumetric flask. Again, dilute to 1,000 ml with 0.001 N $HClO_4$.
b. Equipment
(1) Spectrophotometer
Good-quality instrument is needed. Specifications include the following: linear through 2.0 absorbance units at 350 nm; spectral band width, ≤2 nm (348 to 352 nm); precision (drift) of ≤0.001 Å/h (≤0.0001 nm/h); accuracy of selected wavelength, ≤1 nm.
(2) Cuvettes: square glass cuvettes with a 1-cm light path
Use the same cuvette for all measurements.
(3) Volumetric glassware: two class A 1,000-ml volumetric flasks; one class A 100-ml volumetric pipette; several each class A 1-, 2-, and 10-ml pipettes
(4) Analytic balance: balance capable of measuring to 1.0 mg (0.001 g)
2. Procedure
a. Set spectrophotometer to absorbance mode, using a wavelength of 350 nm.
b. Zero spectrophotometer with diluent (0.001 N $HClO_4$).
c. Consult Table 3.12–A5 to determine appropriate stock solution and diluent to use based on sample volume and cuvette size.
d. Record absorbance values on worksheet 3 (*see* p. 3.12.30).
e. Prerinse the pipettor tip if necessary with distilled water.
f. Aspirate sample by using standard operating procedure for that pipettor (i.e., forward or reverse pipetting). The emphasis throughout is on smoothness, uniform timing, and uniform motion.
g. Deliver appropriate volume of sample (stock solution) into cuvette.
h. Deliver the specified volume of diluent (0.001 N $HClO_4$) into cuvette.
i. Cap the cuvette with Parafilm. Mix solution by inverting cuvette at least three times.
j. Place cuvette in spectrophotometer. Close door.
k. Read absorbance value, and record on worksheet 3 (*see* p. 3.12.30).

Table 3.12–A5 Theoretical absorbance values for test solutions

Vol (µl) of pipettor to be tested	Stock solution to use	Vol (ml) of diluent to use	Theoretical absorbance of test solution[a]
10	I	2	0.532
20	I	10	0.213
50	II	2	0.261
100	II	2	0.510
200	II	10	0.210
250	II	10	0.261
500	II	10	0.509
1,000	II	10	0.972

[a] Adapted from reference 8 with permission.

APPENDIX 3.12–4 *(continued)*

Table 3.12–A6 Suggested tolerance limits for microbiology pipettors

Pipettor use[a]	Inaccuracy (%)	Imprecision (%)
Critical-vol delivery required		
Delivers <50 µl	±2	±2
Delivers >50 µl	±1.5	±1
Critical-vol delivery not required		
Delivering specimen to slide for FA staining	±10	Not necessary
Other uses[b]	±2	±2

[a] See Table 3.12–A3, footnotes *b* and *c*. FA, fluorescent antibody.

[b] Includes Venereal Disease Research Laboratory and rapid plasma reagin tests.

l. Discard test solution from cuvette.
m. Repeat steps III.C.2.f through 1 until 4 (inaccuracy determination) or 10 (imprecision determination) samples have been measured.
n. Rezero spectrophotometer with diluent (0.001 N $HClO_4$). Difference should be <0.001 Å.

3. Calculations
   a. Use worksheet 3 (*see* p. 3.12.30).
   b. Calculate the mean absorbance ($\bar{A}$) by using the individual absorbances (A_i) and the number of absorbance measurements made (n).

$$\bar{A} = \frac{\Sigma A_i}{n}$$

   c. Calculate inaccuracy ($\bar{E}$%) by using the mean absorbance ($\bar{A}$) and the theoretical absorbance (A_0).

$$\bar{E}\% = \frac{\bar{A} - A_0}{A_0} \times 100$$

   d. Calculate imprecision (CV%) by using the individual absorbance (A_i), the mean absorbance ($\bar{A}$), and the number of absorbance measurements (n).

$$\text{CV}\% = 100 \times \frac{\sqrt{\frac{\Sigma(A_i - \bar{A})^2}{n - 1}}}{\bar{A}}$$

D. Tolerance
   1. Tolerance
      a. Different tolerance limits for inaccuracy and imprecision are used for various laboratory applications. Most recommended tolerance limits are designed for pipettors that must deliver critical volumes, such as those used in clinical chemistry laboratories.
      b. Tolerance limits for pipettors and quantitative loops tailored to specific laboratory uses can be determined by each user. Table 3.12–A6 is a list of suggested tolerance limits for common microbiology laboratory uses.
   2. Corrective action
      a. If delivery of pipettor does not fall within the specified tolerance limits, the pipettor must be adjusted (either in-house or by manufacturer), used for less critical delivery, or discarded.
      b. Following pipettor adjustment, 10 weighings must be performed to evaluate both accuracy and precision.

IV. TROUBLESHOOTING

Pipettors that do not deliver volumes within the acceptable tolerance levels even after adjustment may have been calibrated incorrectly or may have the wrong pipettor tips.

APPENDIX 3.12–4 *(continued)*

A. To verify calibration procedure, review carefully, and if necessary, calibrate by a second procedure (in-house or sent out).
B. Different brands of micropipettor tips vary in quality and may contribute to pipetting error. Poorly fitting tips can affect volume of sample aspirated or dispensed. Different brands of tips can be tested for accuracy and precision.

References

1. **Bermes, E. W., and D. T. Forman.** 1976. Basic laboratory principles and procedures, p. 6–15. *In* N. W. Teitz (ed.), *Fundamentals of Clinical Chemistry*, 2nd ed. The W. B. Saunders Co., Philadelphia, PA.
2. **Bio-Rad Laboratories.** 1983. Procedure for comparing precision of pipet tips. *Clin. Lab. Prod.* **12:**15.
3. **Bray, W.** 1995. Software for the gravimetric calibration testing of pipets. *Am. Clin. Lab.* **14:**14–15. (Available on the internet at http://www.labtronics.com/ptM_art.htm)
4. **Connors, M., and R. Curtis.** 1999. Pipetting error: a real problem with a simple solution. Parts I and II. *Am. Lab. Newsl.* **31:**20–22.
5. **Curtis, R. H.** 1994. Performance verification of manual action pipets. Part I. *Am. Clin. Lab.* **12:**8–9.
6. **Curtis, R. H.** 1994. Performance verification of manual action pipets. Part II. *Am. Clin. Lab.* **12:**16–17.
7. **Johnson, B.** 1999. Calibration to dye for: Artel's new pipette calibration system. *Scientist* **13:**14.
8. **NCCLS.** 1984. *Determining Performance of Volumetric Equipment.* Proposed guideline 18-P. NCCLS, Villanova, PA.
9. **Skeen, G. A., and E. R. Ashwood.** 2000. Using spectrophotometry to evaluate volumetric devices. *Lab. Med.* **31:**478–479.
10. **Steiner, P.** 1989. Basic laboratory principles and calculations, p. 19. *In* L. A. Kaplan and A. J. Pesce (ed.), *Clinical Chemistry*, 2nd ed. The C. V. Mosby Co., St. Louis, MO.

Worksheet 1
Colorimetric Quantitative Loop Calibration

Loop size: _____ Date: _____ Scientist initials _____

Loop manufacturer and Lot No. _____ Date put into use_____

Sample No.	Absorbance
1	_____
2	_____
3	_____
4	_____
Sum of 4 readings	_____
Average (divide by 4)	_____

Average minus the 1:1000 absorbance std. (a) _____

Divide (a) by the 1:1000 absorbance std. (b)_____

% inaccuracy: Multiply (b) by 100 (c)_____%

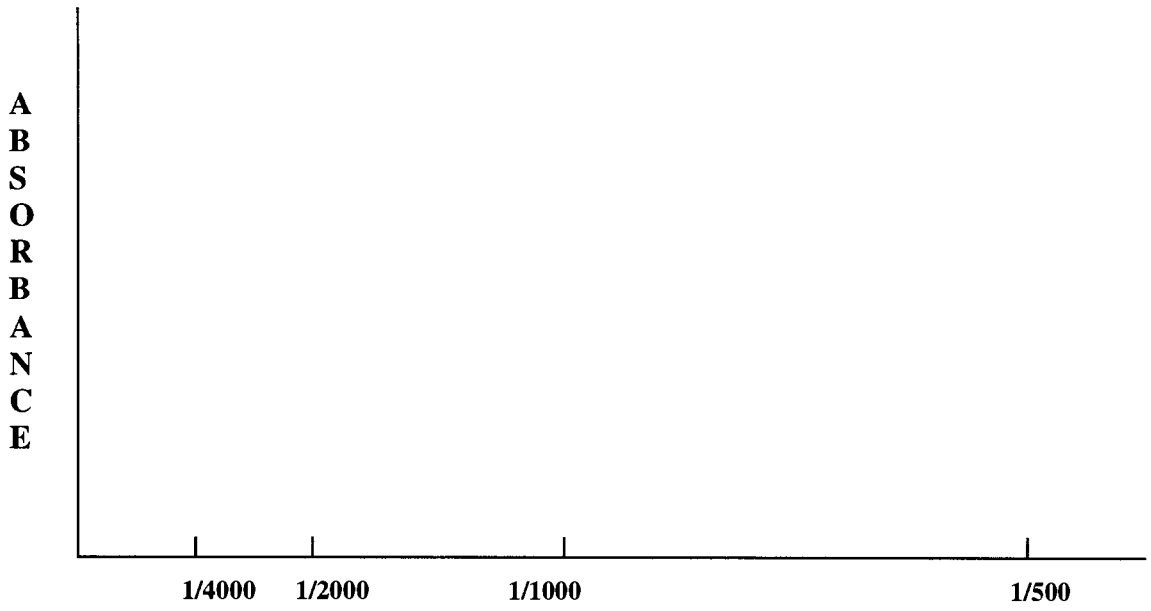
Value (c) must be ± 20%: Acceptable? Yes/No

Corrective action:

_____
_____
_____
_____
_____

Reviewed by:_____ Date:_____

Calibration curve:



Worksheet 2
Pipette Calibration—Gravimetric

Circle frequency:

Initial / Weekly / Monthly / Quarterly

Circle reason for calibration:

Accuracy check / Precision check

Date: _____

Technologist Initials: _____

Pipettor Type: _____

Pipettor Number: _____

Pipettor Size (µl): _____

Z Value: _____

Sample No.	Sample Weight (mg)
1	_____
2	_____
3	_____
4	_____
5	_____
6	_____
7	_____
8	_____
9	_____
10	_____

Record statistics below (see page 3.12.31 for calculations):

	Result	Tolerance Limit
Inaccuracy	_____	_____
Imprecision	_____	_____
Acceptable	Yes / No	

Corrective Action: _____

_____

_____

_____

Reviewed by: _____ Date: _____

_____

Worksheet 3
Pipette Calibration—Spectrophotometric

Circle frequency:

Initial / Weekly / Monthly / Quarterly

Circle reason for calibration:

Accuracy check / Precision check

Date: _____

Technologist Initials: _____

Pipettor Type: _____

Pipettor Number: _____

Pipettor Size (µl): _____

Sample No.	Absorbance
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	

Record statistics below (see page 3.12.31 for calculations):

	Result	Tolerance Limit
Inaccuracy		
Imprecision		
Acceptable	Yes / No	

Corrective Action: _____

_____

_____

_____

Reviewed by: _____ Date: _____

Calculations for worksheet 2

W_i = individual weights
$\bar{W}$ = mean weight
n = number of weighings
$\bar{V}$ = mean volume
V_0 = nominal volume

Mean weight ($\bar{W}$)

$$\bar{W} = \frac{\Sigma W_i}{n}$$

Mean volume ($\bar{V}$)

$$\bar{V} = (\bar{W})(Z)$$

Inaccuracy ($\bar{E}$%): use four weighings.

$$\bar{E}\% = \frac{\bar{V} - V_0}{V_0} \times 100$$

Imprecision (CV%): use 10 weighings.

$$\text{CV}\% = 100 \times \frac{\sqrt{\frac{\Sigma(W_i - \bar{W})^2}{n - 1}}}{\bar{W}}$$

Calculations for worksheet 3

A_i = individual absorbance
n = number of absorbance measurements
$\bar{A}$ = mean absorbance
A_0 = theoretical absorbance

Mean absorbance ($\bar{A}$)

$$\bar{A} = \frac{\Sigma A_i}{n}$$

Inaccuracy ($\bar{E}$%): use four readings.

$$\bar{E}\% = \frac{\bar{A} - A_0}{A_0} \times 100$$

Imprecision (CV%): use 10 readings.

$$\text{CV}\% = 100 \times \frac{\sqrt{\frac{\Sigma(A_i - \bar{A})^2}{n - 1}}}{\bar{A}}$$

3.13.1 Wound/Abscess and Soft Tissue Cultures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

A wide variety of microorganisms that reside on the skin and mucous membranes of the body, as well as those found in the environment, can cause skin and soft tissue infections. These organisms enter the body through breaks in the skin or mucous membranes, through wounds made by trauma or bites (exogenous) or as a complication of surgery or foreign-body implants (endogenous), or they can be spread through the vascular system (hematogenous).

Laboratories may distinguish superficial wound, abscess/fluid, or tissue specimens from those collected from deep body sites. Superficial wound and abscess specimens usually grow primary pathogens causing skin and soft tissue infections. A much broader microbial diversity is usually recovered from deep wound and invasively collected abscess/fluid and tissue specimens (Table 3.13.1–1). A variety of aerobic organisms and anaerobes may be recovered if the appropriate media and cultures are inoculated (*see* Tables 3.13.1–2 and 3.13.1–3).

Acute wound infections are normally caused by external damage to intact skin, such as those produced during surgery or by trauma and bites. Conversely, chronic infections, such as decubiti or foot and leg ulcers, are normally due to complications related to impaired vascular flow or metabolic disease (e.g., diabetes mellitus). Wound colonization and/or infection is often polymicrobic, with both aerobes and anaerobes (*see* section 4) involved.

Tissues collected during surgery or aspirates obtained through intact skin by needle and syringe or by fine-needle biopsy are the best types of specimen to obtain for microbiology culture. If the skin surface and surgical areas are properly disinfected prior to specimen collection, the organisms present can be assumed to be the cause of infection. Interpretation of microbial cultures taken from open skin or abscesses may be compromised, due to the fact that these lesions are often colonized with a large number of indigenous microbiota. Such cultures are indicated only if there are clear signs of infection or if a wound is failing to heal. Proper preparation of the wound prior to specimen collection can minimize contamination. After appropriate debridement and cleansing of the wound, the specimen should be obtained by biopsy from the leading edge of the lesion, where pathogens should be present and colonizing organisms are less likely to occur. Bacterial cultures of purulent material obtained by needle and syringe aspiration can also provide meaningful results. If an aspirate or tissue sample cannot be obtained, swab collections of exudate from the deep portion of lesions can be submitted. Swabs are the least appropriate specimen for microbiology analysis, as the organisms isolated may only be colonizing the area and not involved in the infective process.

The primary agents of skin and tissue infections are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, members of the *Enterobacteriaceae*, beta-hemolytic streptococci, and a variety of anaerobes. In appropriately collected specimens, the presence of one of these organisms may indicate the need for antimicrobial therapy. Since wound infections can be polymicrobic, treatment may initially need to be broad in spectrum, and there is little need to identify and perform antimicrobial susceptibility testing (AST) on all isolates. Tissues and aspirates are acceptable for anaerobic culture, as anaerobes can account for 38 to 48% of the total number of microbial isolates in wound specimens (Table 3.13.1–2 and reference 1). It must be emphasized that wound specimens collected on swabs will be less appropriate than tissues or aspirates for anaerobic culture, provided that the tissues and aspirates are submitted under anaerobic transport conditions.

The accumulation of inflammatory cells and the resultant collection of pus within an abscess or a sinus tract is a hallmark of local infection. Evidence of this process can be documented by the presence of PMNs in the Gram-stained smear. Therefore, the quality of a wound specimen can be assessed by Gram stain, which should be used to guide the extent of microbiology testing. The presence of epithelial cells indicates contamination of the specimen with skin or mucous membrane microbiota and may compromise the significance of the culture results. Quantitative cultures of tissue specimens have been shown to be useful in evaluation of wound healing related to skin grafting (procedure 3.13.2); however, the presence of organisms in the Gram stain of an appropriately collected specimen from an infected wound correlates with a clinically significant count of bacteria (3). In addition, many publications have shown that for acute wounds, a swab culture with enumeration of the organisms present correlates well with quantitative tissue cultures (*see* review by Bowler et al. [1] and refer to procedure 3.13.2 for quantitative methods).

Table 3.13.1–1 Common types of superficial and deep wounds and abscesses

Specimen types	Site or source	Comments
Abscess		
Superficial	Boils, furuncles, infected cysts, skin abscesses, superficial surgical wounds	Defined as an infected space that may drain through the skin but does not extend deeper than the dermis; aerobic culture only required.
Deep	Any site, including deep tissues; usually related to secondary infection of a deep wound, contusion, or hematoma	Defined as a closed infected space that extends deeper than the dermis into deep tissues whose cavity may be encapsulated; aerobic and anaerobic cultures required.
Wound		
Superficial	Abrasion, cut, laceration, or ulcer (any site), plus associated skin diseases (impetigo, folliculitis, cellulites) or burns	Defined as a wound in the skin that does not extend deeper than the dermis; aerobic culture only required
Deep	Typically applies to deep surgical wounds that go across a mucosal surface (e.g., abdominal, pelvic, or chest), bite wounds, deep traumatic wounds (e.g., gunshots, stabs, punctures) third-degree burns due to electrocution	Defined as a wound that penetrates deeper than the dermis of the skin or is located in deep tissues

Table 3.13.1–2 Aerobic and anaerobic isolates from acute and chronic infections[a]

Aerobic and facultative microorganisms	Anaerobic bacteria	Aerobic microorganisms from unusual, specialized, and zoonotic infections	Yeasts
Coagulase-negative staphylococci	*Peptostreptococcus* spp.	*Actinobacillus actinomycetemcomitans*	*Candida albicans*
Staphylococcus aureus	*Clostridium* spp.	*Aeromonas* spp.	*Candida krusei*
Beta-hemolytic streptococci	*Eubacterium limosum*	*Bacillus anthracis*	*Candida parapsilosis*
Enterococcus spp.	*Propionibacterium acnes*	*Bergeyella zoohelcum*	
Streptococcus spp. (viridans group)	*Bacteroides fragilis* group	*Capnocytophaga* spp. and EF-4	
Streptococcus anginosus	*Prevotella* spp.	*Chromobacterium violaceum*	
Corynebacterium spp.[b]	*Porphyromonas asaccharolytica*	*Eikenella corrodens*	
Bacillus cereus	*Fusobacterium necrophorum*	*Erysipelothrix rhusiopathiae*	
	Veillonella spp.	*Francisella tularensis*	
Escherichia coli		*Haemophilus* spp.	
Serratia spp.		*Kingella kingae*	
Klebsiella spp.		NO-1	
Enterobacter spp.		*Pasteurella multocida*	
Citrobacter spp.		*Streptobacillus moniliformis*	
Morganella morganii		*Vibrio vulnificus*	
Providencia stuartii			
Proteus spp.			
Acinetobacter baumannii			
Pseudomonas aeruginosa			
Stenotrophomonas maltophilia			
Sphingobacterium multivorum			

[a] Data revised from reference 1. Wounds include cutaneous abscesses, postsurgical wounds, bites, ulcers, and pressure sores.

[b] *Corynebacterium diphtheriae* causes skin ulceration and should be ruled when *Corynebacterium* spp. are isolated in this clinical context.

Table 3.13.1–3 Commonly encountered superficial and deep-wound/abscess, drainage, and tissue infections

Clinical condition	Location of infection	Primary pathogen(s)	Comments[a]
Superficial infections Boils/carbuncles Cellulitis Cysts Folliculitis Injury (abrasions, first-degree burns, cuts) Subcutaneous abscesses Ulcers	Skin and soft tissues anywhere on the body	*S. aureus*/MRSA Beta-hemolytic streptococci (A, B, C, G) Coagulase-negative staphylococci *Corynebacterium* spp. *P. acnes* Herpes simplex virus	*B. anthracis* may cause a skin eschar.
Deep-wound infections Bites—human Bites—animal Second- or third-degree burns Episiotomy Injury Surgical wounds Ulcer (decubiti, diabetic foot, etc.)	Skin and deeper tissues below the dermis, fascia, and muscle	Animal bites *Pasteurella multocida* *Capnocytophaga* spp. *Eikenella corrodens* Human bites and other deep wounds Mixed aerobes and facultative organisms Anaerobes can also usually be recovered from deep wounds	*Aeromonas* spp., *Vibrio* spp., or *C. violaceum* may be found in wounds exposed to water. Anaerobic culture should be performed on request or when the Gram stain suggests the presence of anaerobes.
Abscesses/fluids	Aspirated from deep body spaces or tissues	Mixed aerobes and facultative organisms Anaerobes can also usually be recovered from deep abscesses.	Anaerobic culture should be performed on request or when the Gram stain suggests the presence of anaerobes.
Drainages[b]	Fluid freshly aspirated from disinfected tubing or area being drained	Mixed aerobes and facultative organisms Anaerobes can also usually be recovered from drainages from deep body spaces or tissues.	Anaerobic culture should be performed on request or when the Gram stain suggests the presence of anaerobes. After broad-spectrum antimicrobial agent treatment, recovery of resistant organisms and yeasts increases.
Tissues	Invasively collected biopsy samples of deep organs and tissues	Mixed aerobes and facultative organisms Anaerobes can also usually be recovered from deep abscesses.	Anaerobic culture should be performed on request or when the Gram stain suggests the presence of anaerobes.

[a] See Table 3.13.1–2 for a complete listing of aerobic and anaerobic isolates from acute and chronic wound/abscess and tissue infections.

[b] See procedure 3.5 and Table 3.5–2 for drainages that are acceptable for culture.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

NOTE: Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for caregivers. It is the responsibility of the laboratory director or designee to educate physicians and other caregivers on proper wound specimen collection.

Observe standard precautions.

A. General considerations

1. *Preferably collect specimen prior to initiation of therapy and only from wounds that are clinically infected or deteriorating or that fail to heal over a long period.*

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

2. Cleanse skin or mucosal surfaces.
   a. For closed wounds and aspirates, disinfect as for a blood culture collection with 2% chlorhexidine or 70% alcohol followed by an iodine solution (1 to 2% tincture of iodine or a 10% solution of povidone-iodine [1% free iodine]). Remove iodine with alcohol prior to specimen collection.
   b. For open wounds, debride, if appropriate, and thoroughly rinse with sterile saline prior to collection.
3. Sample viable infected tissue, rather than superficial debris.
4. Avoid swab collection if aspirates or biopsy samples can be obtained.
5. Containers
   a. Anaerobe transport vial for small tissues
   b. Sterile cup for large tissues with nonbacteriostatic saline on a gauze pad to keep moist
   c. Wound or abscess aspirates
      (1) Samples collected by using a syringe and needle should be placed in a sterile container or blood collection tube without anticoagulant (e.g., Vacutainer or similar type) for submission to the laboratory.
      (2) A portion of the samples should also be placed in a sterile tube containing prereduced anaerobically sterilized (PRAS) medium if an anaerobic culture is required.
      (3) Syringes with the needle attached should not be accepted due to the sharps and biohazard risk to staff. Syringes capped with a Leur-Lok are also not acceptable because the specimen may leak during transport and the samples may also be contaminated during handling.
   d. Broth culture medium in small sterile snap-top microcentrifuge tubes for fine-needle aspirates (FNA). These tubes are ideal for this type of specimen, because the specimen is easily visible and can be minced with a sterile glass rod in the laboratory, if necessary.
   e. Routine culture swabs should be submitted in a transport system with Stuart's or Amies medium to preserve the specimen and keep it moist and to neutralize inhibitory effects of swabs. CultureSwab EZ II (BD Diagnostic Systems) and eswabs (flocked swab in 1 ml of liquid Amies medium; Copan Diagnostics) are best for recovery of aerobes and anaerobes using a single specimen collection device (7; http://www.copanusa.com).

B. Specimen collection after proper disinfection

◩ **NOTE:** Refer to procedure 3.13.2 for quantitative culture methods.

1. Swabs of wounds

   ◩ **NOTE:** Superficial wounds must often be swabbed to collect a sample for culture because there is not enough pus or fluid to aspirate.
   a. Limit swab sampling to wounds that are clinically infected or those that are chronic and not healing.
   b. Superficial or deep wounds, including bites, should be cultured only if there is purulence, chronic drainage, or nonhealing.
   c. Gently roll swab over the surface of the wound approximately five times, focusing on the area where there is evidence of pus or inflamed tissue.
   d. Aerobic and anaerobic culture is indicated for all types of deep wounds and bites that are clinically infected. CultureSwab EZ II (BD Diagnostic Systems) and eswabs (flocked swab in 1 ml of liquid Amies medium; Copan Diagnostics) are best for recovery of aerobes and anaerobes using a single specimen collection device (7; http://www.copanusa.com). Alternatively, separate swabs must be collected for aerobic and anaerobic culture and immediately placed after inoculation back into their respective transport tubes.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

◩ **NOTE:** Organisms may not be distributed evenly in a burn wound, so sampling different areas of the burn is recommended. Blood cultures should be used to monitor patient status.

2. Abscesses

◩ **NOTE:** Abscesses (purulent collections) that are closed off and not yet draining externally should be aspirated and the pus (i.e., purulent fluid) sent for culture.

a. Aspirate infected material with needle and syringe.

b. If the initial aspiration fails to obtain material, inject sterile, nonbacteriostatic saline subcutaneously. Repeat the aspiration attempt.

c. Wound or abscess aspirates collected by using a syringe and needle should be placed in a sterile container or blood collection tube without anticoagulant (e.g., Vacutainer or similar type) for submission to the laboratory. A portion of the samples should also be placed in a sterile tube containing PRAS medium if an anaerobic culture is required.

◩ **NOTE:** Syringes with the needle attached should not be accepted due to the sharps and biohazard risk to staff. Syringes capped with a Leur-Lok are also not acceptable because the specimen may leak during transport and the samples may also be contaminated during handling.

d. A biopsy sample of the abscess capsule or tissue may also be collected and sent for culture during surgical incision and drainage procedures.

3. Drainages

See procedure 3.5 and Table 3.5–2 for drainages that are acceptable for culture. Drainage tube devices should not be cultured. Abdominal, chest tube, and biliary t-tube drainages are the most commonly submitted drainages. Drainage fluids for culture should never be collected from the bag due to organism overgrowth in fluid samples that are not freshly collected, and the concerns about contamination by skin and other normal microbiota in the area being drained. Drainage fluid should be freshly collected by direct aspiration of fluid from the area being drained or by aspiration of fresh fluid in the drainage tube after decontamination of the surface of the device. Abdominal, chest tube, and biliary t-tube drainages are the most commonly submitted drainages. Cultures of surgical drainage from clean surgical procedures are not indicated if there are no signs of infection (4).

a. Disinfect the collection tubing, and aseptically aspirate fresh fluid from the tubing.

b. Submit the drainage fluid in a sterile, leakproof container.

c. Do not inoculate blood culture bottles with drainage fluids, because a Gram stain and culture correlation is needed to determine the clinical significance of drainage fluid isolates.

◩ **NOTE:** Drainage tubes and tubing should be rejected for culture. Physicians may be educated about the proper collection of drainage fluid samples if a comment is included on the report such as “Drainage tubes of this type are not acceptable for culture because of the high rate of contamination by normal skin flora. Drainage fluid is acceptable for culture if a fresh sample is aseptically collected by aspiration into a sterile container after disinfecting the collection tubing.”

4. Tissues and biopsy samples

a. Tissue biopsy samples should be collected from areas within and adjacent to the area of infection. Large enough tissue samples should be collected to perform all of the tests required (i.e., 3- to 4-mm biopsy samples).

b. If anaerobic culture is required, a separate piece of tissue should be submitted in a sterile tube containing PRAS media.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

5. FNA
   a. Insert the needle into the tissue, using various directions, if possible.
   b. If the volume of aspirate is large, remove the needle and submit with Luer-Lok on the syringe.
   c. If the volume is small, aspirate the specimen into the sterile locking microcentrifuge tube containing broth by drawing up and down to release the specimen from the syringe.

◩ **NOTE:** Always use a safety device on the needle. Do not submit needle to the laboratory.

C. Label specimen and requisition.
   1. List demographic information on the patient.
   2. Describe the type of specimen (deep tissue, superficial tissue, decubitus, catheter site, boil, abscess, cellulitis, aspirate, pus, drainage, surgical incision site, etc.)
   3. State anatomic location (arm, leg, etc.)
   4. Record collection time and date.
   5. List diagnosis or ICD9 code, including cause and clinical signs of infection.
   6. List antimicrobial therapy prior to specimen collection.
   7. Choose tests requested, including anaerobic culture, if appropriate.

◩ **NOTE:** To avoid the overuse of full fungal cultures that require incubation periods of greater than 1 week, the laboratory can offer a fungal culture with a shorter (2- to 4-day) incubation period. Such cultures are useful and cost-effective for the diagnosis of nosocomial, foreign-body, and postoperative infections, where the likely pathogen is either bacteria, *Candida* species, or *Aspergillus* species. *Candida* or *Aspergillus* species will grow on routine bacterial culture media within 1 week; however, a selective fungal medium may be indicated for cultures expected to contain mixed microbiota. Full fungal cultures should be reserved for diagnosis of chronic infections, particularly those caused by dematiaceous and biphasic molds, and should be performed only from specimens not submitted on swabs.

D. Deliver aspirates and tissues to the laboratory within 30 min for best recovery.
   1. Keep tissues moist to preserve organism viability.
   2. Do not refrigerate or incubate before or during transport. If there is a delay, keep sample at room temperature, because at lower temperature there is likely to be more dissolved oxygen, which could be detrimental to anaerobes.

E. Rejection criteria
   1. Do not accept specimens for microbiological analysis in container with formalin.
   2. If numerous squamous epithelial cells are present on the Gram stain, especially from swab specimens, request a recollection if there is evidence of infection.
   3. Discourage submission of specimens to determine *if* an infection is present.
   4. Since commercial swab transport systems have been documented to preserve most pathogens for up to 24 h, swabs are generally not STAT transported either in the hospital or in the ambulatory setting.
   5. For multiple requests (acid-fast bacilli, fungal, bacterial, and viral) but little specimen, contact the physician to determine which assays are most important and reject the others as “Quantity not sufficient.”

III. MATERIALS

A. Media

1. BAP

2. CHOC for surgical tissues, closed aspirates, biopsy samples, and FNA specimens or specimens from genital sites to culture for *Neisseria* or *Haemophilus* spp.

3. MAC or EMB, except for clean surgical specimens (e.g., orthopedic cultures). When in doubt, examine Gram stain to determine the likelihood of a mixed culture.

4. Phenylethyl alcohol agar (PEA) or Columbia colistin-nalidixic acid agar (CNA), if source (e.g., gastrointestinal) or *Gram stain indicates that the culture contains gram-negative rods*, which may inhibit gram-positive organisms.

5. Thayer-Martin or similar selective media for genital specimens or for other specimens if *Neisseria gonorrhoeae* is suspected.

6. Anaerobic culture media, if appropriate for site of collection and transport conditions. See section 4.

7. Special media for recovery of certain fastidious or unusual organisms. Refer to the table of contents of this section for procedures listed by specific microorganism names.

8. Tissue broth cultures

Broth culture of deep tissues may increase the detection of small numbers of organisms. Biopsy samples from deep tissues (lung, brain, liver, bone, etc.) and other FNA samples may be inoculated to enrichment broth. Common broth media include the following.

a. Anaerobic BHI or TSB with 0.1% agar with or without yeast extract

b. Fastidious anaerobic broth (Quebec Laboratories, Inc., Montreal, Quebec, Canada)

c. Although THIO is an excellent medium for anaerobe recovery, it is the least suitable for recovery of low numbers of aerobic organisms, including yeasts (6).

◩ **NOTE:** Broth cultures inoculated as an adjunct to direct plating of wounds and fluid samples have been shown to seldom yield results that would alter patient management and could be omitted for most specimens without compromising patient care (5). Broth cultures should also not be done on swabs obtained from superficial or deep wound sites (8).

B. Tissue-homogenizing apparatus

1. Scalpels and petri dishes (Fig. 3.13.1–1)

2. Mortars and pestles. Use only in an anaerobic chamber if anaerobes are suspected, as these devices aerate the specimen (Fig. 3.13.1–2).

3. Automated pummeling instrument (stomacher [Tekmar Co., Cincinnati, OH]; MiniMix [Interscience Laboratories, Inc., Hingham, MA]) (Fig. 3.13.1–3)

4. Commercially available disposable plastic grinding devices (BD Diagnostic Systems; Sage Products Inc., Crystal Lake, IL). These are safer than glass handheld grinders (Fig. 3.13.1–4)

C. Gram stain reagents

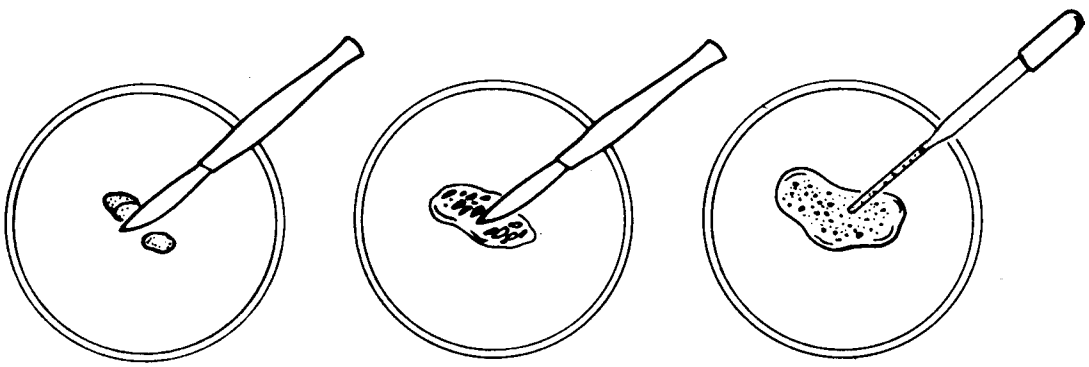


Figure 3.13.1–1 Illustration of sterile-scalpel method of homogenization of tissue.

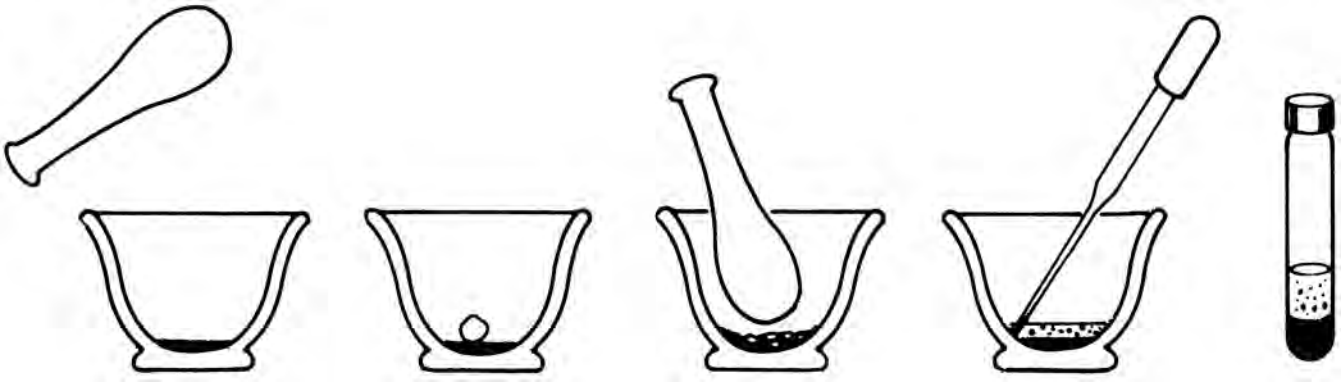


Figure 3.13.1–2 Illustration of mortar-and-pestle method of homogenization of tissue.

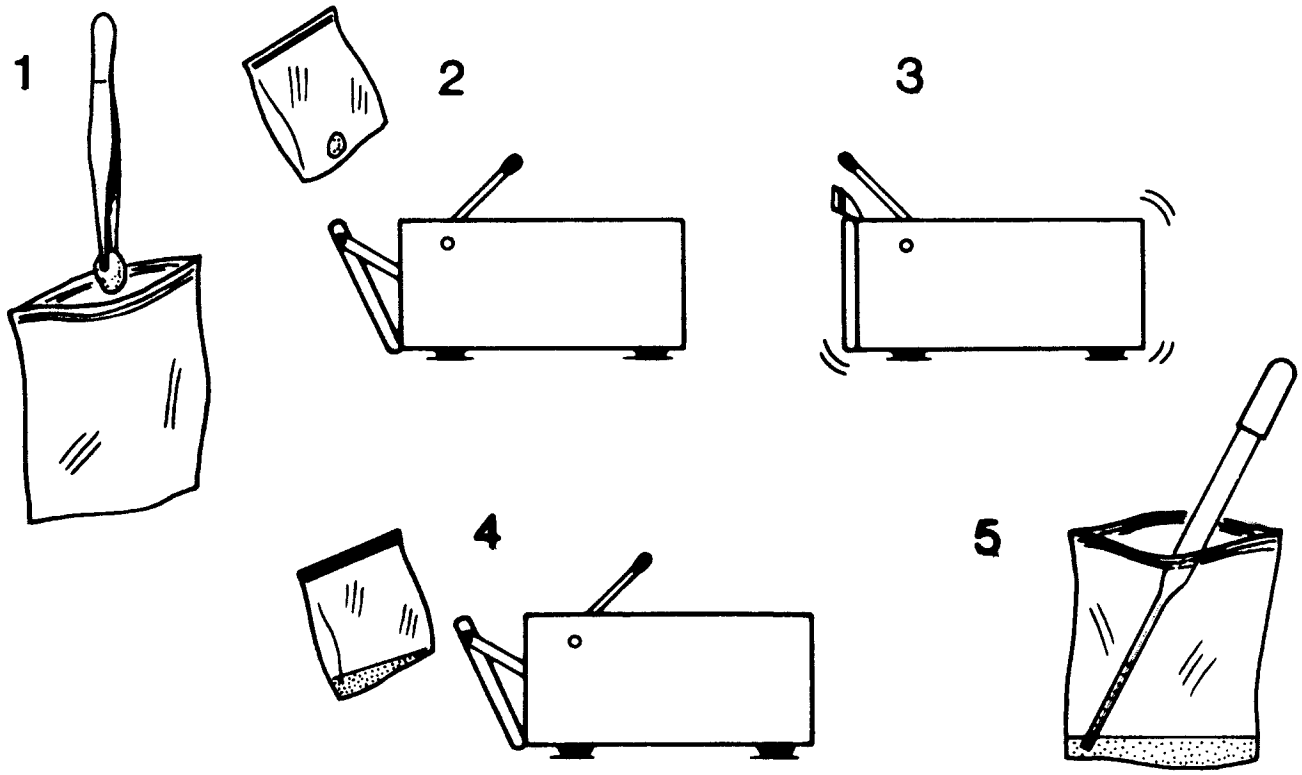


Figure 3.13.1–3 Illustration of stomacher method of homogenization of tissue.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that media meet expiration date and QC parameters per Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M22-A3 (2). See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

B. QC each lot of CHOC (procedure 3.3.1) and *N. gonorrhoeae* selective agar (procedure 3.9.3) using appropriate microorganisms listed in these procedures.

C. Perform other user QC per CLSI document M22-A3 and section 14 of this handbook.

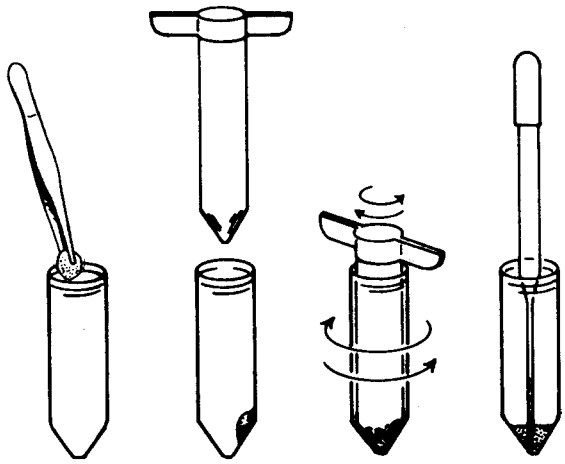


Figure 3.13.1–4 Illustration of tissue-grinding kit method of homogenization of tissue.

V. PROCEDURE

Observe standard precautions.

It is imperative that these cultures be handled in a biological safety cabinet.

A. For safety reasons, as with all microbiological specimens, and to protect the specimen from environmental contamination, place specimen and media in a biological safety cabinet or anaerobic chamber to perform specimen preparation and inoculations.

B. Inoculation

1. Tissues

a. Select a portion of the tissue biopsy sample for culture that is bordering and within the area of infection (i.e., necrotic tissue is usually at the center of infected tissue areas).

b. Perform anaerobic culture first, preferably in an anaerobic chamber.

(1) If the tissue is large enough to be safely handled, place the tissue in a petri dish or specimen cup and cut it in half with a knife. Cut a smaller piece from the half and immediately touch the cut surface to the inoculum area of the anaerobe plates, place the tissue into broth culture medium, and streak the anaerobe plates for isolation.

(2) Alternatively, and for smaller tissue specimens, grind or homogenize tissues in THIO or other reduced broth (Fig. 3.13.1–1 to 3.13.1–4).

(3) Incubate immediately.

c. If the tissue can be easily teased apart (e.g., lung, kidney, and brain tissue), cut a portion of the tissue into several pieces (use a sterile blade and stick or scissors) or gently tease it apart with sterile sticks (Fig. 3.13.1–1). (Save one piece of cut tissue without teasing for the smears.) Inoculate a piece of tissue onto each of the culture plates.

d. If the tissue is hard (e.g., bone and skin)

(1) A sterile scalpel may be used to chip off small bone pieces for culture from a larger specimen. This procedure, however, creates a safety issue for staff, and manipulation of the sample may cause contamination. Alternatively, viable tissue may be removed from the bone biopsy sample, and the tissue and bone may be plated to the same plates after the bone is sonicated.

(2) Alternatively, place some of the tissue in the grinding apparatus (Fig. 3.13.1–2 and 3.13.1–4) and grind with about 0.5 ml of fluid from broth culture medium.

(3) After homogenization, remove the homogenized specimen using a sterile pipette. Inoculate plates and place the rest into broth culture medium.

V. PROCEDURE *(continued)*

◩ **NOTE:** Always examine these specimens for the presence of any soft tissue. If you find any such material, carefully remove it with a sterile surgical scalpel. This tissue may be processed separately.

e. Use an automated pummeling instrument to grind tissues that cannot be easily teased, if available (Fig. 3.13.1–3).

(1) Place a portion of the specimen into a sterile bag along with a small amount of sterile broth. This aids the recovery of organisms from samples that remain intact after pummeling. The tissue and broth should both be cultured.

(2) Insert sample bag between door and paddles in blender, allowing 4 cm of bag to project above top of door.

(3) Pull handle forward to firmly close door, and switch machine on for 1 to 5 min.

(4) Switch machine off, hold bag, open door by lifting handle, and remove bag.

(5) Remove sample from bag by using sterile pipette, and inoculate media.

f. For Gram stain

(1) *Touch preps of the tissue sample are made onto sterile glass slides before the culture is inoculated. If sterile slides are not used for the touch prep smears, then these should be made after the culture has been inoculated to avoid contamination.*

(2) Make a fresh cut of tissue and prepare the smear by touching the tissue to the slide. If the tissue is hard and does not stick to the slide, place the tissue between the two slides and press the slides together. Then separate by drawing the slides against each other (*see* Fig. 3.2.1–1 and 3.2.1–2).

g. If the tissue is large enough, save an intact piece in the refrigerator for up to 7 days, or in the freezer for extended storage.

2. Aspirates and pus

a. Mix the specimen thoroughly. Place a drop of the specimen onto each piece of the medium.

b. If sufficient specimen is submitted, inoculate invasively collected aspirates to broth culture medium to make a 1:10 dilution. If the volume is small, omit broth culture.

c. Prepare smear for Gram stain by placing a drop of specimen on a slide and spreading it to make a thin preparation. If the aspirate fluid is clear, use the cytocentrifuge to concentrate the specimen for the smear.

d. If sufficient specimen is available, save a portion in the refrigerator for up to 7 days for further testing, if indicated.

3. Swabs

a. If an anaerobic culture is to be performed, inoculate anaerobic plates first.

b. Then place swab in 1 to 2 ml of broth and vortex.

c. Squeeze the swab against the side of the broth tube to express remaining fluid and then discard.

d. Inoculate aerobic plates and prepare smear for Gram stain as described for aspirates and pus.

e. Alternatively, the swab can be used for direct specimen plating. Always inoculate media from the least inhibitory to the most inhibitory.

f. Save broth in the refrigerator for up to 7 days for further testing, if indicated.

◩ **NOTE:** Do not culture swabs from superficial wounds or abscesses in broth medium.

V. PROCEDURE *(continued)*

C. Aerobic incubation conditions (*see* section 4 for anaerobic incubation conditions and workup of anaerobic culture)

1. Incubate BAP, CNA or PEA, and CHOC in humidified incubator at 35 to 37°C with 5% CO_2. Incubate for a minimum of 48 h for open wound cultures and for 3 to 4 days for invasively collected specimens with no initial growth. Incubation may be extended to 7 days for invasive specimens (i.e., aspirated fluids and tissues) that remain culture negative after 3 to 4 days of aerobic incubation.

2. Critical deep-wound, abscess, and tissue samples should have anaerobic cultures requested (*see* Table 3.13.1–3 and section 4) in order to recover all of the primary pathogen(s) causing infection in specific clinical conditions (brain abscesses, brain, lung, liver tissue, deep wounds, abscesses, etc.). The laboratory should also routinely do anaerobic cultures on these types of samples when the specimen Gram stain demonstrates purulence (i.e., PMNs) and one or more bacterial morphotypes suggestive of anaerobes.

3. Incubate MAC or EMB plates in ambient air at 35 to 37°C, unless it is inconvenient to keep them separate from the rest of the culture in 5% CO_2.

4. Incubate broth in ambient air at 35 to 37°C for 3 to 4 days. If possible, hold for 1 week at room temperature to ensure that some specimen is available if further testing is indicated.

D. Perform a Gram stain on all specimens and use in the evaluation of culture.

1. Refer to procedure 3.2.1 for details on smear preparation and staining.

2. Record the relative numbers of WBCs, epithelial cells, and bacterial and fungal morphotypes.

a. If clinically important organisms are recognized or suspected (e.g., from a normally sterile site) based on the Gram stain interpretation, telephone or report results to the appropriate caregiver immediately. Report any bacteria seen in a surgically collected specimen from a normally sterile site. Some examples of significant bacteria include the following.

(1) Clostridium-like gram-positive rods seen on specimens from soft tissue infections or aspirates even in the absence of numerous intact PMNs

◩ **NOTE:** Clostridia and other anaerobes produce phospholipases and lipases, and some aerobes (e.g., *S. aureus*, group A *Streptococcus*, *Listeria*, and *Corynebacteium* spp.) produce phospholipases that can destroy host cells, so a reduced or absent number of PMNs may be found in the direct specimen Gram smear.

(2) Numerous PMNs and gram-positive cocci in clusters resembling *Staphylococcus* in an abscess or tissue

(3) Bacteria seen from brain abscess specimens

(4) Gram-positive cocci in chains suggestive of streptococci from endometrial tissue may be found in group A streptococcal puerperal sepsis.

b. *If multiple morphologies are seen on the smear and the culture was not inoculated onto selective agar, go back to the specimen and inoculate it to CNA or PEA and EMB or MAC.*

E. Culture workup

1. Read plates and broth daily. Refer to procedure 3.3.2 for description of colony types, initial reading, testing, and reporting preliminary and final cultures. For identifications refer to Table 3.3.2–5 and procedures 3.18.1 and 3.18.2.

It is imperative that these cultures be handled in a biological safety cabinet.

2. For cultures of lymph nodes, work in a biological safety cabinet, since pathogens can be found in these specimens that are hazardous, e.g., *Francisella, Mycobacterium,* and *Brucella.*

V. PROCEDURE *(continued)*

3. Refer to Table 3.13.1–2 for the list of the most common pathogenic organisms associated with wound infections and Fig. 3.13.1–5 for algorithm for extent of workup of cultures. Follow Fig. 3.13.1–6 and identify any number of the organisms listed.
4. Generally identify up to three microorganisms listed in Table 3.13.1–2 if any of the following is true.
   a. PMNs were present on direct smear.
   b. The specimen was collected from a normally sterile site.
   c. The specimen was of good quality (e.g., no or few epithelial cells present).
   d. The organism was seen on the direct smear.
5. Perform only minimal testing to indicate the type of microbiota present for noninvasively collected specimens with any of the following.
   a. Moderate or numerous epithelial cells present on the smear
   b. No evidence of infection on the smear (no PMNs) and no clinical information accompanying the specimen to indicate an infection
   c. ≥3 Organisms growing in the culture. See exceptions for specific organisms in Fig. 3.13.1–6, which are generally always reported.

   ◩ **NOTE:** Save all culture plates with growth for several days in case further work is requested by the physician. Seven days is usually sufficient, but if space is a problem, transfer isolates to culture tubes for storage or save for a shorter period.
6. Identify any number of microorganisms that only grow on CHOC, and not on BAP *(N. gonorrhoeae, Haemophilus,* and *Francisella).* Identify *Neisseria meningitidis.*
7. Identify *Streptococcus pyogenes* or *Streptococcus agalactiae.*

   ◩ **NOTE:** Notify the physician of the isolation of *S. pyogenes,* as it may represent a life-threatening case of necrotizing fasciitis.
8. *Staphylococcus*
   a. *S. aureus*
      (1) Perform AST from invasively collected specimens and from others, if the Gram stain indicates a good-quality specimen and an infectious process with this organism (e.g., PMNs with few or no squamous epithelial cells and staphylococci seen on specimen Gram stain).
      (2) If an infectious process is not apparent but it is the hospital policy to track nosocomial methicillin-resistant *S. aureus* (MRSA) infections, rule out MRSA on inpatient specimens, unless the patient has a prior positive culture with MRSA. Notify infection control practitioner if MRSA is present, per hospital policy.
   b. When *coagulase-negative staphylococci* are present, perform AST only if they are the only organisms isolated from invasively collected specimens, if they are associated with PMNs in the direct smear, or if they are isolated from multiple cultures. Report as normal cutaneous microbiota if found in mixed cultures in any amount from superficial wound specimens or if numerous epithelial cells are present in the specimen.
9. For *viridans group streptococci* or *enterococci*
   a. Identify at least to the genus level from surgically, invasively collected specimens where the organism is the single or predominant pathogen and the Gram stain indicates infection (the presence of PMNs).
   b. Include in normal microbiota if found in mixed cultures and not predominant.

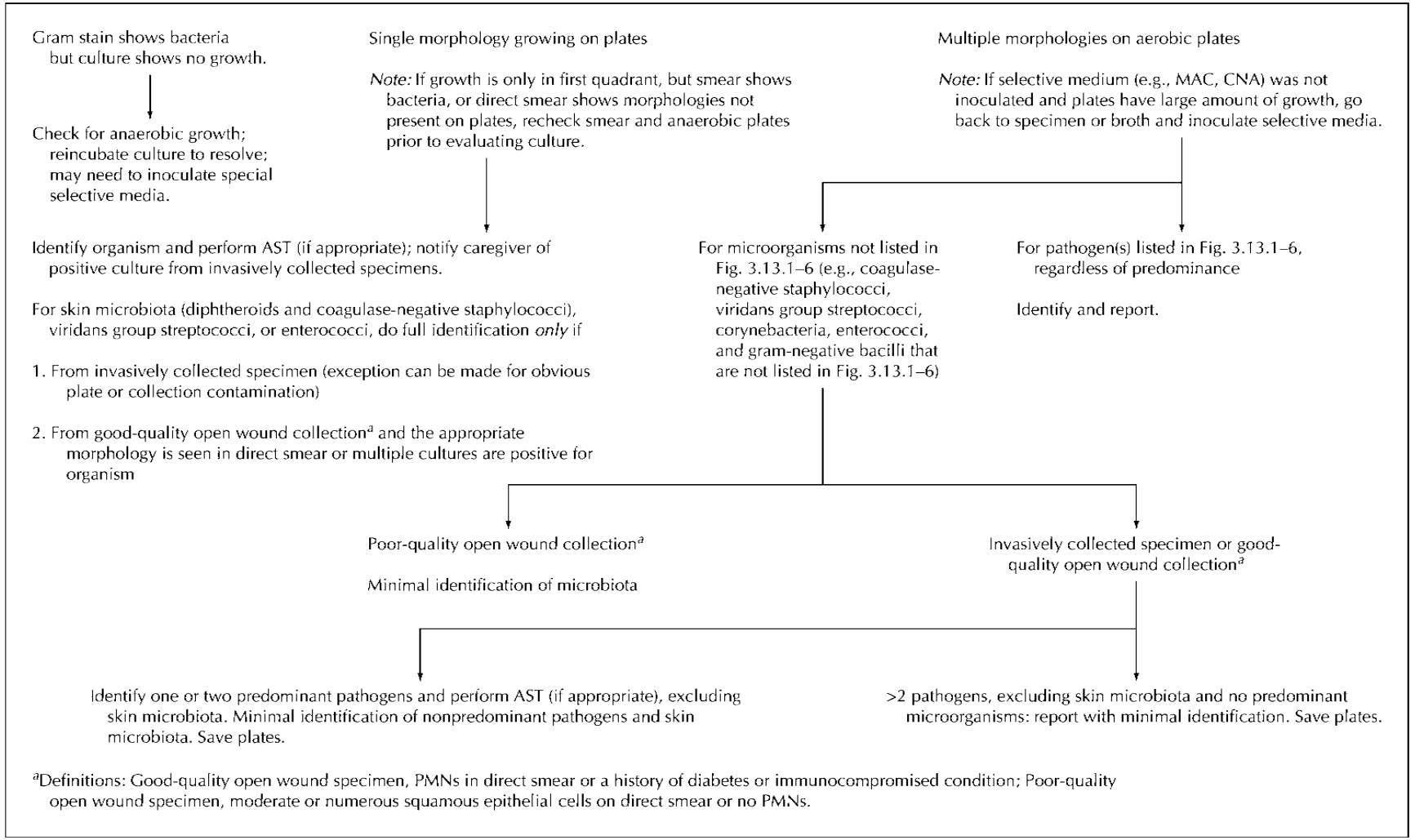

Gram stain shows bacteria but culture shows no growth.
Check for anaerobic growth; reincubate culture to resolve; may need to inoculate special selective media.
Single morphology growing on plates
Note: If growth is only in first quadrant, but smear shows bacteria, or direct smear shows morphologies not present on plates, recheck smear and anaerobic plates prior to evaluating culture.
Identify organism and perform AST (if appropriate); notify caregiver of positive culture from invasively collected specimens.
For skin microbiota (diphtheroids and coagulase-negative staphylococci), viridans group streptococci, or enterococci, do full identification only if
1. From invasively collected specimen (exception can be made for obvious plate or collection contamination)
2. From good-quality open wound collection[a] and the appropriate morphology is seen in direct smear or multiple cultures are positive for organism
Multiple morphologies on aerobic plates
Note: If selective medium (e.g., MAC, CNA) was not inoculated and plates have large amount of growth, go back to specimen or broth and inoculate selective media.
For microorganisms not listed in Fig. 3.13.1–6 (e.g., coagulase-negative staphylococci, viridans group streptococci, corynebacteria, enterococci, and gram-negative bacilli that are not listed in Fig. 3.13.1–6)
For pathogen(s) listed in Fig. 3.13.1–6, regardless of predominance
Identify and report.
Poor-quality open wound collection[a]
Minimal identification of microbiota
Invasively collected specimen or good-quality open wound collection[a]
Identify one or two predominant pathogens and perform AST (if appropriate), excluding skin microbiota. Minimal identification of nonpredominant pathogens and skin microbiota. Save plates.
>2 pathogens, excluding skin microbiota and no predominant microorganisms: report with minimal identification. Save plates.
[a]Definitions: Good-quality open wound specimen, PMNs in direct smear or a history of diabetes or immunocompromised condition; Poor-quality open wound specimen, moderate or numerous squamous epithelial cells on direct smear or no PMNs.

Figure 3.13.1–5 Initial evaluation of positive wound cultures for organisms growing aerobically. *Note:* For lymph nodes, perform all work using a biological safety cabinet.

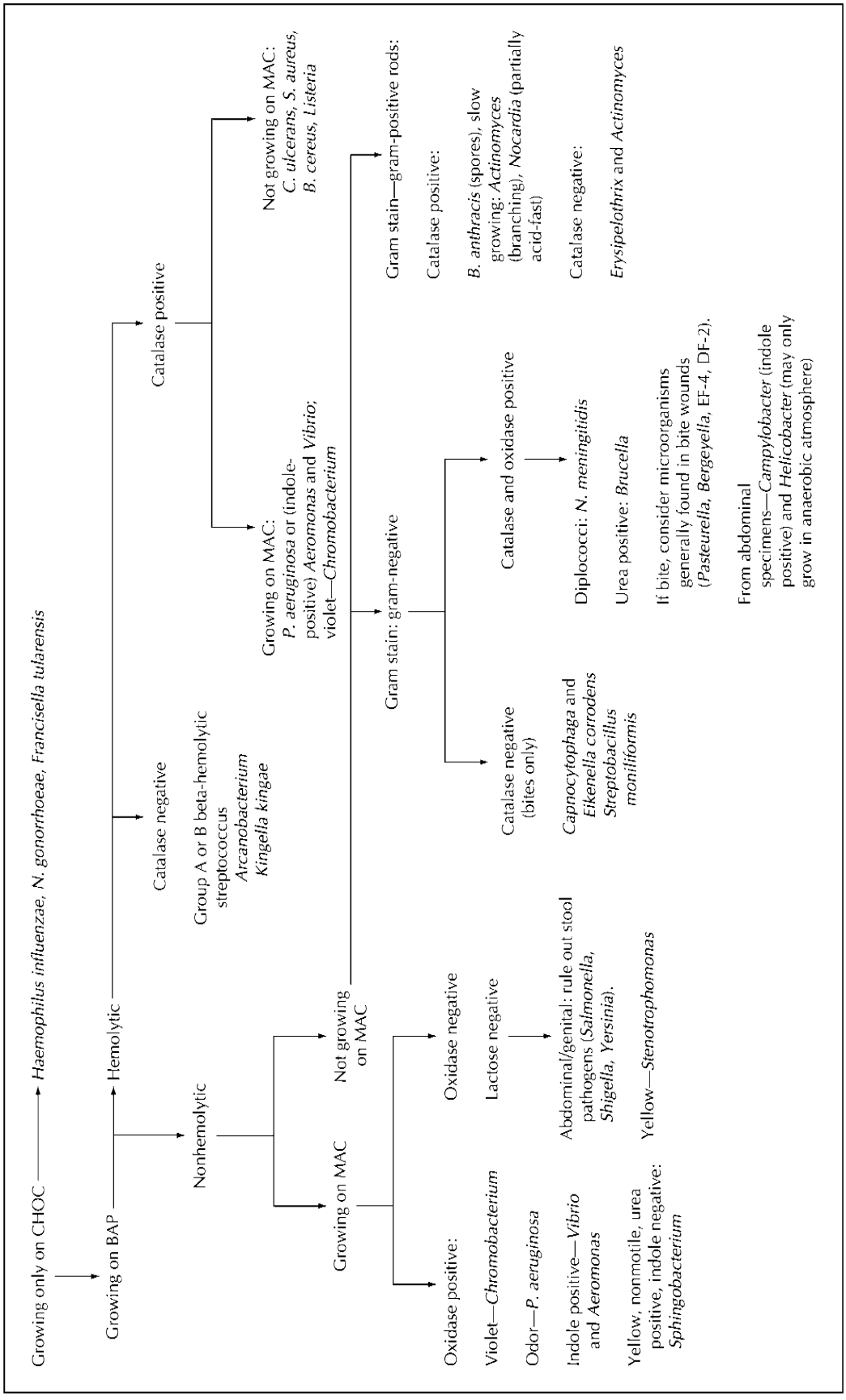

Growing only on CHOC → *Haemophilus influenzae, N. gonorrhoeae, Francisella tularensis*
Growing on BAP
Hemolytic
Catalase positive
Catalase negative
Nonhemolytic
Group A or B beta-hemolytic streptococcus
Arcanobacterium
Kingella kingae
Growing on MAC
Not growing on MAC
Growing on MAC: *P. aeruginosa* or (indole-positive) *Aeromonas* and *Vibrio*; violet—*Chromobacterium*
Not growing on MAC: *C. ulcerans, S. aureus, B. cereus, Listeria*
Gram stain: gram-negative
Gram stain—gram-positive rods:
Oxidase positive:
Oxidase negative
Catalase positive:
Violet—*Chromobacterium*
Lactose negative
Catalase negative (bites only)
Catalase and oxidase positive
B. anthracis (spores), slow growing: *Actinomyces* (branching), *Nocardia* (partially acid-fast)
Odor—*P. aeruginosa*
Abdominal/genital: rule out stool pathogens (*Salmonella, Shigella, Yersinia*).
Capnocytophaga and *Eikenella corrodens*
Streptobacillus moniliformis
Diplococci: *N. meningitidis*
Catalase negative:
Indole positive—*Vibrio* and *Aeromonas*
Urea positive: *Brucella*
Erysipelothrix and *Actinomyces*
Yellow, nonmotile, urea positive, indole negative: *Sphingobacterium*
Yellow—*Stenotrophomonas*
If bite, consider microorganisms generally found in bite wounds (*Pasteurella, Bergeyella*, EF-4, DF-2).
From abdominal specimens—*Campylobacter* (indole positive) and *Helicobacter* (may only grow in anaerobic atmosphere)

Figure 3.13.1–6 Algorithm to rapidly detect aerobic and facultatively aerobic microorganisms usually considered significant, even in low numbers or in mixed cultures. See procedures 3.18.1 and 3.18.2 for other tests needed to confirm suspected identifications.

V. PROCEDURE *(continued)*

c. If determined to be a significant isolate or if indicated by infection control policies, perform a vancomycin screen on enterococci from inpatients and from transplant and oncology outpatients. Perform AST only if isolate is from normally sterile site (e.g., bone, brain) in pure or almost pure culture.

10. For *gram-positive rods,* if specimen is from a normally sterile site or biopsy sample, rule out *Listeria, Erysipelothrix, Bacillus cereus, Bacillus anthracis, Arcanobacterium, Corynebacterium diphtheriae, Corynebacterium ulcerans, Nocardia,* and *Actinomyces.* Identify other gram-positive rods if numerous or seen as predominant in smear. Otherwise include these in skin microbiota.

11. Include *yeasts* as part of normal microbiota unless predominant or numerous. Except for specimens from normally sterile sites, generally identify only *Candida albicans* to the species level.

12. For predominant or moderate to numerous amounts of *enteric gram-negative rods*

a. If only one or two species are present or predominant and *an indication of infection is seen on smear,* identify and perform AST. For specimens from the abdominal cavity, the aerobic plates may contain only a few *Escherichia coli* organisms but the smear appears to represent mixed morphologies. In such cases, do not set up AST on the *E. coli* until the results of the anaerobic culture can be evaluated. Potentially the anaerobic microbiota may be the significant, predominant pathogen(s).

b. If enteric bacilli are few in amount or not predominant, or if >2 species are present with no predominant strain, report as "mixed GI [for gastrointestinal] microbiota."

(1) Rule out fecal pathogens (*Salmonella, Shigella, Campylobacter,* and *Yersinia* spp.) for specimens from abdominal abscesses.

(2) Generally save a representative plate for up to 7 days in case further work is requested.

c. Identify and perform susceptibility tests on multiple morphologies of enteric gram-negative rods only on special request after consultation with the laboratory director, designee, or physician.

◩ **NOTE:** When cultures contain a variety of enteric rods, treatment must include a combination of antimicrobial agents which are known to eradicate normal intestinal microbiota. Examination of a culture with fecal contamination to detect and separate each species is futile and not helpful for overall treatment decisions.

It is imperative that these cultures be handled in a biological safety cabinet.

13. For gram-negative rods that are not of the *Enterobacteriaceae* family

a. Rule out organisms which are always considered pathogenic (e.g., *Brucella, Haemophilus, Pasteurella, Francisella*). Generally these organisms are recognized because they do not grow on MAC or EMB. These organisms are not uncommon in dog and cat bite wounds. Work in a biological safety cabinet, and see procedure 3.18.2 for identification flowcharts and tables.

■ Francisella *can be found in lymph node biopsy samples and is extremely infectious. It is a tiny coccobacillus that grows slowly and is catalase positive or weak and oxidase negative. It can ferment glucose, but it is negative for other biochemical tests. It is beta-lactamase positive. Refer to procedure 16.8 for other details.*

b. Identify obvious *P. aeruginosa* (characteristic odor and beta-hemolytic colonies) and *Stenotrophomonas maltophilia* (yellow and oxidase negative). If in pure culture or significant amounts and the Gram stain suggests an infective process, perform AST.

V. PROCEDURE *(continued)*

c. Identify oxidase-positive, indole-positive organisms (Table 3.18.2–8) which are likely to be *Aeromonas* or *Vibrio.* Also examine for the pigmented gram-negative rods *Chromobacterium violaceum* and *Sphingobacterium.*

d. Identify and perform AST on other gram-negative rods (*Pseudomonaceae, Acinetobacter,* and related non-glucose-fermenting rods) by following the algorithm in Fig. 3.13.1–5.

14. Refer to procedure 3.3.2 for details on handling of broth cultures.

F. Hold positive culture plates at room temperature or in the refrigerator for several days (generally 7 days) after the culture is completed for additional work if requested by the physician.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report Gram stain results as soon as possible, generally within 1 h for specimens from critical sites.

B. Report all negative cultures as "No growth in _____ days."

C. Report individually those organisms that are always considered pathogenic (Fig. 3.13.1–6) with enumeration, using a preliminary identification initially and the genus and species (if applicable) as the final identification, if applicable.

D. Due to their known virulence factors, indicate the presence of the following species.

1. Beta-hemolytic streptococci

2. *S. aureus*

3. *P. aeruginosa*

4. *Clostridium perfringens*

5. Report "Pigmented anaerobes," *Bacteroides* spp., and "Mixed anaerobes" without further identification. (*See* section 4 for identification methods.)

E. Report other pathogens, as indicated in Fig. 3.13.1–5, with either definitive or minimal identification, depending on quantitation, number of species present, and Gram stain results. (*See* Table 3.13.1–4 for examples of reports.)

F. Report AST on gram-negative rods, enterococci, or *S. aureus,* using the flowchart in Fig. 3.13.1–5. Generally do not perform AST on microorganisms that are not predominant, are in mixed cultures, or are skin microbiota or if culture does not show evidence of an infectious process. Make exceptions to this general policy if requested to do so by physician caring for the patient or for infection control purposes.

G. When multiple morphologies are present, report with minimal identification.

Example: "Culture yields growth of >3 colony types of enteric gram-negative bacilli. Consult microbiology laboratory if more definitive studies are clinically indicated."

See Table 3.13.1–4 for other examples.

H. Additionally, if mixed microbiota are cultured with no predominant microorganism, report as GI, oronasal, skin, or genital microbiota. Use of selective media is helpful in evaluation of polymicrobic nature of culture.

I. For further details on reporting, refer to procedure 3.3.2.

VII. INTERPRETATION

A. Continuous dialogue between the clinician or nurse and the microbiology laboratory should be encouraged for proper interpretation of results.

B. The results of wound cultures will only be as valuable as the quality of the specimen submitted, its transport, and expedient processing.

Table 3.13.1–4 Examples of mixed wound culture reporting[a]

Microbiological observation	Examples of specimen submissions			
	Example 1	Example 2	Example 3	Example 4
PMNs in Gram stain[b]	−	2+	3+	1+
Bacteria in Gram stain	3+ gram-negative rods	3+ gram-negative rods	3+ gram-positive and -negative rods	No organisms seen
Bacteria in culture				
S. aureus	1+	2+	1+	−
P. aeruginosa	−	3+	−	−
Beta-hemolytic streptococci	−	−	−	1+ group A
Enterobacteriaceae (agents of gastroenteritis ruled out)	3+ pure	1+	2+	1+
Peptostreptococcus spp.	−	1+	1+	1+
Clostridium spp.	1+	−	3+	−
Pigmented gram-negative anaerobes	−	−	3+	−
Nonpigmented gram-negative anaerobes	1+	−	1+	−
Information provided on microbiology report	3+ *E. coli;* 1+ anaerobic GI microbiota; 1+ *S. aureus*	2+ *S. aureus* (AST); 3+ *P. aeruginosa* (AST); 1+ mixed skin and enteric microbiota	4+ mixed aerobic and anaerobic GI microbiota; 1+ *S. aureus*	1+ *S. pyogenes;* 1+ mixed skin and enteric microbiota
Notes	May rule out MRSA for infection control purposes. Save plates; may need to do AST on *E. coli* if diabetic, etc.		May rule out MRSA for infection control purposes.	

[a] Revised from reference 1.

[b] Caution must be used in using the presence of absence of PMNs to report the presence of certain pathogens from mixed wound cultures. Clostridia and other anaerobes produce phospholipases and lipases, and some aerobes (e.g., *S. aureus*, group A *Streptococcus*, *Listeria*, and *Corynebacterium* spp.) produce phospholipases that can destroy host cells, so a reduced or absent number of PMNs may be found in the direct specimen Gram smear.

VII. INTERPRETATION *(continued)*

C. Reporting selected organisms in mixed cultures can lead to erroneous interpretation of the number and variety of infecting pathogens.

D. Performance of AST is not indicated in cases of mixed microbiota indicative of infection of the abdominal cavity with bowel contents. Treatment should include broad-spectrum coverage for normal intestinal microbiota.

E. *Use of the Gram stain can improve the accuracy of evaluating the importance of each potential pathogen. Organisms present in the Gram stain of an appropriately collected specimen correlate with* $\geq 10^5$ *organisms per g of tissue (3, 4).*

F. Clinical studies have demonstrated that the microbial load in an acute wound can predict delayed healing or infection. The more numerous the organisms, the more likely they are to be indicative of infection (1).

G. Many wound infections are polymicrobic, and the isolation of an organism in culture may or may not correlate with infection of the wound.

VIII. LIMITATIONS

A. The microbiologist plays a critical role in the treatment of wound infections because practitioners often consider the report from the laboratory as definitive proof of infection. Providing inappropriate identifications and susceptibility results can prompt unnecessary treatment.

B. The presence of PMNs is an indication of an inflammatory or infectious process, while the presence of epithelial cells indicates surface contamination of the specimen. Specimens containing numerous epithelial cells yield culture results of questionable accuracy in the diagnosis of the infectious process, and one can consider rejection of these specimens for culture.

C. If a patient is immunocompromised or has poor vascular supply, inflammatory cells may not be present in the specimen as a guide to the extent of workup of the culture.

D. Low levels of organisms or fastidious organisms that grow poorly on the direct plates may be missed in culture.

E. Unusual treatment considerations may alter the usual policies of the laboratory in reporting AST.

F. The lack of isolation of a pathogen does not necessarily mean that the laboratory was unable to detect the agent. Other inflammatory diseases can have the same presentations as infectious diseases, including the presence of PMNs on the Gram stain.

REFERENCES

1. **Bowler, P. G., B. I. Duerden, and D. G. Armstrong.** 2001. Wound microbiology and associated approaches to wound management. *Clin. Microbiol. Rev.* **14:**244–269.
2. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
3. **Levine, N. S., R. B. Lindberg, A. D. Mason, Jr., and B. A. Pruitt, Jr.** 1976. The quantitative swab culture and smear: a quick, simple method for determining the number of viable aerobic bacteria on open wounds. *J. Trauma* **16:**89–94.
4. **Magee, C., B. Haury, G. Rodeheaver, J. Fox, M. T. Edgerton, and R. F. Edlieh.** 1977. A rapid technique for quantitating wound bacterial count. *Am. J. Surg.* **133:**760–762.
5. **Morris, A. J., S. J. Wilson, C. E. Marx, M. L. Wilson, S. Mirrett, and L. B. Reller.** 1995. Clinical impact of bacteria and fungi recovered only from broth cultures. *J. Clin. Microbiol.* **33:**161–165.
6. **Rinehold, C. E., D. J. Nickolai, T. E. Piccinni, B. A. Byford, M. K. York, and G. F. Brooks.** 1988. Evaluation of broth media for routine culture of cerebrospinal and joint fluid specimens. *Am. J. Clin. Pathol.* **89:**671–674.
7. **Roelofsen, E., M. van Leeuwen, G. J. Meijer-Severs, M. H. Wilkinson, and J. E. Degener.** 1999. Evaluation of the effects of storage in two different swab fabrics and under three different transport conditions on recovery of aerobic and anaerobic bacteria. *J. Clin. Microbiol.* **37:**3041–3043.
8. **Silletti, R. P., E. Ailey, S. Sun, and D. Tang.** 1997. Microbiologic and clinical value of primary broth cultures of wound specimens collected with swabs. *J. Clin. Microbiol.* **35:**2003–2006.

SUPPLEMENTAL READING

Bartlett, R. C. 1974. *Medical Microbiology: Quality Cost and Clinical Relevance.* John Wiley & Sons, Inc., New York, NY.

Bartlett, R. C., M. Mazens-Sullivan, J. Z. Tetreault, S. Lobel, and J. Nivard. 1994. Evolving approaches to management of quality in clinical microbiology. *Clin. Microbiol. Rev.* **7:**55–88.

Hindiyeh, M., V. Acevedo, and K. C. Carroll. 2001. Comparison of three transport systems (Starplex StarSwab II, the new Copan Vi-Pak Amies agar gel collection and transport swabs, and BBL Port-A-Cul) for maintenance of anaerobic and fastidious aerobic organisms. *J. Clin. Microbiol.* **39:**377–380.

Miller, J. M. 1996. *A Guide to Specimen Management in Clinical Microbiology*, 2nd ed. ASM Press, Washington, DC.

Perry, J. L. 1997. Assessment of swab transport systems for aerobic and anaerobic organism recovery. *J. Clin. Microbiol.* **35:**1269–1271.

Sharp, S. E. 1999. Algorithms for wound specimens. *Clin. Microbiol. Newsl.* **21:**118–120.

Wilson, M. 2005. *Microbial Inhabitants of Humans: Their Ecology and Role in Health and Disease.* Cambridge University Press, New York, NY.

3.13.2 Quantitative Cultures of Wound Tissues

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Quantitative culturing is a patient management tool that can be used with a limited variety of specimen types. However, as indicated in the review by Bowler et al. (2), several publications have demonstrated a correlation between quantitative tissue biopsy cultures and the semiquantitative method of enumeration of organism growth (*see* Table 3.3.2–2) in a qualitative swab culture. Tissues from acute wounds, such as those from trauma and burn patients, and duodenal aspirates are the specimen types that may be used for quantitative microbiological analysis. This procedure describes collection and processing of tissue specimens and determination of bacterial counts. The presence of bacteria in tissue in significant amounts is one of a number of factors which have been associated with delayed healing and has also been correlated with infection. When tissue is not readily available, a swab sample may be a convenient substitute for a tissue biopsy sample, and in a quantitative culture, it may similarly be an indicator of an infectious process. However, semiquantitative swab culture is generally sufficient for patient management.

Quantitative cultures for anaerobic bacteria are problematic and thus less meaningful. Anaerobic microorganims tend to live in microbial synergy with other organisms in the culture and do not grow well when diluted.

Quantitation of bacteria in duodenal aspirates can predict defects in mobility of the intestines. See procedure 13.15 for details. For quantitative culture of specimens from bronchoscopy, refer to the respiratory procedure (Appendix 3.11.2–1).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. Tissues

a. Submit 2- by 1-cm or larger tissue sample collected after cleansing and/or surgical debridement to yield the most useful information. (Refer to procedure 3.13.1 for collection details). This will yield approximately 500 mg of tissue, depending on the density (4).

b. Submit as soon as possible without transport medium, but keep moist in a sealed, sterile container.

2. Swab collection

a. Refer to procedure 3.13.1 for collection.

b. *Use only alginate swabs for quantitative culture.*

B. Rejection criteria

1. If insufficient specimen is received for quantitation, process the specimen for qualitative culture only.

2. Do not process dry swabs.

III. MATERIALS

A. Media
1. BAP or CHOC
2. EMB or MAC
3. THIO or saline for dilutions

B. Reagents
1. Stain reagents for Gram stain
2. 0.85% sterile NaCl
3. Sterile Ringer's solution (available from suppliers of intravenous solutions) containing the following per liter
   - **a.** 8.5 g of NaCl
   - **b.** 0.3 g of KCl
   - **c.** 0.33 g of $CaCl_2$

C. Other supplies
1. Loop method: use either platinum or sterile plastic disposable loops to deliver 0.001 ml (1 µl) or 0.01 ml (10 µl).
2. Pipettor method: sterile pipette tips and pipettor to deliver 10 or 1 µl
3. Sterile bent glass or plastic disposable sterile rods to spread inoculum (Excel Scientific, Wrightwood, CA; [760] 249-6371)
4. Sterile pipettes
5. Analytical balance
6. Polytron omnimixer (Brinkman Instruments, Westburg, NY) or automated pummeling instrument (stomacher; Tekmar Co., Cincinnati, OH)
7. CO_2 incubator at 35 to 37°C
8. Anaerobic atmosphere (optional)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that media meet expiration date and QC parameters per Clinical and Laboratory Standards Institute (formerly NCCLS) document M22-A3 (3). See procedures 14.2 and 3.3.1 for further procedures, especially for in-house-prepared media.

B. Validate the method of quantitation using known cultures of various colony counts. Refer to Appendixes 3.12–3 and 3.12–4 for QC for loops and pipettors, respectively.

V. PROCEDURE

Observe standard precautions.

A. Specimen processing

1. Tissues
   - **a.** Weigh the tube containing the tissue on an analytical balance.
   - **b.** Remove the tissue by using aseptic technique, and place it in 5 ml of sterile 0.85% NaCl or THIO (5, 6, 8). Use THIO only if anaerobic culture is performed. This is a 1:5 dilution of tissue.
   - **c.** Reweigh the now empty original specimen tube, and subtract to determine the weight of the tissue in grams or milligrams.
   - **d.** Homogenize the tissue for 15 to 30 s. Keep the homogenate cool during processing. See procedure 3.13.1 for detailed tissue homogenization methods.
   - **e.** Plate 0.1 ml of the original homogenate. Label plate "10^{-1}" for amount of dilution of original homogenate (which is a 1:5 dilution of the specimen).
   - **f.** Make one to three serial 1:10 dilutions of the homogenate with 0.5-ml aliquots and 4.5 ml of sterile 0.85% NaCl per aliquot.
   - **g.** Plate 0.1 ml of each dilution onto BAP or CHOC and EMB or MAC. Evenly distribute the inoculum with a sterile bent rod. Label plates "10^{-2}," "10^{-3}," and "10^{-4}." Alternatively, for the 10^{-3} and 10^{-4} dilutions, inoculate 0.01 and 0.001 ml of the first dilution with 10- and 1-µl loops, respectively.
   - **h.** Repeat inoculations onto anaerobic BAP, if desired.

V. PROCEDURE *(continued)*

i. Incubate aerobic plates at 35°C in 5% CO_2 for 18 to 24 h. Use an anaerobic atmosphere for anaerobic plates.

2. Swabs

a. Place swab in 5 ml of Ringer's solution. Vortex and remove remaining swab material. (Swab should dissolve.)

b. Beginning with item V.A.1.e above, serially make 10-fold dilutions in 0.85% NaCl and process as for tissue.

B. Gram stains

1. Prepare Gram stain of tissue specimens by spreading a 0.01-ml aliquot of the homogenate on a 1- by 1-cm area of a glass slide (7).

2. Allow the slide to dry, fix with methanol, and Gram stain.

3. Examine 10 fields under a 100× oil immersion objective (*see* procedure 3.2.1 for reporting guidelines).

4. Organisms are visible in Gram stains when at least 10^5 organisms per g of tissue are present in the specimen (7).

C. Examination of cultures: tissue and swabs

1. Determine the number of organisms per gram of tissue by counting the colonies on the plate that grew between 30 and 300 colonies.

◩ **NOTE:** Only use MAC or EMB if overgrowth on BAP or CHOC precludes accurate counting of gram-negative bacilli.

2. Calculate the total number of organisms by using the colony count times the dilution factor (use 5 as the factor for the homogenate dilution and the dilutions labeled on the plates for subsequent dilutions) divided by the original weight of the tissue. There is no weight of the tissue for swab specimens. Use only dilution factors.

Example: Tissue weighed 0.3 g.
Count was 50 on plate labeled "10^{-3}."

$$\frac{50 \text{ CFU} \times 5 \text{ (homogenate dilution)} \times 10^3 \text{ (plate dilution)}}{0.3 \text{ g}} = \frac{2.5 \times 10^5 \text{ organisms}}{0.3 \text{ g}} = 8.3 \times 10^5 \text{ CFU/g}$$

3. Report total count "per gram of tissue" or "per swab."

4. Work up pathogenic organisms for identification and antimicrobial susceptibility testing (AST) according to procedure 3.13.1 when the count is $>10^5$ organisms per g. Special consideration must be made case by case for counts of $<10^5$ organisms per g, depending especially on the identity of the isolate(s) and the type of disease of the patient.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report direct results of smear of tissues with enumeration of each morphotype.

B. Report wound cultures as total number of organisms per gram of tissue.

C. See procedure 3.13.1 for reporting predominant species and AST.

VII. INTERPRETATION

A. The probability that tissue from a traumatic wound or burn is infected or will fail to heal can be predicted from cultures showing $>10^5$ organisms per g (2, 4–6).

B. Colony counts of approximately 10^5 CFU/g of tissue were found to be equivalent to colony counts of 10^3 bacteria/ml of specimen obtained on a moist *alginated* swab (1).

VIII. LIMITATIONS

A. These tests should be used only under certain circumstances and after consultation with the physician or clinical service.

B. Loops are not as accurate as pipettes to deliver the inoculum.

C. Spreading with a loop is not as accurate as spreading with a bent rod.

REFERENCES

1. **Bornside, G. H., and B. B. Bornside.** 1979. Comparison between moist swab and tissue biopsy methods for quantitation of bacteria in experimental incisional wounds. *J. Trauma* **19:**103–105.
2. **Bowler, P. G., B. I. Duerden, and D. G. Armstrong.** 2001. Wound microbiology and associated approaches to wound management. *Clin. Microbiol. Rev.* **14:**244–269.
3. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Edlich, R. F., G. T. Rodeheaver, T. R. Stevenson, C. M. Magee, J. G. Thaeker, and M. T. Edgerton.** 1977. Management of the contaminated wound. *Compr. Ther.* **3:**67–74.
5. **Lawrence, J. C., and H. A. Lilly.** 1972. A quantitative method for investigating the bacteriology of skin: its application to burns. *Br. J. Exp. Pathol.* **53:**550–558.
6. **Loebl, E. C., J. A. Marvin, E. L. Heek, P. W. Curreri, and C. R. Baxter.** 1974. The use of quantitative biopsy cultures in bacteriologic monitoring of burn patients. *J. Surg. Res.* **16:**1–5.
7. **Magee, C., B. Haury, G. Rodeheaver, J. Fox, M. T. Edgerton, and R. F. Edlich.** 1977. A rapid technique for quantitating wound bacterial count. *Am. J. Surg.* **133:**760–762.
8. **Rodeheaver, G. T., J. Hiebert, R. F. Edlich, and M. Spengler.** 1980. Practical bacteriologic monitoring of the burn patient. *Curr. Concepts Trauma Care* **3:**8–15.

SUPPLEMENTAL READING

Heggers, J. R., and M. C. Robson (ed.). 1991. *Quantitative Bacteriology: Its Role in the Armamentarium of the Surgeon.* CRC Press, Boca Raton, FL.

3.14 *Leptospira* Culture

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Leptospirosis is a spirochetal zoonosis caused by the genus *Leptospira.* Human-pathogenic *Leptospira* infects about 160 wild and domesticated mammalian species worldwide, which excrete the organism in their urine. The human disease is more commonly associated with occupations or recreational activities associated with direct skin or mucous membrane contact either with the animal reservoir or with water, soil, or sewage contaminated with the animal urine. The genus *Leptospira* was previously divided into two species, *Leptospira interrogans* and *Leptospira biflexa,* which were differentiated by a number of biochemical tests (9, 10, 15). Most reports of human infection are based on this phenotypic classification system, and *L. interrogans* comprised all pathogenic strains, while *L. biflexa* was thought to include only saprophytic environmental strains (15). Leptospires are also assigned a serovar based on agglutination after cross-absorption with homologous antigen (8. 10). *L. interrogans* comprises 218 serovars, while *L. biflexa* comprises more than 60 serovars (8, 10). *Leptospira* serovars that are antigenically related are further grouped into serogroups, primarily for epidemiological purposes when interpreting serologic test results to distinguish closely related species.

A genotypic classification based on DNA relatedness has more recently replaced the older phenotypic one, and 17 genomospecies are now recognized (3, 14, 15, 17; *see* Table 2 in reference 9). The genomospecies also do not correlate to the previous two species *(L. interrogans* and *L. biflexa)* (3, 14, 15, 17). In addition, both pathogenic and saprophytic commensal serovars occur within some species (3, 9, 10, 17). Moreover, the genetypic *Leptospira* classification system does not correlate with the results of strain serologic testing, so multiple species are classified within some serovars (3, 9, 10, 17). Species identification is thus limited to special reference laboratories, such as the CDC.

The disease can present as a febrile illness with or without meningitis or as Weil's syndrome, a potentially fatal illness that presents as hemorrhage, renal failure, and jaundice. Symptoms in milder disease include fever, malaise, muscle aches, and headache. Clinical recognition of leptospirosis is difficult because leptospires can affect many different organ systems, resulting in a nonspecific clinical presentation. Consequently, leptospirosis is often misdiagnosed as influenza, aseptic meningitis, encephalitis, dengue fever, hepatitis, or gastroenteritis. Effective treatment and preventive measures are available, if the disease is diagnosed in a timely manner.

Diagnosis is generally made by serologic testing (11), which is available from specialized reference laboratories, such as the Zoonoses and Select Agent Laboratory or the Bacterial Zoonoses Branch, CDC (1-800-CDC-INFO or http://www.cdc.gov). The microscopic agglutination test (MAT) is the "gold standard" serologic test to which others are compared. Because a fourfold rise in titer between acute- and convalescent-phase samples is necessary for serologic confirmation, the MAT is not useful for guiding clinical management early in the course of the patient's illness.

Several serologic tests have been evaluated by the CDC and others, including a microplate immunoglobulin M (IgM) ELISA (PanBio, Windsor, Brisbane, Australia; 61-7-3357-1177), an indirect hemagglutination assay (MRL Diagnostics, Cypress, CA), and an IgM dot ELISA dipstick test (DST; Integrated Diagnostics Inc., Baltimore, MD), as rapid alternatives to the MAT (1, 12). The CDC conclude that the ease of use and significantly high sensitivity and specificity of DST and ELISA make these good choices for diagnostic testing.

Leptospires are thin, spiral-shaped bacteria requiring special techniques for isolation. They are aerobic bacteria that can be cultivated at 30°C in media containing either rabbit serum (Fletcher's medium) or bovine serum albumin and fatty acids (leptospira or Ellinghausen-McCullough-Johnson-Harris [EMJH] medium [5, 6]). See Appendix 3.14–1 for further descriptions of media. Growth is observed in broth in a few days to 4 weeks. Culture of spinal fluid and blood during the first week of illness and of urine after the first week can be helpful in confirming the diagnosis. However, rapid methods, such as PCR, are more helpful since culture is not timely for treatment of serious infections (13).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. CSF (collect during first week of illness)
2. Urine (collect during second week of illness)
   - **a.** Inoculate into culture medium within 1 h of collection *or*
   - **b.** Dilute 1:10 in 1% bovine serum albumin and store at 5 to 20°C for longer (a few days) stability (9).
3. Dialysis fluid
4. Blood
   - **a.** Collect with sodium polyanethol sulfonate (SPS) or inoculate directly into culture medium.
   - **b.** In SPS, leptospires are stable for at least 6 days (9).
   - **c.** Protect from excessive heat or cold (9).

B. Specimen timing

1. Alert laboratory that specimen is being submitted for *Leptospira* culture, so that media can be available prior to collection and/or arrangements for PCR testing can be made.
2. Collect blood and CSF only in the first week of illness, prior to initiation of treatment.
3. Collect urine during the second week of illness or later. Organisms can be present for up to several months.
4. Culture multiple specimens taken at least 1 day apart to increase likelihood of a positive result.

C. Rejection criteria

1. Urine received greater than 1 h after collection unless it has been diluted in albumin
2. Blood or CSF from patients who have been symptomatic for greater than 10 days
3. Direct dark-field smears are not recommended since protoplasmic extrusions are too easily mistaken for spirochetes.

III. MATERIALS

A. Media

1. Leptospira EMJH base and enrichment medium
   - **a.** Purchase dehydrated powdered media from BD Diagnostic Systems (4). Follow manufacturer's instructions.
   - **b.** Other manufacturers may refer to medium as bovine serum albumin-Tween 80 medium.
   - **c.** EMJH medium can be prepared in-house; see Appendix 3.14–1 (5, 6).

   ◩ **NOTE:** Bovine serum albumin-Tween 80 medium is recommended.
2. Fletcher's medium (4) (beef extract and peptone with rabbit serum and 1.5% agar) with and without 5-fluorouracil can be purchased in prepared tubes from Remel, Inc., or BD Diagnostic Systems.
3. Dispense medium in 5-ml amounts into sterile screw-cap tubes.
4. For urine specimens, add 5-fluorouracil to the medium to inhibit contaminants (2, 7).
   - **a.** Preparation of 5-fluorouracil
     - (1) Add 1.0 g of 5-fluorouracil to 50 ml of sterile distilled water.
     - (2) Add 1 to 2 ml of 2 N NaOH, and heat gently (less than 56°C) for 1 to 2 h to dissolve.
     - (3) Adjust to pH 7.4 with 1 N HCl, and bring volume to 100 ml with sterile distilled water.
     - (4) Filter sterilize, and store at 2 to 8°C.
   - **b.** Descriptions of this reagent do not give a shelf life (7). Keep for no longer than 1 year. Write expiration date on the label.
   - **c.** Add 0.1 ml of solution described above to 5 ml of medium for a final concentration of 200 μg/ml.

III. MATERIALS *(continued)*

5. Store medium at 2 to 8°C, or add 1.5 g of agar per liter to the base to make medium semisolid. Then store at room temperature.
6. Shelf life is as long as 36 months for EMJH medium (9).

B. Other supplies

1. Fluorescent antiserum to *Leptospira* (Animal and Plant Health Inspection Service, U.S. Department of Agriculture, Ames, IA)
2. Dark-field microscope
3. Fluorescent microscope
4. Incubator at 28 to 30°C

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Organisms needed

1. *Leptospira interrogans* subsp. *canicola* (ATCC 23470)
2. *Leptospira interrogans* subsp. *grippotyphosa* (ATCC 23604)

B. Procedure

1. Inoculate *L. interrogans* subsp. *canicola* into one tube of medium and *L. interrogans* subsp. *grippotyphosa* into another tube of medium.
2. Incubate at 28 to 30°C (or at room temperature). Medium should give good growth with typical microscopic morphology by 7 days (4).
3. Check the performance of the medium every 6 months, or keep strains viable by subculturing 0.5 ml monthly to new media.

V. PROCEDURE

Observe standard precautions.

It is imperative that these cultures be handled in a biological safety cabinet.

A. Inoculation

1. Process specimen as soon as received.

   ◩ **NOTE:** Use of biosafety cabinet will avoid contamination of the culture or specimen as well as protect laboratory processing personnel.
2. Inoculate media.
   - **a.** Blood
     (1) Inoculate four tubes of bovine serum albumin-Tween 80 medium with 1 or 2 drops of fluid per tube (two tubes with 1 drop and two tubes with 2 drops).
     (2) If it is not possible to inoculate with freshly drawn blood, the blood sample can be taken with sodium oxalate or heparin as well as with SPS. Citrate may be inhibitory (16).
   - **b.** CSF or dialysis fluid
     (1) Inoculate up to 0.5 ml per 5-ml tube.
     (2) Inoculate several tubes if enough specimen is available, using various amounts of inoculum per tube.
   - **c.** Urine
     (1) Prepare 1:10 and 1:100 dilutions of urine with bovine serum albumin-Tween 80 medium. This will dilute out growth-inhibiting substances that may be present in the urine (9). It may also dilute out contaminating organisms.
     (2) For each of the following dilutions, inoculate two tubes of bovine serum albumin-Tween 80 medium.
       (a) Urine undiluted, 1 drop per tube
       (b) Urine diluted 1:10, 1 drop per tube
       (c) Urine diluted 1:100, 1 drop per tube
     (3) Duplicate these inoculations with medium containing 5-fluorouracil.
     (4) Using this protocol, inoculate a total of 12 tubes for one urine specimen.

V. PROCEDURE *(continued)*

B. Incubate the tubes in the dark at 28 to 30°C (or at room temperature).

C. Using a fluorescent microscope, directly examine specimen with specific fluorescent antiserum, if available (Animal and Plant Health Inspection Service, U.S. Department of Agriculture).

D. Examination of cultures

1. Examine all culture tubes weekly for signs of growth (turbidity, haze, or a ring of growth).

2. Examine all tubes microscopically each week.

a. Take a small drop from 1 cm below the surface, and examine it with dark-field illumination. Use ×400 magnification. Leptospires will be seen as tightly coiled spirochetes about 1 μm wide and 6 to 20 μm long. They rotate rapidly on their long axes and usually have hooked ends.

b. Positive culture tubes generally have a ring of growth (Dinger's ring) several centimeters below the surface of the semisolid medium.

3. If the specimen is positive, subculture one of the tubes to two tubes of fresh medium. Transfer about 0.5 ml taken from the area of growth.

a. Cultures can remain viable for at least 8 weeks in semisolid medium (9).

b. Submit positive cultures to a reference laboratory for confirmation and possible serologic typing.

c. Securely seal in tube of bovine serum albumin-Tween 80 medium for submission of culture.

4. Incubate cultures for 6 weeks before reporting them as negative.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report negative cultures as "No *Leptospira* isolated after 6 weeks."

B. If all tubes inoculated with a specimen become contaminated before 6 weeks, report as "Culture contaminated; no further examination possible."

C. Report positive cultures as "Leptospires isolated."

D. Document notification of physician of positive findings.

VII. INTERPRETATION

A. A positive culture indicates infection with the organism.

B. A negative result does not rule out leptospirosis.

C. Susceptibility testing is rarely performed. The treatments of choice are penicillin and doxycycline.

VIII. LIMITATIONS

A. False-negative cultures can result from contamination of the urine with other microbiota.

B. Culture is the definitive test but has a low sensitivity and may take up to 6 weeks for a positive result.

REFERENCES

1. **Bajani, M. D., D. A. Ashford, S. L. Bragg, C. W. Woods, T. Aye, R. A. Spiegel, B. D. Plikaytis, B. A. Perkins, M. Phelan, P. N. Levett, and R. S. Weyant.** 2003. Evaluation of four commercially available rapid serologic tests for diagnosis of leptospirosis. *J. Clin. Microbiol.* **41:**803–809.
2. **Blair, E. B.** 1970. Media, test procedures and chemical reagents, p. 791–857. *In* H. L. Bodily, E. L. Updyke, and J. O. Mason (ed.), *Diagnostic Procedures for Bacterial, Mycotic and Parasitic Infections*, 5th ed. American Public Health Association, New York, NY.
3. **Brenner, D. J., A. F. Kaufmann, K. R. Sulzer, A. G. Steigerwalt, F. C. Rogers, and R. S. Weyant.** 1999. Further determination of DNA relatedness between serogroups and serovars in the family *Leptospiraceae* with a proposal for *Leptospira alexanderi* sp. nov. and four new *Leptospira* genomospecies. *Int. J. Syst. Bacteriol.* **49:**839–858.
4. **Difco Laboratories.** 1984. *Difco Manual*, 10th ed., p. 513. Difco Laboratories, Detroit, MI.

REFERENCES *(continued)*

5. **Ellinghausen, H. C., Jr., and W. G. McCullough.** 1965. Nutrition of *Leptospira pomona* and growth of 13 other serotypes: fractionation of oleic albumin complex (OAC) and a medium of bovine albumin and polysorbate 80. *Am. J. Vet. Res.* **26:**45–51.
6. **Johnson, R. C., and V. G. Harris.** 1967. Differentiation of pathogenic and saprophytic leptospires. 1. Growth at low temperatures. *J. Bacteriol.* **94:**27–31.
7. **Johnson, R. C., and P. Rogers.** 1964. 5-Fluorouracil as a selective agent for growth of leptospirae. *J. Bacteriol.* **87:**422–426.
8. **Kmety, E., and H. Dickens.** 1993. *Classification of the Species* Leptospira interrogans *and History of Its Serovars.* University Press Groningen, Groningen, The Netherlands.
9. **Levett, P. N.** 2007. *Leptospira,* p. 963–970. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M.A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
10. **Levett, P. N.** 2001. Leptospirosis. *Clin. Microbiol. Rev.* **14:**296–326.
11. **Levett, P. N.** 2003. Usefulness of serologic analysis as a predictor of the infecting serovar in patients with severe leptospirosis. *Clin. Infect. Dis.* **36:**447–452.
12. **Levett, P. N., S. L. Branch, C. U. Whittington, C. N. Edwards, and H. Paxton.** 2001. Two methods for rapid serological diagnosis of acute leptospirosis. *Clin. Diagn. Lab. Immunol.* **8:**349–351.
13. **Levett, P. N., R. E. More, R. L. Galloway, D. E. Turner, A. G. Steigerwalt, and L. W. Mayer.** 2005. Detection of pathogenic leptospires by real-time quantitative PCR. *J. Med. Microbiol.* **54:**45–59.
14. **Ramadass, P., B. D. W. Jarvis, R. J. Corner, M. Cinco, and R. B. Marshall.** 1990. DNA relatedness among strains of *Leptospira biflexa. Int. J. Syst. Bacteriol.* **40:**231–235.
15. **Ramadass, P., B. D. W. Jarvis, R. J. Corner, D. Penny, and R. B. Marshall.** 1992. Genetic characterization of pathogenic *Leptospira* species by DNA hybridization. *Int. J. Syst. Bacteriol.* **42:**215–219.
16. **Wolff, J. W.** 1954. *The Laboratory Diagnosis of Leptospirosis.* Charles C Thomas, Publisher, Springfield, IL.
17. **Yasuda, P. H., A. G. Steigerwalt, K. B. Sulzer, A. E. Kaufmann, F. Rogers, and D. J. Brenner.** 1987. Deoxyribonucleic acid relatedness beween serogroups and serovars in the family *Leptospiraceae* with proposal for seven new *Leptospira* species. *Int. J. Syst. Bacteriol.* **37:**407–415.

SUPPLEMENTAL READING

Binder, W. D., and L. A. Mermel. 1998. Leptospirosis in an urban setting: case report and review of an emerging infectious disease. *J. Emerg. Med.* **16:**851–856.

Farr, R. W. 1995. Leptospirosis. *Clin. Infect. Dis.* **21:**1–6.

APPENDIX 3.14–1

Leptospira Ellinghausen-McCullough-Johnson-Harris Base and Enrichment Medium (1, 2)

A. Basal medium

1. Ingredients

Na_2HPO_4, anhydrous	1.0 g
KH_2PO_4, anhydrous	0.3 g
NaCl	1.0 g
NH_4Cl, 25% aqueous	1.0 ml
thiamine hydrochloride, 0.5% aqueous	1.0 ml
sodium pyruvate, 10% aqueous	1.0 ml
glycerol, 10% aqueous	1.0 ml
distilled water	996.0 ml

2. Procedure
   a. Dissolve salts in 996 ml of distilled water, add stock solutions (ammonium chloride, thiamine hydrochloride, sodium pyruvate, and glycerol), and adjust to pH 7.4.
   b. Autoclave at 121°C for 20 min.

APPENDIX 3.14–1 *(continued)*

B. Albumin-fatty acid supplement

1. Ingredients

bovine albumin fraction V	20.0 g
$CaCl_2 \cdot 2H_2O$, 1.0% aqueous	2.0 ml
$MgCl_2 \cdot 6H_2O$, 1.0% aqueous	2.0 ml
$ZnSO_4 \cdot 7H_2O$, 0.4% aqueous	2.0 ml
$CuSO_4 \cdot 5H_2O$, 0.3% aqueous	0.2 ml
$FeSO_4 \cdot 7H_2O$, 0.5% aqueous	20.0 ml
vitamin B_{12}, 0.2% aqueous	2.0 ml
Polysorbate (Tween) 80, 10% aqueous	25.0 ml
distilled water	to 200.0 ml

2. Procedure

◩ **NOTE:** Aqueous stock solutions must be prepared prior to formulation of medium.

a. Add the bovine albumin slowly, with careful stirring to avoid foaming, to 100 ml of water.
b. Slowly add the remaining ingredients, stirring constantly.
c. Adjust the pH to 7.4, and add water to make a 200-ml final volume.
d. Filter sterilize through a Seitz or membrane filter (0.2- to 0.3-μm porosity).

C. Prepare the complete medium by combining 1 part supplement with 9 parts basal medium.

References

1. **Ellinghausen, H. C., Jr., and W. G. McCullough.** 1965. Nutrition of *Leptospira pomona* and growth of 13 other serotypes: fractionation of oleic albumin complex (OAC) and a medium of bovine albumin and polysorbate 80. *Am. J. Vet. Res.* **26:**45–51.
2. **Johnson, R. C., and V. G. Harris.** 1967. Differentiation of pathogenic and saprophytic leptospires. 1. Growth at low temperatures. *J. Bacteriol.* **94:**27–31.

3.15 *Mycoplasma pneumoniae*, *Mycoplasma hominis*, and *Ureaplasma* Cultures from Clinical Specimens

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Mycoplasma pneumoniae is a common cause of upper and lower respiratory infections in persons of all ages. Tracheobronchitis is the most common clinical syndrome, but pneumonia commonly occurs and extragenital infections have been described. A review of human disease caused by *M. pneumoniae* (5) contains more detailed information about clinical aspects of infections due to this organism.

Specimens obtained from the upper and lower respiratory tract are appropriate to culture for the presence of *M. pneumoniae.* Since the organism has also been known to cause invasive disease in other body sites, it may also be appropriate in some circumstances to culture sterile body fluids such as CSF, synovial fluid, or other types of clinical specimen, depending on the type of infection suspected. Growth and presumptive identification of *M. pneumoniae* can be accomplished by demonstration of glucose hydrolysis in SP-4 broth and development of spherical 10- to 100-µm colonies on SP-4 agar media after 4 to 20 days or more of incubation. Definitive organism identification has been accomplished using a variety of tests such as hemadsorption, tetrazolium reduction, or growth inhibition in the presence of specific antiserum. More recently, the PCR assay has been used to distinguish *M. pneumoniae* from several species of commensal mycoplasmas that often inhabit the human respiratory tract (1, 7). Since culture may not detect *M. pneumoniae* in some specimens, alternative methods such as the PCR assay and serologic testing should be considered for optimum detection, even if culture is attempted.

Mycoplasma hominis and *Ureaplasma* species are the predominant mycoplasmal species recoverable by culture from the human urogenital tract. Even though these organisms may occur as commensals, they have been associated with a number of human urogenital infections in adults and can also cause extragenital infections involving the lungs and sterile body sites such as synovial fluid or CSF, most commonly in immunosuppressed children or adults or in preterm neonates. Detailed descriptions of specific diseases that may be caused by these organisms have been published (6, 8). *Mycoplasma genitalium* causes nongonococcal urethritis and has been associated with endometritis and cervicitis (1, 8), and *M. fermentans* is another species that may be a pathogen in some settings. These species are best detected by PCR because cultivation conditions are not well established (1–3). The Gen-Probe *M. genitalium* transmission-mediated amplification assay is currently available for research use only, but recent studies demonstrate its utility in accurately detecting this pathogen in urine samples and vaginal swabs (1). Therefore, cultivation techniques for them are not described in this handbook. The species *Ureaplasma urealyticum* (the only pathogenic *Ureaplasma* of humans) was recently divided into two separate species, *Ureaplasma urealyticum* and *Ureaplasma parvum* sp. nov. However, it is not practical or necessary to distinguish between them for diagnostic purposes.

A single liquid growth medium such as 10B broth can be used for cultivation of *M. hominis* and *Ureaplasma* species. Evidence of growth in liquid medium is based on an alkaline shift due to arginine hydrolysis by *M. hominis* or urea by *Ureaplasma* species. Definitive identification of *Ureaplasma* spp. can be established based on characteristic 15- to 60-µm brown granular colonies that develop in 1 to 3 days. Presumptive identification of *M. hominis* can be determined by typical 200- to 300-µm "fried-egg" colonies that develop in 2 to 4 days, but definitive species identification requires additional tests such as growth inhibition with homologous antiserum or the PCR assay (*see* procedure 12.2.3, part 10).

A variety of serologic assays are commercially available to test for the presence of antibodies against *M. pneumoniae.* Due to the organism's slow growth in culture and the limited availability of PCR in diagnostic laboratories, demonstration of seroconversion (fourfold change in titer) using quantitative assays for immunoglobulin M (IgM) and IgG antibodies has been the primary means for diagnosis of *M. pneumoniae* respiratory infections. Commercially available serologic tests for *M. pneumoniae* include both qualitative and quantitative assays and utilize EIA, immunofluorescence, and particle agglutination technologies. These products are discussed in greater detail elsewhere (9, 10). Evaluation of commercial diagnostic kits and media can be found in *Cumitech 34* (9).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

◩ **NOTE:** Refer to procedure 3.3.1 for additional details. The following can be copied for preparation of a specimen collection instruction manual for physicians and caregivers. It is the responsibility of the laboratory director or designee to educate physicians and caregivers on proper specimen collection.

A. Specimen collection

1. Fluid specimens (e.g., pleural fluid, bronchoalveolar lavage fluid, sputum, endotracheal aspirates, CSF, synovial fluid, amniotic fluid, pouch of Douglas fluid, semen, prostatic secretions, and urine)

◩ **NOTE:** Urethral swabs from men and vaginal or cervical swabs from women are preferred over urine specimens for detection of genital mycoplasmas.

a. Collect in a sterile container.

b. If greater than a 1-h delay in inoculation is anticipated, and/or volume of fluid obtained is very small and susceptible to drying, as in the case of neonatal endotracheal aspirates, place fluid in 0.3 to 1.5 ml of transport medium, described below and in Appendix 3.15–1.

c. Collect urine specimens from women by urethral catheterization, for more meaningful result. A first-void urine specimen should be collected from men with nongonococcal urethritis.

2. Blood

a. Collect in a vial of mycoplasma broth culture medium without anticoagulant in at least a 1:5 or 1:10 ratio.

b. Collect at least 10 ml for adults, but smaller volumes comparable to those obtained for conventional bacterial blood cultures are acceptable for neonates and children.

c. Do not use any medium containing sodium polyanethol sulfonate (SPS) anticoagulant, which is present in most commercial blood culture bottles, since it is inhibitory to mycoplasmas and ureaplasmas.

3. Tissues (e.g., lung, placenta, endometrium, fallopian tube, bone chips, and urinary calculi)

Collect in a sterile container with sufficient transport medium to prevent drying.

4. Swab specimens (e.g., nasopharynx, throat, urethra, vagina, cervix, and wound)

a. Use only Dacron or polyester swabs with aluminum or plastic shafts.

(1) Do not use cotton swabs because the fibers and wooden sticks are inhibitory to mycoplasmas.

(2) Do not use vaginal lubricants or antiseptics prior to collection of urogenital specimens, as they are inhibitory to mycoplasmas.

b. Collect throat, nasopharyngeal, vaginal, or wound specimens by swabbing back and forth over the mucosa or surface of wound to maximize recovery of cells.

◩ **NOTE:** Vaginal swabs are somewhat more likely to contain genital mycoplasmas and ureaplasmas than cervical or urethral swabs from women.

c. Collect specimens from the male or female urethra or female cervix by inserting a urethral swab at least 1 cm into the urethra and rotating it 360°.

d. After collection of swab specimen, swirl swab in a vial containing transport medium, express excess fluid by pressing the swab against the inside of the vial, and then discard swab.

B. Timing of specimen collection

1. Collect early-morning specimens of expectorated sputum.

2. Obtain all specimens prior to initiation of antimicrobial therapy when possible.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

C. Specimen transport

1. Transport specimens to the laboratory as soon as possible because of the fastidious nature of mycoplasmas and potential loss of mycoplasmal viability if specimens are allowed to dry out.

a. Add 0.3 to 1.5 ml of transport medium to original fluid specimens if more than 1 h is likely to elapse before a specimen can be transported to the laboratory. Always collect swab specimens, blood, and tissues in transport medium.

b. Refrigerate specimen in transport medium at 4°C if delay in transportation to the laboratory is anticipated, but this should be kept to a minimum.

c. Freeze the original specimen in transport medium at −70°C or in liquid nitrogen if specimen will be shipped to a reference laboratory for processing or if storage will exceed 24 h.

d. Ship frozen specimens in transport medium on dry ice to a reference laboratory for processing.

e. Do not store at −20°C for even short periods, since this will result in loss of viability.

2. Transport media

a. Broths used for mycoplasmal cultivation are most desirable for use as transport media. Refer to Appendix 3.15–1 for instructions for preparing these media.

(1) 10B or SP-4 (Remel, Inc.) for genital mycoplasmas

(2) SP-4 (Remel, Inc.) for *M. pneumoniae*

b. Other commercially available media for genital mycoplasmas

(1) Mycotrans (Irvine Scientific, Santa Ana, CA, [800] 437-5706, http://www.irvinesci.com)

(2) A3B (Remel, Inc.)

(3) Arginine broth (Remel, Inc.)

c. For transport of culture specimens and for sample preparation for PCR assays

(1) 2-SP with 10% (vol/vol) heat-inactivated fetal calf serum with 0.2 M sucrose in 0.02 M phosphate buffer (pH 7.2)

(2) TSB with 0.5% (vol/vol) bovine serum albumin

3. Limits at receiving site

(1) Process all specimens immediately after receipt in the laboratory.

(2) If this is not possible, add transport medium to the specimen, if not already present, and store as described above.

D. Request submission

In addition to the usual essential information, including identifying information for the patient and body site cultured, clearly specify which organisms are to be sought (e.g., *M. pneumoniae* or genital mycoplasmas), since cultivation procedures differ.

E. Rejection criteria

1. Perform diagnostic cultures for mycoplasmas for patients who are suspected of having a condition known to be associated with or caused by these organisms, but not in circumstances in which there has been no such association. Screening the lower urogenital tract of asymptomatic adults for genital mycoplasmas is not recommended.

2. Reject specimens received in the laboratory >24 h after collection that have not been frozen at −70°C in transport medium, because the likelihood of finding viable mycoplasmas is significantly reduced.

3. If time of specimen collection and/or storage conditions cannot be documented after consultation with the ordering physician or clinic from which the specimen was obtained, do not process the specimen.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

4. If an improperly collected, transported, or handled specimen cannot be replaced, and it is deemed critical for patient management, perform the culture and document in the final report that specimen quality may have been compromised in the event that no organisms are detected.
5. Do not process blood, tissues, or swab specimens that are not received in transport medium.

III. MATERIALS

A. Media (from Remel, Inc., or see instructions for medium preparation and formulations in Appendix 3.15–1)

1. SP-4 broth and agar—a glucose-based medium appropriate for cultivation of *M. pneumoniae* and *M. hominis*

   ◩ **NOTE:** This medium can also be used for cultivation of the more fastidious species, e.g., *M. genitalium* and *M. fermentans*, but as stated earlier, non-culture-based techniques such as the PCR assay are recommended for detection of these organisms.

   a. Add agar to SP-4 broth for preparation of solid medium.
   b. Add arginine as a metabolic substrate if *M. hominis* is being sought.
2. 10B broth—an enriched urea- and arginine-containing medium for cultivation of ureaplasmas and *M. hominis*
   a. Add clindamycin or lincomycin (10 μg/ml) to make selective for ureaplasmas.
   b. Add erythromycin (10 μg/ml) to select for *M. hominis*.
3. A8 agar—a differential agar medium useful for isolating *M. hominis* and ureaplasmas

   ◩ **NOTE:** Urea and $CaCl_2$ are provided to allow differentiation of ureaplasmas from non-urea-hydrolyzing mycoplasmas.

B. Diagnostic kits (Irvine Scientific)

1. Mycoscreen GU for genital mycoplasmas
2. Mycotrim GU triphasic flask system for genital mycoplasmas
3. Mycotrim RS for *M. pneumoniae*

◩ **NOTE:** Consult the *Manual of Commercial Methods in Clinical Microbiology* (10) and *Cumitech 34* (9) for a more comprehensive discussion of these products.

C. Stain reagents

1. Direct specimens

   ◩ **NOTE:** Mycoplasmas lack a cell wall and are not visualized by Gram stain.

   a. Giemsa stain for examination of body fluids. Results can be difficult to interpret because debris and artifacts can be confused with mycoplasmas because of their very small size.
   b. DNA fluorochrome stains, such as Hoechst 33258 (ICN Biomedicals, Inc., Costa Mesa, CA, [800] 854-0530), for clinical specimens or culture do not distinguish mycoplasmas from other bacteria. Refer to procedure 3.2.2 for acridine orange stain.
2. Stains to distinguish mycoplasmal colonies on agar from artifacts (Sigma Diagnostics, St. Louis, MO)
   a. Diene's methylene blue stain—10% in distilled water or ethanol
   b. Neutral red stain
3. Urease stain for ureaplasmas
   a. Add 0.2 ml of $CaCl_2$ solution to isolated colonies.
   b. Observe whether colonies change from colorless to dark brown within 15 min.

   ◩ **NOTE:** Incorporation of $CaCl_2$ directly into A8 agar eliminates the need for a separate test to detect urease production to identify colonies as *Ureaplasma* spp.

D. Strains

Purdue University Mollicutes Collection contains a large variety of ATCC type strains, clinical isolates, and diagnostic reagents for use in mycoplasmal culture.

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III. MATERIALS *(continued)*

E. Immunological reagents
Plain and fluorescein isothiocyanate-conjugated antisera for the identification of mollicutes can be obtained from the University of Florida Mollicutes Collection.

F. Other supplies
1. Pipetting device and sterile pipette tips
2. Sterile 2-ml screw-cap vials suitable for freezing organisms isolated in culture
3. Racks for broth tubes
4. Parafilm, American National Can
5. Candle jar or anaerobe jar with GasPak (BD Diagnostic Systems, Sparks, MD) if CO_2 incubator is unavailable
6. Fluorescent microscope for fluorescent stains

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Shelf life
1. Store broths and agars containing inhibitory antimicrobials at 4°C, where they should usually provide satisfactory growth parameters over a 4-week period.
2. Store broths in tubes that are tightly capped.
3. Store agar plates in sealed plastic bags.

◩ **NOTE:** Some commercial broth media are available in small lyophilized vials designed to be used for individual cultures that can be rehydrated as needed. This can substantially prolong shelf life.

B. Perform sterility checks on all new batches or lots of broth and agar by overnight incubation of an aliquot of broth and an agar plate at 37°C. Note and record the appearance of agar plates and verify clarity, surface, and color of media.

C. If any of the commercial media or kits are used for diagnostic purposes
1. Confirm with the manufacturer the extent of QC performed prior to shipment.
2. Perform rigorous QC on-site, since many products have not been subjected to extensive comparative studies to document their abilities to detect mycoplasmas in clinical specimens.

D. QC testing of a new batch of broth
1. Include type strains of each species to be cultivated in the broth, a low-passage clinical isolate of each species, and a minimum of two strains of *Ureaplasma* species. Recommended choices are serotypes 3 and either 5, 7, or 8 since some ureaplasmas are more fastidious than others. Some choices include the following.
   a. *Ureaplasma* serotype 3 ATCC 27815
   b. *Ureaplasma* serotype 5 ATCC 27817
   c. *Ureaplasma* serotype 7 ATCC 27819
   d. *Ureaplasma* serotype 8 ATCC 27618
   e. *M. hominis* ATCC 23114
   f. *M. pneumoniae* ATCC 15531
2. Thaw frozen stock cultures of the desired organisms, mix on a vortex mixer, and serially dilute each strain 1:10 (0.l ml of culture into 0.9 ml of broth) in the reference and the new batch out to 10^{-9}.
3. Incubate broths at 37°C under atmospheric conditions for up to 72 h for *M. hominis* and *Ureaplasma* spp. and 7 days for *M. pneumoniae.*
4. Compare the number of tube dilutions with color change (color changing units) between the reference broth and the new batch.
5. Consider new lots or batches of broth satisfactory if the numbers of all strains tested grow within 10-fold (1 dilution) of the number in the reference batch.

IV. QUALITY CONTROL *(continued)*

6. If growth in the new broth is >1 dilution less than the reference batch, repeat the test. If results are still outside the acceptable range, do not use the broth for specimens.
7. Maintain records of all media QC tests (refer to section 14 for general QC principles).

E. QC testing of a new batch of agar

1. Include the same assortment of mycoplasmas and ureaplasmas and record keeping as described above for testing broths.
2. Use one new and one reference plate for each group of three strains to be tested.
3. Prewarm agar plates bottom side up for 5 to 10 min in a 37°C incubator prior to testing.
4. Thaw organisms as described above and serially dilute (0.1 ml of culture into 0.9 ml of broth) in the appropriate broth (e.g., 10B when checking A8 agar and SP-4 when checking SP-4 agar) so that there will be approximately 30 to 300 colonies on each plate after incubation. The number of dilutions necessary to yield plates with this colony density will depend on the number of organisms in the original culture. Plating the 10^{-2}, 10^{-3}, and 10^{-4} dilutions will usually yield this number of colonies.
5. Plate 0.02 ml of the 10^{-2} to 10^{-4} dilutions of each strain onto the test agar and the reference agar, allow to dry, and incubate at 37°C under 5% CO_2 in air. Colonies of *M. hominis* and *Ureaplasma* spp. may be visible in 48 to 72 h, whereas *M. pneumoniae* will take 7 days or more.
6. Count colonies and determine the number of CFU per milliliter.
7. Calculate the percentage of growth on new medium compared to old medium by dividing the CFU per milliliter of the new test plate by that of the old test plate. Agar plates should differ by not more than 1/2 log in the number of colonies from the reference batch.
8. If >1/2-log difference occurs for any of the test strains, repeat the QC test. If a second test is still out of the acceptable range, do not use the agar for diagnostic purposes.

V. PROCEDURES

◩ **NOTE:** Refer to Fig. 3.15–1 for flowchart descriptions of culture techniques for *M. pneumoniae* and Fig. 3.15–2 for *M. hominis* and *Ureaplasma* spp.

A. Direct nucleic acid amplification by the PCR assay is important for detection of *M. pneumoniae* in clinical specimens, since culture may fail to detect the organism in some instances even when done properly.

◩ **NOTE:** Laboratories may perform in-house PCR testing for *M. pneumoniae,* and procedures using available primers have been described and are included in procedure 12.2.3, part 10. Alternatively, commercial multiplex PCR assays are available for the detection of *M. pneumoniae* and *Chlamydia pneumoniae* (ProPneumono-1; Prodesse, Inc.) for research use only. The Pneumoplex (Prodesse, Inc.) PCR assay is also available and detects the previous respiratory pathogens as well as *Legionella pneumophila*, *Legionella micdadei*, and *Bordetella pertussis* (4). Other multiplex PCR assays which detect *M. pneumoniae*, *C. pneumoniae*, and *L. pneumophila* in development include ProPneumo-2 (Prodesse, Inc.) and a BD ProbeTec System assay (BD Diagnostic Systems). Nonamplified direct antigen detection kits for mycoplasmas or ureaplasmas are no longer available commercially in the United States.

It is imperative that these cultures be handled in a biological safety cabinet.

B. Culture methods

1. Inoculation
   a. Mix fluid specimens by gently rotating the container and then centrifuge at 600 × *g* for 15 min. Use the pellet for medium inoculation.

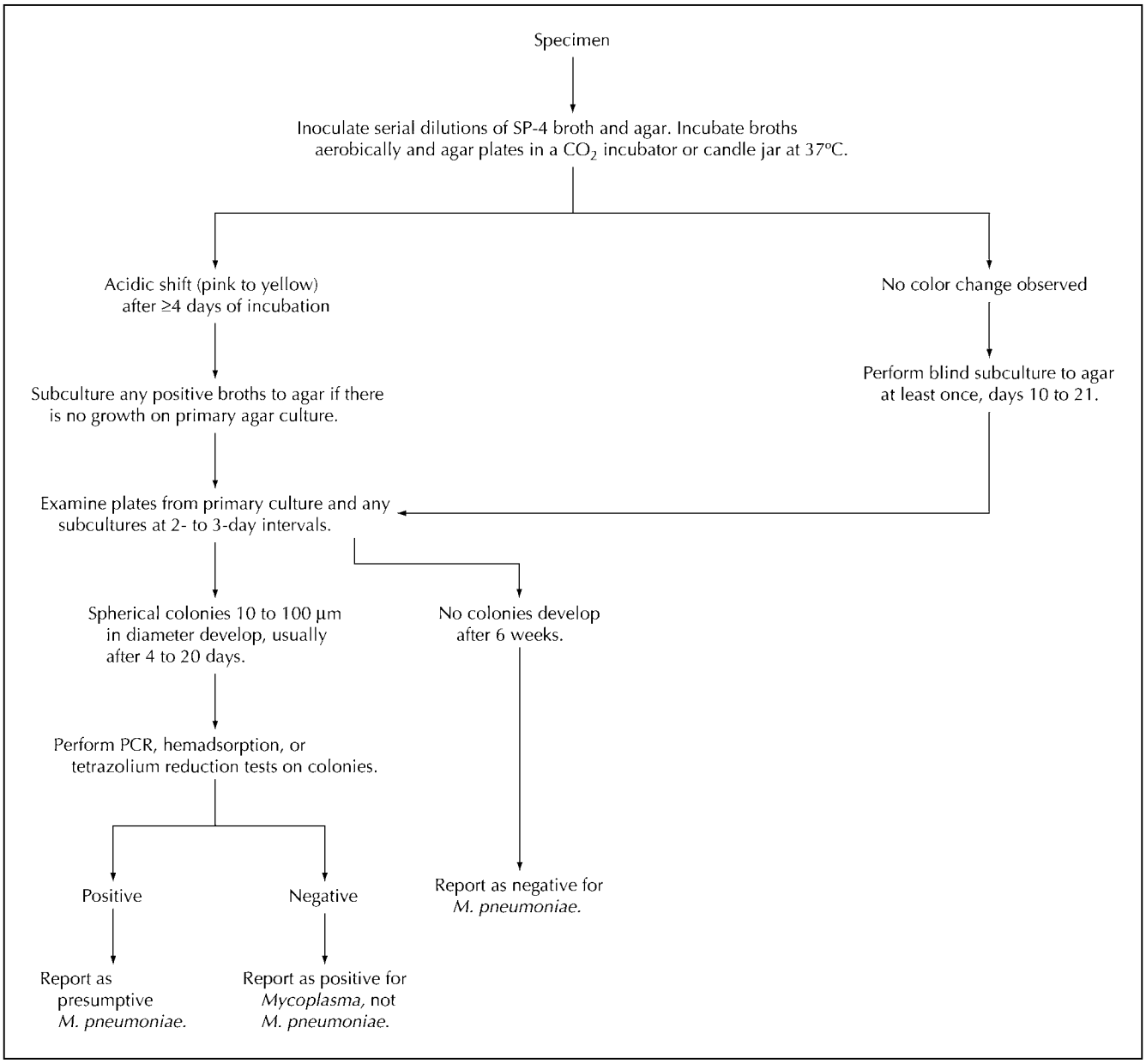

Specimen
Inoculate serial dilutions of SP-4 broth and agar. Incubate broths aerobically and agar plates in a CO_2 incubator or candle jar at 37°C.
Acidic shift (pink to yellow) after ≥4 days of incubation
No color change observed
Subculture any positive broths to agar if there is no growth on primary agar culture.
Perform blind subculture to agar at least once, days 10 to 21.
Examine plates from primary culture and any subcultures at 2- to 3-day intervals.
Spherical colonies 10 to 100 µm in diameter develop, usually after 4 to 20 days.
No colonies develop after 6 weeks.
Perform PCR, hemadsorption, or tetrazolium reduction tests on colonies.
Positive
Negative
Report as negative for *M. pneumoniae.*
Report as presumptive *M. pneumoniae.*
Report as positive for *Mycoplasma,* not *M. pneumoniae.*

Figure 3.15–1 Algorithm for isolation of *M. pneumoniae* from clinical specimens.

V. PROCEDURES *(continued)*

b. Mince tissues with a sterile scalpel blade prior to inoculation. Mincing is preferable to grinding since it minimizes release of tissue inhibitors that may affect growth.

c. Perform serial 10-fold dilutions (0.1 ml of specimen into 0.9 ml of appropriate broth) out to at least 10^{-3} in order to overcome inhibitors that may be present in original specimen.

d. Label each plate with patient's name and laboratory accession number.

e. Mark the bottom of the appropriate agar plate with a felt tip pen into at least four sections: one for the original specimen and three for the three 10-fold dilutions.

f. Inoculate agar plate with 0.02 ml of the original specimen and each dilution.

g. Seal agar plates with Parafilm to prevent desiccation.

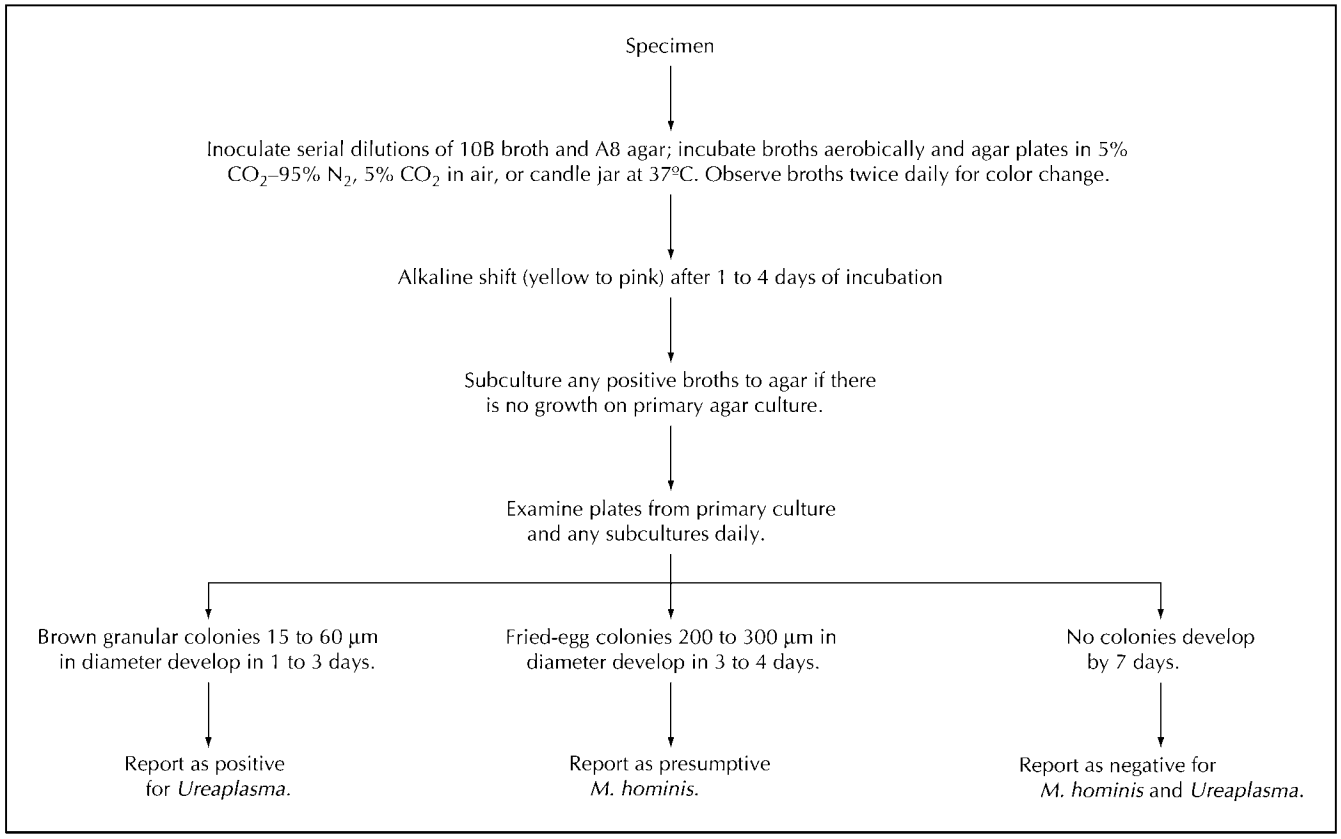

Specimen
Inoculate serial dilutions of 10B broth and A8 agar; incubate broths aerobically and agar plates in 5% CO_2–95% N_2, 5% CO_2 in air, or candle jar at 37ºC. Observe broths twice daily for color change.
Alkaline shift (yellow to pink) after 1 to 4 days of incubation
Subculture any positive broths to agar if there is no growth on primary agar culture.
Examine plates from primary culture and any subcultures daily.
Brown granular colonies 15 to 60 µm in diameter develop in 1 to 3 days.
Fried-egg colonies 200 to 300 µm in diameter develop in 3 to 4 days.
No colonies develop by 7 days.
Report as positive for *Ureaplasma*.
Report as presumptive *M. hominis*.
Report as negative for *M. hominis* and *Ureaplasma*.

Figure 3.15–2 Algorithm for isolation of *M. hominis* and *Ureaplasma* spp. from clinical specimens.

V. PROCEDURES *(continued)*

2. Incubation
- **a.** Incubate broths at 37°C under atmospheric conditions in a humidified incubator. Agitate broths on a mechanical rotator to shorten time by about 1 day for detection of *M. pneumoniae*.
- **b.** Incubate agar plates for genital mycoplasmas at 37°C in an atmosphere of 95% N_2–5% CO_2 or room air plus 5% CO_2. Incubate agar plates for *M. pneumoniae* in room air plus 5% CO_2. Use candle jars or anaerobe jars with a CO_2-generating system, if a CO_2 incubator is not available.

3. Examination of culture media
- **a.** *M. pneumoniae* cultures
  - (1) Visually inspect SP-4 broth cultures daily for acidic shift (pink to yellow).
  - (2) Subculture 0.02 ml of positive SP-4 broths to SP-4 agar if there has been no growth on primary agar culture, and incubate as described above.
  - (3) Perform blind subculture of broth to agar at least once after 10 to 21 days of incubation if no color change has occurred.
  - (4) Using a stereomicroscope, examine agar plates from primary culture and any subcultures at 2- to 3-day intervals for the presence of spherical colonies of *M. pneumoniae* that are 10 to 100 µm in diameter (Fig. 3.15–3).
  - (5) Incubate specimens for 6 weeks before discarding.
- **b.** *M. hominis* and *Ureaplasma* cultures
  - (1) Visually inspect 10B broths daily for basic shift (yellow to pink).

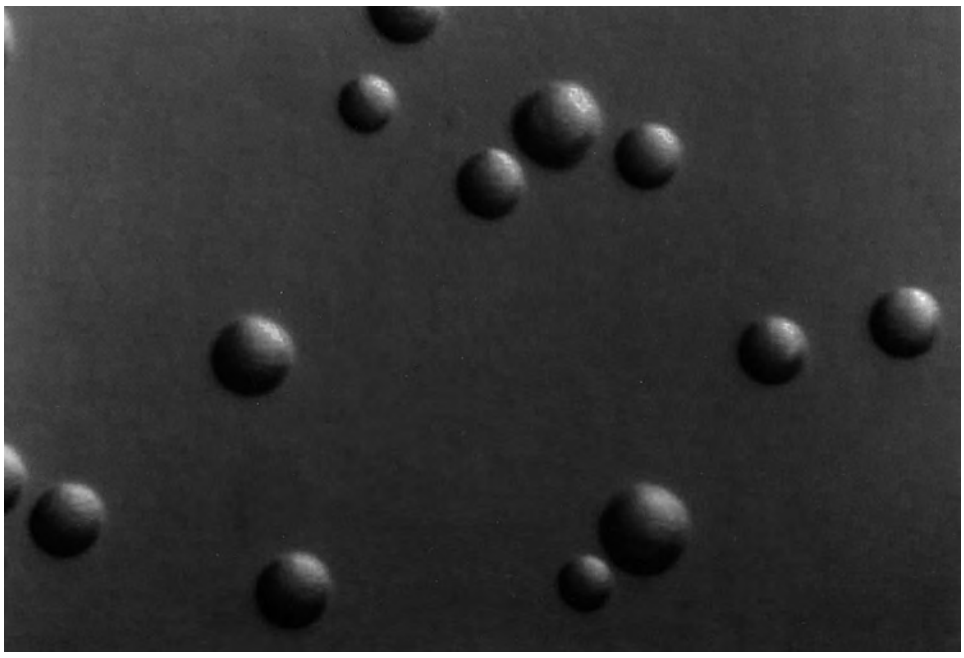


Figure 3.15–3 Spherical colonies of *M. pneumoniae* up to 100 μm in diameter growing on SP-4 agar. Magnification, ×126.

V. PROCEDURES *(continued)*

(2) Subculture 0.02 ml of positive broths to A8 agar if there is no growth on primary agar culture.
(3) Using a stereomicroscope, examine agar plates from primary culture and any subcultures daily.
(a) Brown granular colonies 15 to 60 μm in diameter characteristic of *Ureaplasma* spp. develop in 1 to 3 days (Fig. 3.15–4).
(b) Fried-egg colonies 200 to 300 μm in diameter typical of *M. hominis* develop in 2 to 4 days (Fig. 3.15–4).
(4) Incubate plates for 7 days before discarding.

4. Further workup of positive cultures
a. Enumerate each type of mycoplasma or ureaplasma present by colony counts, and record results.
b. Perform procedures as described below if definitive species identification is desired for large-colony mycoplasmas.

5. Freeze an aliquot of the original specimen at −70°C in case additional procedures are necessary.

6. Freeze any positive mycoplasma broths detected by culture at −70°C in case further workup is required or antimicrobial susceptibility tests are to be performed.

◩ **NOTE:** It is important to freeze genital mycoplasmas soon after color change occurs in broth due to a rapid death phase in liquid medium.

a. To remove mycoplasmal colonies from agar for subculture or freezing, use a sterile scalpel or Pasteur pipette to cut out a block of agar that contains the colonies.
b. Immerse block in a small vial containing approximately 1 to 1.5 ml of broth, and mix on a vortex mixer.
c. Incubate or freeze the broth, depending on the need.

7. If broth is turbid, indicating bacterial contamination, filter original liquid specimens through a 0.45-μm-pore-size filter to remove bacteria and allow the smaller mycoplasmas to pass through. Inoculate media and repeat culture.

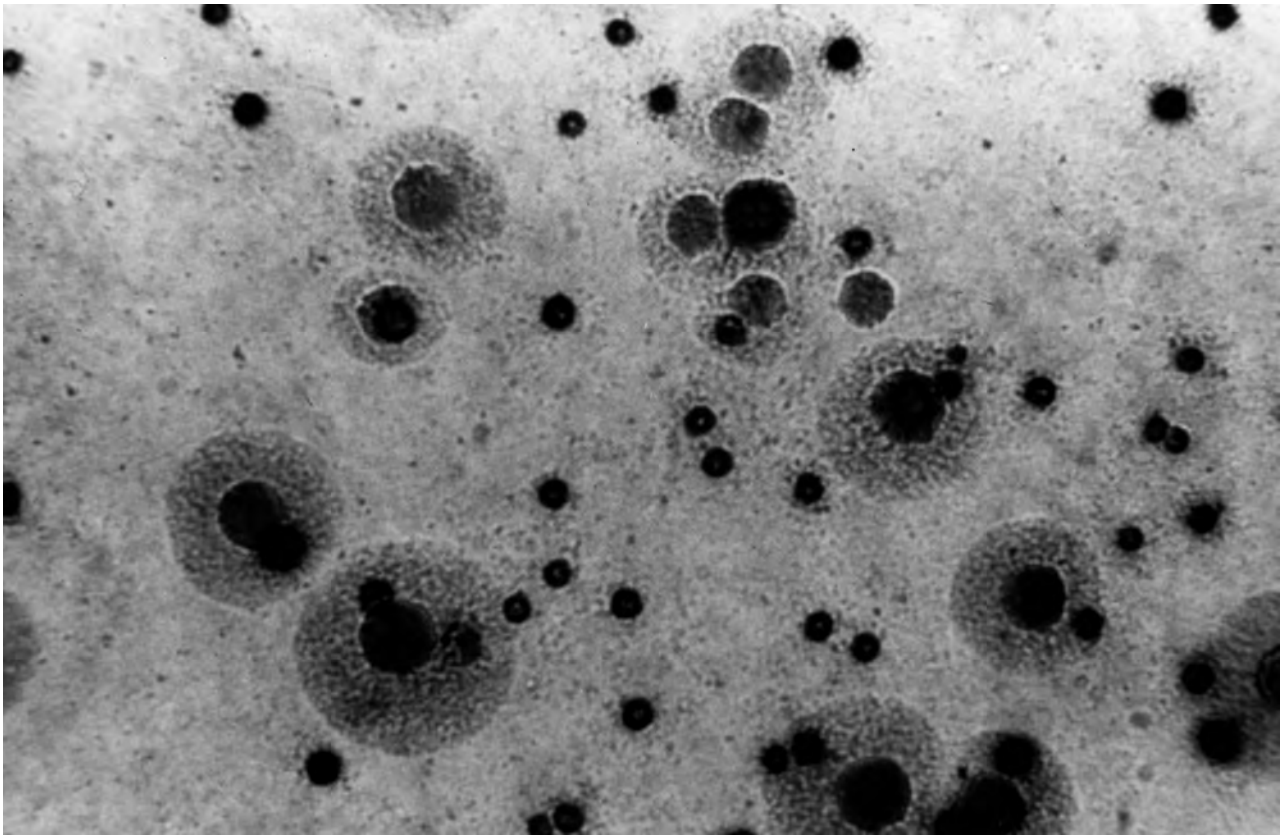


Figure 3.15–4 Small colonies typical of *Ureaplasma* spp. and larger fried-egg colonies of *M. hominis.* Diene's stain; magnification, ×100. Reprinted from **M. G. Gabridge.** 1981. *Pathogenic Mycoplasma*, part 2, no. 6702 (teaching set). American Society for Microbiology, Washington, DC.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report presumptive *M. pneumoniae* based on spherical colonies that will be evident on SP-4 agar after 4 to 20 days or more of incubation and on glucose utilization. Definitive species identification requires supplemental tests.

1. Perform additional procedures, such as tetrazolium reduction, hemadsorption, or PCR assay, for definitive identification of *M. pneumoniae* to distinguish it from commensal mycoplasmas that commonly inhabit the respiratory tract.

2. Refer isolate to a reference laboratory or see procedures for PCR assay (procedure 12.2.3, part 10) and hemadsorption activity (Appendix 3.15–2).

B. Report at the genus level for *Ureaplasma* spp. Definitive identification sufficient for clinical purposes is based on characteristic granular brown colonies on A8 agar within 1 to 3 days of incubation, due to urease production in the presence of the $CaCl_2$ indicator in A8 agar.

C. Report presumptive *M. hominis* based on fried-egg colonies on A8 agar within 2 to 4 days of incubation and on arginine utilization. Definitive identification requires additional procedures such as PCR, agar growth inhibition in the presence of homologous antisera, or colony epiimmunofluorescence. Refer to procedure 12.2.3, part 10, or submit to reference laboratory for confirmation.

VI. REPORTING RESULTS *(continued)*

D. Identification to the species level of large-colony mycoplasmas is generally not clinically indicated, unless the infection occurs in a normally sterile site such as CSF and/or the patient is immunocompromised. Submit organisms to a reference laboratory, such as the UAB Diagnostic Mycoplasma Laboratory (University of Alabama at Birmingham, Birmingham, [205] 934-9142) unless a laboratory has molecular diagnostic capabilities for performance of the PCR assay.

E. Document all testing in hard copy and computerized work card, according to the laboratory information system in use.

VII. INTERPRETATION

A. Isolation of *M. pneumoniae* from respiratory tract specimens is clinically significant in most instances, but it should be correlated with the presence of clinical respiratory disease since a small portion of asymptomatic carriers may exist. Detection of *M. pneumoniae* in normally sterile body fluids is always clinically significant.

B. Isolation of ureaplasmas in any quantity in normally sterile body fluids or tissue is significantly associated with disease. The presence of fewer than 10^4 organisms in the male urethra is less likely to be significant.

C. Isolation of *M. hominis* in any quantity in normally sterile body fluids or tissue is significantly associated with disease, but reporting the quantities of organisms present in nonsterile body sites may be of value in interpreting the significance of its presence.

D. Treatment considerations (*see* Appendix 3.15–3 for methods)

1. *M. pneumoniae:* macrolides, including erythromycin, azithromycin, and clarithromycin; tetracyclines; fluoroquinolones, including levofloxacin, moxifloxacin, and gatifloxacin

2. *M. hominis:* clindamycin; fluoroquinolones, including levofloxacin, moxifloxacin, and gatifloxacin; tetracyclines (some strains may be resistant)

3. *Ureaplasma* spp.: macrolides, including erythromycin, azithromycin, and clarithromycin; tetracyclines (some strains may be resistant); fluoroquinolones, including levofloxacin, moxifloxacin, and gatifloxacin

VIII. LIMITATIONS

A. Culture is the method of choice for detection of organisms such as *M. hominis* and *Ureaplasma* spp. that can be isolated rapidly and easily from clinical specimens, and it has the advantage of being able to provide quantitative results and an isolate for antimicrobial susceptibility testing.

B. Culture alone is not optimum for fastidious and/or slow-growing species such as *M. genitalium, M. fermentans,* and, to some degree, *M. pneumoniae.* Serology has been used for many years for detection of *M. pneumoniae* infection, and more recently PCR assays have been developed for human mycoplasmal species of clinical importance.

C. In some circumstances, particularly in systemic infections of sterile sites such as CSF or synovial fluid, for immunocompromised hosts or neonates it may be appropriate to collect a follow-up specimen after treatment has been initiated or completed to determine whether the mycoplasma is still present. This may be particularly relevant in the event that the patient has not responded to antimicrobial therapy in a satisfactory manner and because mycoplasmas tend to produce chronic infections that can be difficult to eradicate.

D. Due to the fastidious nature of mycoplasmas, it is not uncommon for batches of culture medium to fail to support the growth of some strains. If this occurs, it is necessary to systematically evaluate each individual component of the medium, if self-prepared, and to choose a different lot if medium is commercially obtained.

REFERENCES

1. **Hardick, J., J. Giles, A. Hardick, Y. H. Hsich, T. Quinn, and C. Gaydos**. Performance of the Gen-Probe transmission-mediated amplification research assay compared to that of a multitarget real-time PCR for *Mycoplasma genitalium* detection. *J. Clin. Microbiol.* **44:**1236–1240.
2. **Jensen, J. S., E. Björnelius, B. Dohn, and P. Lidbrink.** 2004. Use of TaqMan 5′ nuclease real-time PCR for quantitative detection of *Mycoplasma genitalium* DNA in males with and without urethritis who were attendees at a sexually transmitted disease clinic. *J. Clin. Microbiol.* **42:**683–692.
3. **Jurstand, M., J. S. Jensen, H. Fredlund, L. Falk, and P. Molling.** 2005. Detection of *Mycoplasma genitalium* in urogenital specimens by real-time PCR and by conventional PCR assay. *J. Med. Microbiol.* **54:**23–29.
4. **Khanna, M., J. Fan, K. Pehler-Harrington, C. Waters, P. Douglass, J. Stallock, S. Kehl, and K. J. Henrickson.** 2005. The Pneumoplex assays, a multiplex PCR-enzyme hybridization assay that allows simultaneous detection of five organisms, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Legionella pneumophila*, *Legionella micdadei*, and *Bordetella pertussis*, and its real-time counterpart. *J. Clin. Microbiol.* **43:**565–571.
5. **Talkington, D. F., K. B. Waites, S. B. Schwartz, and R. E. Besser.** 2002. Emerging from obscurity: understanding pulmonary and extrapulmonary syndromes, pathogenesis, and epidemiology of human *Mycoplasma pneumoniae* infections, p. 57–84. *In* W. M. Scheld, W. A. Craig, and J. M. Hughes (ed.), *Emerging Infections 5*. ASM Press, Washington, DC.
6. **Taylor-Robinson, D.** 1996. Infections due to species of *Mycoplasma* and *Ureaplasma:* an update. *Clin. Infect. Dis.* **23:**671–684.
7. **Templeton, K. E., S. A. Scheltinga, A. W. Graffelman, J. M. van Schie, J. W. Crielaard, P. Sillekens, P. J. van den Broek, H. Goossens, M. F. C. Beersma, and E. C. J. Claas**. 2003. Comparison and evaluation of real-time PCR, real-time nucleic acid sequence-based amplification, conventional PCR, and serology for diagnosis of *Mycoplasma pneumoniae*. *J. Clin. Microbiol.* **41:**4366–4371.
8. **Waites, K. B., and D. Taylor-Robinson.** 2007. *Mycoplasma* and *Ureaplasma*, p. 1004–1020. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
9. **Waites, K. B., C. M. Bébéar, J. A. Robertson, D. F. Talkington, and G. E. Kenny.** 2001. *Cumitech 34, Laboratory Diagnosis of Mycoplasmal Infections.* Coordinating ed., F. S. Nolte. American Society for Microbiology, Washington, DC.
10. **Waites, K. B., D. F. Talkington, and C. M. Bébéar.** 2002. Mycoplasmas, p. 201–224. *In* A. L. Truant (ed.), *Manual of Commercial Methods in Clinical Microbiology*. ASM Press, Washington, DC.

SUPPLEMENTAL READING

Baum, S. G. 2005. Introduction to mycoplasma diseases, p. 2269–2271. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), *Principles and Practice of Infectious Diseases*, 6th ed. Elsevier Churchill Livingstone, Philadelphia, PA.

Baum, S. G. 2005. *Mycoplasma pneumoniae* and atypical pneumonia, p. 2271–2279. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), *Principles and Practice of Infectious Diseases*, 6th ed. Elsevier Churchill Livingstone, Philadelphia, PA.

Kenney, G. E. 2005. Genital mycoplasmas: *Mycoplasma genitalium, Mycoplasma hominis*, and *Ureaplasm*a species, p. 2280–2283. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), *Principles and Practice of Infectious Diseases*, 6th ed. Elsevier Churchill Livingstone, Philadelphia, PA.

APPENDIX 3.15–1

Medium Formulations for Cultivation of Mycoplasmas from Humans

A. A8 agar

1. Purpose. A8 is a differential agar medium useful for isolating genital mycoplasmas. Urea is included in this medium to enhance differentiation of *Ureaplasma* spp. from non-urea-hydrolyzing mycoplasmas.
2. Ingredients
   a. Base
      (1) 825 ml of ultrapurified water (Milli-Q; Millipore, Inc.)
      (2) 0.15 g of $CaCl_2$ dihydride (dissolve before adding other ingredients)
      (3) 24 g of TSB (BD Diagnostic Systems)
      (4) 2 g of yeast extract (Difco, BD Diagnostic Systems)
      (5) 1.7 g of putrescine dihydrochloride
      (6) 0.2 g of DNA
      (7) 10.5 g of Select Agar (BD Diagnostic Systems)

APPENDIX 3.15–1 *(continued)*

b. Supplements
Filter sterilize (0.2-μm-pore-size filter) each supplement separately, if not received sterile from manufacturer.
(1) 200 ml of horse serum (HyClone, Logan, UT)
(2) 5 ml of IsoVitaleX (BD Diagnostic Systems)
(3) 10 ml of 10% urea
(4) 1 ml of GHL tripeptide solution (Calbiochem-Novabiochem International, Inc., La Jolla, CA)
(5) 5 ml of 2% L-cysteine (prepare fresh on day of use)
(6) 1,000 IU of penicillin per ml to prevent bacterial overgrowth

3. Preparation
a. Mix the base ingredients in a flask in the order specified above to prepare 1 liter.
b. Adjust pH to 5.5 with 2 N HCl.
c. Autoclave for 15 min at 121°C. Cool in a 56°C water bath before adding supplements.
d. After preparing and filter sterilizing each supplement separately, mix together and add supplements to autoclaved and cooled base agar.
e. Adjust pH to 6.0.
f. Pour plates, and after 2 h, invert and keep at room temperature overnight. (Small petri plates [60 mm] that can be used for agar require 10 ml, whereas standard 100-mm plates require 20 ml.)
g. Place plates in sealed plastic bags and refrigerate at 4°C.

4. Limitation
Plates will usually perform in a satisfactory manner for at least 4 weeks. Loss of inhibitory effect of antimicrobial agents when plates older than 4 weeks are used may result in bacterial overgrowth and inability to detect mycoplasmas when culturing specimens from nonsterile sites.

B. 10B broth

1. Purpose
10B is an enriched broth medium useful for cultivation of *Ureaplasma* spp. and *Mycoplasma hominis.*

2. Ingredients
a. Base
(1) 825 ml of ultrapurified water (Milli-Q; Millipore Inc.)
(2) 14 g of mycoplasma broth base without crystal violet (BD Diagnostic Systems)
(3) 2 g of arginine
(4) 0.2 g of DNA
(5) 1 ml of 1% phenol red (prepare fresh monthly)

b. Supplements
Filter sterilize (0.2-μm-pore-size filter) each supplement separately, if not received sterile from manufacturer.
(1) 200 ml of horse serum (HyClone) (heat-inactivated fetal bovine serum can also be used instead of horse serum)
(2) 100 ml of 25% yeast extract (Difco, BD Diagnostic Systems)
(3) 5 ml of IsoVitaleX (BD Diagnostic Systems)
(4) 4 ml of 10% urea
(5) 2.5 ml of 4% L-cysteine (prepare fresh on day of use)
(6) 1,000 IU of penicillin per ml to prevent bacterial overgrowth

3. Preparation
a. Mix the base ingredients in a flask in the order specified above to prepare 1 liter.
b. Adjust the pH to 5.5 with 2 N HCl.
c. Autoclave for 15 min at 121°C. Cool in a 56°C water bath before adding supplements.
d. After preparing and filter sterilizing each supplement separately, mix together and add supplements to autoclaved and cooled base agar.
e. Adjust the pH to 5.9 to 6.1.
f. Dispense broth aseptically into sterile tubes each containing 0.9 to 1.0 ml.

4. Limitation
10B broth will usually perform in a satisfactory manner for at least 4 weeks when stored at 4°C. Loss of inhibitory effect of antimicrobial agents when broths older than 4 weeks are used may result in bacterial overgrowth and inability to detect mycoplasmas.

APPENDIX 3.15–1 *(continued)*

C. SP-4 broth and agar
1. Purpose
   SP-4 broth is an enriched growth medium used for cultivation of many *Mycoplasma* species, including *Mycoplasma pneumoniae*. Agar may be added to SP-4 broth for preparation of solid medium and glucose and/or arginine and urea may be added as metabolic substrates, depending on which mycoplasmas are being sought.
2. Ingredients
   a. Base
      (1) 643 ml of ultrapurified water (Milli-Q; Millipore Inc.)
      (2) 3.5 g of mycoplasma broth base without crystal violet (BD Diagnostic Systems)
      (3) 10 g of tryptone (Difco; BD Diagnostic Systems)
      (4) 5.3 g of peptone (Difco; BD Diagnostic Systems)
      (5) 2 g of arginine (only if medium is to be used to isolate *M. hominis*) (Sigma)
      (6) 2 ml of 1% phenol red (prepare fresh monthly)
      (7) 0.2 g of DNA
      (8) 15 g of Noble agar (only if preparing SP-4 agar) (Difco, BD Diagnostic Systems)
   b. Supplements
      (1) 50 ml of 10× CMRL 1066 (formula no. 01-0127DJ, in 10-liter volumes; Invitrogen Life Technologies, Carlsbad, CA)
      (2) 35 ml of 25% yeast extract (Difco, BD Diagnostic Systems)
      (3) 100 ml of 2% yeastolate (Difco, BD Diagnostic Systems)
      (4) 170 ml of fetal bovine serum (HyClone) (heat inactivate at 56°C for 30 min)
      (5) 10 ml of 50% glucose
      (6) 1,000 IU of penicillin per ml to prevent bacterial overgrowth
3. Preparation
   a. Mix the base ingredients in a flask in the order specified above to prepare 1 liter.
   b. Autoclave for 15 min at 121°C and cool before adding supplements.
   c. For agar, cool in a 56°C water bath. For broth, cool to room temperature.
   d. After preparing and filter sterilizing each supplement separately, mix together and add supplements to autoclaved and cooled base.
   e. Adjust the pH to 7.4 to 7.6.
   f. If agar is added, pour plates, and after 2 h, invert and keep at room temperature overnight. (Small petri plates [60 mm] that can be used for agar require 7.5 ml, whereas standard 100-mm plates require 10 ml.) Place plates in plastic bags and refrigerate at 4°C.
   g. Dispense broth in sterile tubes. (The volume of broth required for individual cultures is dictated by the length of incubation required, e.g., 1.8 to 4.5 ml for *M. pneumoniae* and 0.9 to 1 ml for the genital mycoplasmas.)
4. Limitation
   Plates will usually perform in a satisfactory manner for at least 4 weeks. Loss of inhibitory effect of antimicrobial agents when broths or agar plates older than 4 weeks are used may result in bacterial overgrowth and inability to detect mycoplasmas when culturing specimens from nonsterile anatomic sites.

D. Contact information for manufacturers not listed in procedure 3.1
1. Calbiochem-Novabiochem International, Inc., La Jolla, CA, (800) 854-3417, http://www.calbiochem.com
2. HyClone, Logan, UT, (800) 492-5663, http://www.hyclone.com
3. Millipore, Inc., Billerica, MA, (978) 715-4321

APPENDIX 3.15–2

Hemadsorption Test for Identification of *Mycoplasma pneumoniae*

I. PRINCIPLE

This procedure involves flooding colonies on agar with a dilute suspension of washed guinea pig erythrocytes, incubating and washing them, and examining them microscopically for adherent erythrocytes. Hemadsorption is unique to *Mycoplasma pneumoniae* and *Mycoplasma genitalium* among mycoplasmas isolated from humans. Since *M. genitalium* is much more fastidious and very slow-growing and has not been reliably isolated from humans using culture methods described for *M. pneumoniae*, a positive hemadsorption test provides a strong basis for an organism's identification as *M. pneumoniae.*

II. MICROORGANISMS TESTED

Unidentified *Mycoplasma* colonies <10 days old on SP-4 agar

APPENDIX 3.15–2 *(continued)*

III. SUPPLIES
- A. Guinea pig erythrocytes (Biowhittaker, Walkersville, MD)
- B. Sterile phosphate-buffered saline (PBS) (pH 7.2)
- C. 15-ml conical centrifuge tubes
- D. Pipetting device and sterile disposable pipette tips

IV. QUALITY CONTROL
- A. Use *M. pneumoniae* ATCC 15531 (positive control) and *Mycoplasma hominis* ATCC 23114 (negative control) grown for <10 days on SP-4 agar.
- B. The *M. pneumoniae* positive control should show hemadsorption. The *M. hominis* negative control should not show any hemadsorption.

V. PROCEDURE
- A. Prepare a 10% working suspension of washed guinea pig erythrocytes by transferring 5 to 8 ml of the blood and an equal volume of sterile PBS (pH 7.2) aseptically into a 15-ml conical centrifuge tube.
- B. Centrifuge at 900 × *g* for 5 min at room temperature. Discard supernatant, and resuspend cell pellet in 10 ml of PBS by gently pipetting cells up and down several times.
- C. Centrifuge at 900 × *g* for 5 min at room temperature, and repeat washing procedure.
- D. Discard supernatant and measure packed-cell volume. Add a volume of PBS equal to nine times the packed-cell volume to yield a 10% suspension.
- E. Store the suspension at 4°C and use within 7 days. Do not use if there is evidence of hemolysis or bacterial contamination.
- F. Use this stock solution to prepare a 0.5% working solution by combining 1 part 10% suspension to 19 parts sterile PBS.
- G. Flood agar plates containing the unknown mycoplasma and positive and negative controls with 0.5% guinea pig erythrocytes.
- H. Cover plates and incubate at room temperature for 30 min.
- I. Tilt plates and aspirate the cell suspension from the agar surface.
- J. Flood plate with PBS to wash the agar surface, rocking the plate back and forth.
- K. Aspirate and discard the PBS.
- L. Observe the plate macroscopically and microscopically under a stereomicroscope for absorption of erythrocytes to the surface of the colonies.
- M. *M. pneumoniae* will hemadsorb; *M. hominis* and other large-colony mycoplasmas that are cultivable on SP-4 agar in 4 to 20 days will not do so.

APPENDIX 3.15–3 Performance of Antimicrobial Susceptibility Testing for Mycoplasmas

I. PRINCIPLE

Susceptibility testing is useful mainly for systemic infections, particularly in immunosuppressed hosts, caused by *Mycoplasma hominis* or *Ureaplasma* spp. Testing is limited to antimicrobial agents that are commonly used to treat such infections, such as the tetracyclines, to which the organism may be resistant. Broth microdilution, agar dilution, and agar gradient diffusion (Etest technique) have been described for testing of antimicrobial agents against mycoplasmas and ureaplasmas in vitro (3). Broth microdilution is the most widely used method and the one most amenable to use in diagnostic laboratories for testing multiple antimicrobials. At present, there are no Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) standardized methods or approved MIC breakpoints for testing mycoplasmas. However, the broth microdilution method described below has been endorsed by the Chemotherapy Working Group of the International Research Program for Comparative Mycoplasmology (2).

II. SUPPLIES
- A. Reference powders of antimicrobial agents of known purity
- B. Conical centrifuge tubes (10 and 50 ml)
- C. 96-well microtiter plates with covers
- D. Acetate sealers for microtiter plates
- E. Multichannel pipetting device and sterile pipette tips
- F. Plastic bags
- G. Paper towels

APPENDIX 3.15–3 *(continued)*

H. SP-4 broth for testing *Mycoplasma* spp. or 10B broth for testing *Ureaplasma* spp. without antimicrobials added (Remel, Inc., or Appendix 3.15–1)
I. SP-4 or A8 agar plates (Remel, Inc., or Appendix 3.15–1)

III. QUALITY CONTROL

A. Results are valid only if the inoculum is 10^4 to 10^5 CFU/ml based on the assay performed at the time the microtiter plate is inoculated.
B. Results are invalid if the positive control does not show color change, the drug or broth controls show color change, or there is turbidity indicating bacterial contamination.
C. Read MICs when the positive control first shows evidence of color change, because the value tends to shift over time because of nonspecific color change after prolonged incubation.
D. There are no type strains with expected MIC ranges for specific drugs that have been recommended for universal QC by the CLSI. Always include an isolate for which the MICs of the drugs being used are reproducible to ensure validity of results for the test organisms, until reliable type strains have been designated for this purpose.
E. When testing a new antimicrobial agent, test a type strain of a reference bacterium such as *Staphylococcus aureus* against the drug simultaneously in cation-adjusted Mueller-Hinton broth according to CLSI standards (1) and in 10B or SP-4 broth to determine whether there is any interaction between the enriched mycoplasmal media and the antimicrobial being tested. The MICs should agree within ± 1 two-fold dilution. Commonly tested drugs that are used for testing against mycoplasmas and ureaplasmas do not show such an interaction.

Observe standard precautions.

IV. PROCEDURE

A. Test the following drugs.
   1. A fluoroquinolone such as levofloxacin, gatifloxacin, or moxifloxacin
   2. Tetracycline or doxycycline
   3. A macrolide such as erythromycin, azithromycin, or clarithromycin (for *Mycoplasma pneumoniae* and *Ureaplasma* spp.)
   4. Clindamycin (for *M. hominis*)
   5. Optional: test chloramphenicol on isolates from CSF.
B. Preparation of stocks
   1. Weigh out an appropriate amount of powdered drug to prepare 10 ml of a stock solution containing 2,048 μg/ml.
   2. Dissolve antimicrobial agents according to manufacturer's instructions and in accordance with CLSI guidelines (1).
   3. Depending on drug stability, store most stock solutions at -70°C. Some agents work best if prepared on the day of assay.
C. Prepare a stock strain of known titer by incubating a stock culture of each strain to be tested, including controls, in the appropriate broth medium (10B for ureaplasmas and SP-4 for mycoplasmas) until color change occurs, and then freeze immediately at -70°C in ≤1-ml volumes.
   1. The approximate number of organisms in the frozen vials is determined by serially diluting a thawed vial.
      a. Place 0.1 ml of culture into 0.9 ml of appropriate broth.
      b. Make dilutions to at least 10^{-8} to achieve an endpoint titer.
      c. Incubate tubes and count the dilutions that exhibit color change, indicative of mycoplasmal growth.
   2. Thaw vials of the organisms to be tested on the day the assay is to be performed and dilute in appropriate media to yield concentrations of 10^4 to 10^5 CFU/ml, based on the original predetermined inoculum.
   3. Prepare a total of 40 ml of each isolate for testing up to six drugs.
   4. Incubate the inoculated broth at 37°C for 2 h prior to use to allow organisms to become metabolically active prior to inoculating microtiter plates.
D. Prepare two microtiter plates for every three drugs tested to allow testing a full range of drug concentrations from 0.008 to 256 μg/ml.
   1. If fewer concentrations are required, all testing for an individual drug can be performed in a single microtiter plate and the concentrations adjusted accordingly.

APPENDIX 3.15–3 *(continued)*

2. Test each drug in duplicate, skipping a row between drugs. (Drug one, rows A and B; drug two, rows D and E; drug three, rows G and H.)
3. Add 0.025 ml of appropriate medium in rows 2 to 12 of the first microtiter plate and rows 1 to 4, 6, and 10 of the second microtiter plate.
4. Add 0.175 ml of appropriate medium to well 10 (total of 0.2 ml) for the broth control and row 8 (total of 0.175 ml) for the drug control.
5. Add 0.025 ml of drug 1 to wells 1 and 2 in rows A and B in the first microtiter plate and well 8 in rows A and B of the second microtiter plate for drug control. (The other drugs to be tested will be added the same way in their respective rows.)
6. Dilute the antimicrobial agent through all wells in the first microtiter plate using a 0.025-ml multichannel pipette, beginning at the second well and continuing through wells 1 to 4 in the second microtiter plate. Discard the final 0.025 ml.
7. Add 0.175 ml of inoculated medium that has been prewarmed for 2 h to each well in rows 1 to 12 in the first microtiter plate and rows 1 to 4 and to the positive organism control (well 6) in the second microtiter plate as soon as the dilutions are completed for each drug to be tested. Start with row 6, move to row 4, and work backwards to row 1 in first microtiter plate to prevent drug carryover.
8. Repeat the above procedure for each drug and organism to be tested.

E. Perform a final determination of color changing units (CCU) by serial dilution of the cultures, as described above, at this time to check that a proper dilution was made and that the inoculum contains 10^4 to 10^5 CCU/ml.

F. Place plastic covers over plates being tested against *M. pneumoniae*, and seal them in plastic bags with a piece of paper towel moistened with water to prevent desiccation of the broth. Cover plates being tested for *Ureaplasma* spp. and *M. hominis* with adhesive-back acetate sealers before placing lid to prevent urea by-products escaping to other wells and causing color change. Incubate aerobically at 37°C.

G. Read the microtiter plates daily for color change in the organism control wells.
1. Ureaplasmas will normally grow after 12 to 18 h of incubation. A positive reaction for *Ureaplasma* in 10B broth will be evidenced by a color change from yellow to pink.
2. *M. hominis* may take 1 to 3 days. A positive reaction for *M. hominis* in SP-4 broth will be evidenced by a color change from pink to deeper red.
3. *M. pneumoniae* may take 5 to 10 days for completion of the assay. A positive reaction for *M. pneumoniae* in SP-4 broth will be evidenced by a color change from pink to yellow.

H. Record the MIC as the concentration of antimicrobial agent inhibiting color change in broth medium at the time when the organism control well first shows color change.

I. Determine minimal bactericidal concentrations (MBC), if desired, by subculturing 0.3 ml of fluid from wells of the microbroth dilution system that do not show color change into a volume of fresh broth sufficient to dilute the antimicrobial beyond the MIC. Usually a 1:100 dilution is sufficient.
1. Incubate broths and examine for color change.
2. Subculture to agar if color change occurs.
3. Subculture fluid from the growth control to make certain organisms have remained viable in the absence of antimicrobials.
4. Determine the MBC as the lowest concentration of antimicrobial at which there is no evidence of color change and no colonies formed on subculture to agar.

V. REPORTING ANTIMICROBIAL SUSCEPTIBILITY RESULTS

A. Report actual MICs since there are no breakpoints specified for mycoplasmas or ureaplasmas.

B. In addition, interpretive breakpoints developed for conventional bacteria may be reported for mycoplasmas, but *add a comment* to the report indicating that breakpoints used for interpretation of results have not been developed specifically for mycoplasmas and may not be valid.

C. Ureaplasmas must be tested at an acidic pH (6.0 to 6.5) in order for organisms to grow adequately. MICs for some macrolides such as erythromycin will be 2 to 3 dilutions higher at this pH than at neutral pH, making these organisms appear less susceptible in vitro than they may be in vivo. Include an explanatory comment with all MIC reports for macrolides when testing ureaplasmas.

APPENDIX 3.15–3 *(continued)*

D. Correlation of MICs for tetracycline with the presence or absence of the *tet*M transposon, which mediates resistance to this drug, has indicated that the MICs for *tet*M-containing strains of *M. hominis* or *Ureaplasma* spp. will be ≥8 μg/ml, whereas the MICs for strains that do not contain this resistance determinant will be ≤2 μg/ml, with no overlap between these two distinct populations.

References

1. **Clinical and Laboratory Standards Institute.** 2006. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*, 7th ed. Approved standard M7-A7. Clinical and Laboratory Standards Institute, Wayne, PA.
2. **Waites, K. B., C. M. Bébéar, J. A. Robertson, D. F. Talkington, and G. E. Kenny.** 2001. *Cumitech 34, Laboratory Diagnosis of Mycoplasmal Infections.* Coordinating ed., F. S. Nolte. American Society for Microbiology, Washington, DC.
3. **Waites, K. B., K. C. Canupp, and G. E. Kenny.** 1999. In vitro susceptibilities of *Mycoplasma hominis* to six fluoroquinolones determined by Etest. *Antimicrob. Agents Chemother.* **43:**2571–2573.

3.16 Guidelines for Biochemical Identification of Aerobic Bacteria

On the following pages are 48 biochemical procedures in alphabetical order, including both simple, rapid test procedures and standard conventional methods, for the identification of gram-positive and gram-negative bacteria. The biochemical tests have been selected that are most useful to laboratories, with emphasis on rapid testing. The list of tests includes the generally accepted tests that all laboratories should be able to perform to identify the clinically important microorganisms encountered in the laboratory, as well as some for use by reference or referral laboratories. Smaller laboratories may choose to perform fewer tests and refer cultures when less common microorganisms are found in culture. When identification to the species level is not clinically important, tests to separate these species are not included. When there is a choice of different tests that can be performed, both are presented and the user can choose which is preferable for use in the laboratory.

Rapid and overnight commercial identification systems, containing many tests, provide very accurate identifications (Tables 3.16–1 to 3.16–4) (1, 2) with little hands-on time. The tests listed here are intended to supplement those systems, not replace them. For more detailed instructions on the performance of those test systems, refer to the instruction manual received with the product. If individual tests are available on a kit system, then the separate purchase of that biochemical test may not be needed. However, commercial systems can be expensive, and rapid spot tests can often be a substitute or can be used to screen in order to suggest which commercial system might yield an identification. Serologic tests are not listed and should be a part of the identification methods used in most laboratories for various bacteria. See section 11.

As a policy, manufacturers are not listed if the product is available from most vendors. When a test has unique names depending on the manufacturer, attempts are made to list each of the product names. Product suppliers, addresses, and websites are listed in the introduction to this section, procedure 3.1. Often useful, detailed procedural information can be found on the vendor's website.

Generally, the formulas are presented, if it is likely that a laboratory might make the media or reagents in-house or the ingredients are of interest for the purchase or use of the test. Otherwise, only the key ingredients are listed for informational purposes, since good-quality commercial products are readily available and preparation of media in most laboratories is not cost-effective.

Below are a few reminders for performing the biochemical tests described in the following procedures. (i) Use only fresh cultures (18 to 24 h) as inoculum. (ii) Most reactions are aerobic. Keep caps loose, and incubate at 35°C without CO_2 unless otherwise indicated. (iii) Perform the oxidase, indole, and sometimes catalase tests on gram-negative bacteria before selecting additional tests. Perform a catalase and Gram stain on gram-positive bacteria prior to inoculation of other tests. (iv) Properly identify all material, prepared or purchased, with a legible label. Include the expiration date on the label and also log in a work record or on a receipt record. (v) Perform QC testing to ensure proper functioning of each reagent and of the test procedure with a negatively and a positively reacting microorganism prior to use of each new lot and possibly more often, depending on local regulations and on the frequency of usage. (vi) Although specific ATCC strains are listed for each procedure, any strain that will give the same result is acceptable. (vii) Record on each package, tube, or vial or in the QC records the date the reagent or test medium was initially put into use. (viii) Inoculum can be important in the use of medium and kit identifications. When appropriate, this is indicated with the appropriate McFarland standard. Refer to Appendix 3.16–1 for details on McFarland standards. (ix) Perform spot tests only from BAP or CHOC; *do not use selective media with dyes or biochemical tube agars for spot testing.*

Information presented in the following procedures was taken in part from HUGO, the product information manual from Hardy Diagnostics, with permission.

Table 3.16–1 Summary of bacterial identification systems

System	Manufacturer	Database	Storage temp (°C)	No. of tests	Length of incubation (h)	Automated
Enterobacteriaceae						
API 20E	bioMérieux	See Table 3.16–2a	2–8	21	24–48	No
BBL CRYSTAL E/NF	BD Diagnostic Systems	See Table 3.16–2a	2–25	30	18–20	Reader only
Enterotube II	BD Diagnostic Systems	*Enterobacteriaceae*	2–8	15	18–24	No
GN2 Microplate	Biolog	See Table 3.16–2b	2–8	95	4–24	Yes
Microbact 12A, 12E[a]	Oxoid	*Enterobacteriaceae* and miscellaneous gram-negative bacilli	2–8	12	18–48	No
Microbact 12B[a]	Oxoid	Gram-negative bacilli not detected by Microbact 12A	2–8	12	24–48	No
Microbact 24E[a]	Oxoid	*Enterobacteriaceae* and miscellaneous gram-negative bacilli (Table 3.16–2)[a]	2–8	24	24–48	No
Micro-ID	Remel	*Enterobacteriaceae*	2–8	15	4	No
NEG ID type 2	Dade MicroScan (Siemens)	See Table 3.16–2b	2–25	32	16–18	Yes
PASCO GN MIC/ID	BD Diagnostic Systems	Gram-negative bacteria	−70–20	27	16–20	Reader only
Phoenix NID[c]	BD Diagnostic Systems	*Enterobacteriaceae* and other gram-negative bacilli (Table 3.16–2b)	RT[b]	45	2–12	Yes
Rapid NEG ID Type 3	Dade MicroScan (Siemens)	See Table 3.16–2b	2–8	36	2.5	Yes
RapID ONE	Remel	See Table 3.16–2a	2–8	19	4	No
r/b Enteric Differential System	Remel	*Enterobacteriaceae*	2–8	15	18–24	No
Sensititre GNID	TREK Diagnostic Systems, Inc.	*Enterobacteriaceae* and nonfermenting gram-negative bacteria (Table 3.16–2b)	RT	32	5 (Aris); 18–24 offline	Yes
UID-1/UID-3	bioMérieux	Urinary tract pathogens directly from urine	2–8	9	1–13	Yes
Vitek 2 ID-GNB	bioMérieux	*Enterobacteriaceae* and nonfermenting gram-negative bacteria	2–8	41	3	Yes
Vitek GNI+	bioMérieux	See Table 3.16–2b	2–8	28	2–12	Yes
Vitek 2 GN	bioMérieux	See Table 3.16–2b	2–8	47	3–10	Yes
Nonfermenting or oxidase-positive gram-negative rods						
API 20 NE	bioMérieux	Nonfermenting gram-negative bacteria	2–8	20	24–48; aerobic	No
BBL CRYSTAL E/NF	BD Diagnostic Systems	*Enterobacteriaceae* and nonfermenting gram-negative bacteria	2–25	30	18–20	Reader only
NEG ID type 2	Dade MicroScan (Siemens)	*Enterobacteriaceae* and nonfermenting gram-negative bacteria	2–25	32	16–18	Yes
Rapid NEG ID type 3	Dade MicroScan (Siemens)	*Enterobacteriaceae* and nonfermenting gram-negative bacteria;	2–8	36	2.5	Yes
Oxi-Ferm III	BD Diagnostic Systems	Gram-negative, oxidase-positive glucose fermenters	2–8	14	48	No
Phoenix NID[c]	BD Diagnostic Systems	*Enterobacteriaceae* and other gram-negative bacilli	RT	45	2–12	Yes
RapID NF Plus	Remel	Nonfermenting and selected fermenting gram-negative bacteria	2–8	17	4	No
Sensititre GNID	TREK Diagnostic Systems, Inc.	*Enterobacteriaceae* and nonfermenting gram-negative bacteria	RT	32	5 (Aris); 18–24 offline	Yes

(continued)

Table 3.16–1 Summary of bacterial identification systems *(continued)*

System	Manufacturer	Database	Storage temp (°C)	No. of tests	Length of incubation (h)	Automated
Vitek GNI +	bioMérieux	*Enterobacteriaceae* and nonfermenting gram-negative bacteria	2–8	28	2–12	Yes
Vitek 2 GN	bioMérieux	*Enterobacteriaceae* and nonfermenting gram-negative bacteria	2–8	47	3–10	Yes
Uni-N/F Tek Plate	Remel	Gram-negative nonfermenting bacteria	2–8	13	18–48	No
Haemophilus and *Neisseria* and other fastidious gram-negative bacteria						
API NH	bioMérieux	*Haemophilus* and *Neisseria* spp. and *Moraxella catarrhalis*	2–8	12	2; aerobic	No
BBL CRYSTAL N/H	BD Diagnostic Systems	*Neisseria, Haemophilus, Moraxella,* and other fastidious bacteria	2–8	29	4	No
NHI	bioMérieux	*Neisseria, Haemophilus,* and other fastidious bacteria	2–8	15	4	No
RapID NH	Remel	*Neisseria, Haemophilus,* and other fastidious bacteria	2–8	13	4; 1 for gonococci	No
Staphylococci and related catalase-positive cocci						
API Staph	bioMérieux	Staphylococci and micrococci (Table 3.16–3)	2–8	20	4–24; aerobic	No
BBL CRYSTAL Gram-Positive	BD Diagnostic Systems	Gram-positive cocci and bacilli (Table 3.16–4)	2–8	29	18–24	Reader only
BBL CRYSTAL Rapid Gram-Positive	BD Diagnostic Systems	Gram-positive cocci and bacilli (Table 3.16–4)	2–8	29	4	No
GPI	bioMérieux	Gram-positive cocci and bacilli (Table 3.16–3)	2–8	29	2–15	Yes
Microbact Staph 12S	Oxoid	Staphylococci	2–8	12	24	No
PASCO GP MIC/ID	BD Diagnostic Systems	Gram-positive bacteria	− 70–20	27	16–20	Reader only
Phoenix PID[c]	BD Diagnostic Systems	Gram-positive bacteria (Table 3.16–4)	RT	45	2–16	Yes
Pos ID 2	Dade MicroScan (Siemens)	Gram-positive cocci and *Listeria* spp. (Table 3.16–3)	2–25	27	16–18	Yes
Rapid POS ID	Dade MicroScan (Siemens)	Gram-positive cocci and *Listeria* spp. (Table 3.16–3)	2–8	34	2.5	Yes
Sensititre GPID	TREK Diagnostic Systems, Inc.	Gram-positive bacteria (Table 3.16–4)	RT	32	24	Yes
Vitek 2 GP	bioMérieux	Gram-positive cocci and bacilli (Table 3.16–3)	2–8	43	3–8	Yes
Streptococci and related catalase-negative genera						
API 20 Strep	bioMérieux	Streptococci and enterococci (Table 3.16–3)	2–8	20	4–24; aerobic	No
BBL CRYSTAL Gram-Positive	BD Diagnostic Systems	Gram-positive cocci and bacilli (Table 3.16–4)	2–8	29	18–24	Reader only
BBL CRYSTAL Rapid Gram-Positive	BD Diagnostic Systems	Gram-positive cocci and bacilli (Table 3.16–4)	2–8	29	4	No
GPI	bioMérieux	Gram-positive cocci and bacilli (Table 3.16–3)	2–8	29	2–15	Yes
Phoenix PID[c]	BD Diagnostic Systems	Gram-positive bacteria (Table 3.16–4)	RT	45	2–16	Yes
Pos ID 2	Dade MicroScan (Siemens)	Gram-positive cocci and *Listeria* spp. (Table 3.16–3)	2–25	27	16–18	Yes

(continued)

Table 3.16–1 *(continued)*

System	Manufacturer	Database	Storage temp (°C)	No. of tests	Length of incubation (h)	Automated
Rapid POS ID	Dade MicroScan (Siemens)	Gram-positive cocci and *Listeria* spp. (Table 3.16–3)	2–8	34	2.5	Yes
RapID STR	Remel	Streptococci and related bacteria (Table 3.16–4)	2–8	14	4	No
Sensititre GPID	TREK Diagnostic Systems, Inc.	Gram-positive bacteria (Table 3.16–4)	RT	32	24	Yes
Vitek 2 GP	bioMérieux	Gram-positive cocci and bacilli (Table 3.16–3)	2–8	43	3–8	Yes
Corynebacterium and related genera						
API Coryne	bioMérieux	Corynebacteria and corynebacterium-like bacteria	2–8	20	24; aerobic	No
BBL CRYSTAL Gram-Positive	BD Diagnostic Systems	Gram-positive cocci and bacilli	2–8	29	18–24	Reader only
GP microplate	Biolog	Aerobic gram-positive bacteria	2–8	95	4–24	Yes
Phoenix PID[c]	BD Diagnostic Systems	Gram-positive bacteria	RT	45	2–16	Yes
RapID CB Plus	Remel	Coryneform bacilli	2–8	18	4	No
Sensititre GPID	TREK Diagnostic Systems, Inc.	Gram-positive bacteria	RT	32	24	Yes
Vitek 2 ANC[d]	bioMérieux	Anaerobes and *Corynebacterium* spp.	2–8	64	18–24	Yes

[a] Microbact 12A and 12B are in strip format; the 12E and 24E have microplate formats.

[b] RT, room temperature.

[c] Combination identification and susceptibility panels are also available: NMIC/ID and PMIC/ID for gram-negative and gram-positive organisms, respectively.

[d] See reference 3 for performance data for the new Vitek 2 ANC card.

Table 3.16–2a Database entries of the *Enterobacteriaceae* (human isolates) for manual systems[a]

Organism	API 20E, version 4.0	BBL CRYSTAL E/NF, version 4.0	RapID ONE, version 1.93	Microbact 24E [f]	MIDI, version 5.0
Budvicia aquatica				×	
Buttiauxella agrestis	×			×	
Cedecea davisae	×	×	×	×	
Cedecea lapagei	×	×	×	×	
Cedecea neteri		×	×	×	
Cedecea sp. 3			×	×	
Cedecea sp. 5			×	×	
Citrobacter amalonaticus	× *(Citrobacter koseri)*[b]	×	×	×	×
Citrobacter braakii	×			×	×
Citrobacter farmeri	× *(Citrobacter koseri)*			×	×
Citrobacter freundii	×	×	×	×	×
Citrobacter gillenii					×
Citrobacter koseri	× *(Citrobacter amalonaticus)*	×	×	×	×
Citrobacter murliniae					
Citrobacter sedlakii				×	
Citrobacter werkmanii				×	
Citrobacter youngae	×			×	
Edwardsiella hoshinae	×	×	×	×	
Edwardsiella ictaluri				×	
Edwardsiella tarda	×	×	×	×	×
Enterobacter aerogenes	×	×	×	×	×
Enterobacter amnigenus group 1	×		×		
Enterobacter amnigenus group 2	×				
Enterobacter asburiae	×	×	×		
Enterobacter cancerogenus	×	× *(Enterobacter taylorae)*[d]	×		×
Enterobacter cloacae	×	×	×	×	×
Enterobacter gergoviae	×	×	×	×	×
Enterobacter hormaechei					
Enterobacter intermedius	×		×		×
Enterobacter sakazakii	×	×	×	×	×
Escherichia coli	×	×[e] (O111, O157)	×	×	×
Escherichia fergusonii	×	×	×		
Escherichia hermannii	×	×	×		×
Escherichia vulneris	×	×	×	×	×
Ewingella americana	×	×	×	×	
Hafnia alvei	×	×	×	×	×
Klebsiella oxytoca	×	×	×	×	×
Klebsiella planticola			×	×	
Klebsiella pneumoniae subsp. *ozaenae*	×	×	×	×	×
Klebsiella pneumoniae subsp. *pneumoniae*	×	×	×	×	×
Klebsiella pneumoniae subsp. *rhinoscleromatis*	×	×	×	×	×
Klebsiella terrigena	×			×	×
Kluyvera ascorbata	#[c]	×	×	×	×

Kluyvera cryocrescens	#[c]	×	×	×	×
Leclercia adecarboxylota	×	×	×	×	×
Leminorella grimontii			×	×	
Leminorella richardii			×	×	
Moellerella wisconsensis	×	×	×	×	
Morganella morganii	×	×	×	×	×
Pantoea agglomerans	#[c]	×	×		×
Pantoea dispersa	#[c]				
Proteus mirabilis	×	×		×	×
Proteus penneri	×	×	×	×	×
Proteus vulgaris	×	×	×	×	×
Providencia alcalifaciens	× *(Providencia rustigianii)*	×	×	×	×
Providencia heimbachae				×	×
Providencia rettgeri	×	×	×	×	×
Providencia rustigianii	× *(Providencia alcalifaciens)*	×	×	×	×
Providencia stuartii	×	×	×	×	×
Rahnella aquatilis	×	×	×	×	×
Raoultella ornithinolytica	× *(Klebsiella)*[d]	× *(Klebsiella)*[d]	× *(Klebsiella)*[d]	× *(Klebsiella)*[d]	
Salmonella	× (six groups)	× (four groups)	× (seven groups)	× (eight groups)	× (four groups)
Serratia ficaria	×	×		×	
Serratia fonticola	×	×		×	×
Serratia liquefaciens	×	×	×	×	×
Serratia marcescens	×	×	×	×	×
Serratia odorifera group 1	×	×	×	×	×
Serratia odorifera group 2	×	×	×	×	
Serratia plymuthica	×	×	×	×	×
Serratia rubidaea	×	×	×	×	×
Shigella sp.	× *(Shigella sonnei)*	× (two groups)	#[c] *(Shigella sonnei)*	× (two groups)	× (six groups)
Taturnella ptyseos		×	×	×	
Yersinia enterocolitica	×	× (group)[g]	×	×	×
Yersinia frederiksenii	× *(Yersinia intermedia)*		×	×	×
Yersinia intermedia	× *(Yersinia frederiksenii)*		×	×	×
Yersinia kristensenii	×		×	×	×
Yersinia pestis	×			×	×
Yersinia pseudotuberculosis	×	×	×	×	×
Yersinia ruckeri				×	
Yokenella regensburgei		×	×	×	

[a] Reproduced from reference 2.

[b] Some products give a choice between two species; the alternate species is indicated in parentheses.

[c] #, genus-level identification only.

[d] Previous taxonomic designation.

[e] Able to differentiate O111 from O157.

[f] Also contains 10 unnamed enteric groups in the database.

[g] "Group" indicates *Yersinia* group.

Table 3.16–2b Database entries of the *Enterobacteriaceae* (human isolates) for automated systems[a]

Organism	Vitek GNI+, version 10.01	Vitek 2 GN, version 4.01	Dade MicroScan (Siemens)		MicroLog, version 6.01/6.02	Sensititre, version 2.6	Phoenix
			Conventional LabPro, version 1.6	Rapid LabPro, version 1.6			
Budvicia aquatica	×				×		
Buttiauxella agrestis		×			×		
Cedecea davisae	×	×	×	×	×	×	×
Cedecea lapagei	×	×	×	×	×	×	×
Cedecea neteri			×	×	×	×	×
Cedecea sp. 3			×	"3/5"			
Cedecea sp. 5			×	"3/5"			
Citrobacter amalonaticus	×	×	×	×	×	×	×
Citrobacter braakii	×	×		× *(Citrobacter braakii/ freundii/sedlakii)*	×		×
Citrobacter farmeri		×		×	×		×
Citrobacter freundii	×	×	×	× *(Citrobacter braakii/ freundii/sedlakii)*	×	×	×
Citrobacter gillenii					×		
Citrobacter koseri	×	×	×	×	×	×	×
Citrobacter murliniae					×		×
Citrobacter sedlakii		×		× *(Citrobacter braakii/ freundii/sedlakii)*	×		×
Citrobacter werkmanii				× *(Citrobacter werkmanii/ C. youngae)*	×		×
Citrobacter youngae		×		× *(Citrobacter werkmanii/ C. youngae)*	×		×
Edwardsiella hoshinae	×	×		×	×	×	×
Edwardsiella ictaluri					×	×	×
Edwardsiella tarda	×	×	×	×	×	×	×
Enterobacter aerogenes	×	×	×	×	×	×	×
Enterobacter amnigenus group 1	×	×	×	×	×	×	×
Enterobacter amnigenus group 2	×	×	×	×			×
Enterobacter asburiae		×	×	×	×	×	×
Enterobacter cancerogenus	×	×	×	×	×	×	×
Enterobacter cloacae	×	×	×	×	×	×	×
Enterobacter gergoviae	×	×	×	×	×	×	×
Enterobacter hormaechei	×			×	×		×
Enterobacter intermedius	×	×	×	×	×	×	×
Enterobacter sakazakii	×	×	×	×	×	×	×
Escherichia coli	×	× (O157)[b]	×	× (O157:H7)[b]	×	×	×
Escherichia fergusonii	×	×	×	×	×		×
Escherichia hermannii	×	×	×	×	×	×	×
Escherichia vulneris	×	×	×	×	×	×	×
Ewingella americana	×	×	×	×	×	×	×
Hafnia alvei	×	×	×	×	×	×	×
Klebsiella oxytoca	× *(Klebsiella pneumoniae)*	×	×	×	×	×	×
Klebsiella pneumoniae subsp. *ozaenae*	×	×	×	×	×	×	×
Klebsiella pneumoniae subsp. *pneumoniae*		×	×	×	×	×	×
Klebsiella pneumoniae subsp. *rhinoscleromatis*	×	×	×	×	×	×	×
Kluyvera ascorbata	#[c]	×	×	#	×	×	×

Kluyvera cryocrescens	#	×	×	#	×	×	×
Leclercia adecarboxylota	×	×	×	×	×	×	×
Leminorella grimontii			#	#	×	#	×
Leminorella richardii			#	#	×	#	×
Moellerella wisconsensis	×	×	×	×	×	×	×
Morganella morganii		×	×	×	×	×	×
Pantoea agglomerans	×	×	× *(Enterobacter agglomerans)*	× *(Enterobacter agglomerans)*	×	×	×
Pantoea dispersa					×		
Pragia frontium					×		×
Proteus mirabilis	×	×	×	×	×	×	×
Proteus penneri	× *(Proteus vulgaris)*	× *(Proteus vulgaris)*	×	×	× *(Proteus vulgaris)*	×	×
Proteus vulgaris	× *(Proteus penneri)*	× *(Proteus penneri)*	×	×	× *(Proteus penneri)*	×	×
Providencia alcalifaciens	×	×	×	×	×	×	×
Providencia heimbachae					×		
Providencia rettgeri	×	×	×	×	×	×	×
Providencia rustigianii		×	×	×	×		×
Providencia stuartii	×	×	×	×	×	×	×
Rahnella aquatilis	×	×			×		×
Raoultella ornithinolytica	×	×	×	×	× *(Raoultella planticola)*		×
Raoultella planticola	×	×	×	×	× *(Raoultella ornithinolytica)*		×
Raoultella terrigena					×	×	
Salmonella	#[a] and *Salmonella enterica* serovar Typhi	× (five groups)	× (four groups)	× (four groups)	× (twelve groups)	× (thirteen groups)	× (seven groups)
Serratia ficaria		×		×	×		×
Serratia fonticola	×	×	×	×	×	×	×
Serratia liquefaciens	×	×	×	×	× *(Serratia grimesii)*	×	×
Serratia marcescens	×	×	×	×	×	×	×
Serratia odorifera group 1	×	×	×	×	×	×	×
Serratia odorifera group 2			×	×			×
Serratia plymuthica	×	×	×	×	×	×	×
Serratia rubidaea	×	×	×	×	×	×	×
Shigella sp.	× (four groups)	× (two groups)	× *(Shigella sonnei), #*	× *(Shigella sonnei), #*	× *(four groups)*	× *(four groups)*	× *(four groups)*
Taturnella ptyseos			×	×	×	×	×
Trabulsiella guamensis					×		
Yersinia enterocolitica	×	×	× (group)[d]	× (group)	×	×	×
Yersinia frederiksenii	×		× (group)	× (group)	×	×	×
Yersinia intermedia	×		× (group)	× (group)	×	×	×
Yersinia kristensenii	×		× (group)	× (group)	×	×	×
Yersinia pestis	×	×	×	×	×	×	×
Yersinia pseudotuberculosis	×	×	×	×	×	×	×
Yersinia ruckeri		×	×	×	×		×
Yokenella regensburgei	×	×	×	×	×	×	×

[a] Reproduced from reference 2. Some products give a choice between two or more species; the alternate species is in parentheses.

[b] Includes the ability to differentiate between serogroups O111 and O157.

[c] #, genus designation only.

[d] "Group" indicates *Yersinia* group.

Table 3.16–3 Database entries of the gram-positive organisms (human isolates) for bioMérieux and Dade MicroScan products[a]

Organism	API		VITEK GPI, version 10.01	VITEK 2 GP, version 4.01	MicroScan	
	Staph, version 4.0	20 Strep, version 6.0			Conventional Pos ID, LabPro version 1.6	Rapid Pos ID, LabPro version 1.6
Listeria monocytogenes		×	#[b]	×	×	×
Micrococcaceae						
Kocuria (Micrococcus) kristinae	×			×	×	×
Micrococcus luteus	*Micrococcus* species			× *(Micrococcus lylae)*	*Micrococcus* species	*Micrococcus* species
Micrococcus lylae				× *(Micrococcus luteus)*	*Micrococcus* species	*Micrococcus* species
Kocuria (Micrococcus) rosea	× *(Micrococcus varians)*			×	*Micrococcus* species	×
Kocuria varians	× *(Micrococcus rosea)*			×	*Micrococcus* species	*Micrococcus* species
Micrococcus sedentarius					*Micrococcus* species	*Micrococcus* species
Rothia dentocariosa						
Rothia mucilaginosa				×		
Staphylococcus arlettae				×		×
Staphylococcus aureus	×		×	×	×	×
Staphylococcus auricularis	×		×	×	×	×
Staphylococcus capitis subsp. *capitis*	×		×	×	×	×
Staphylococcus capitis subsp. *ureolyticus*					×	
Staphylococcus caprae	×			×		×
Staphylococcus carnosus	×			×		×
Staphylococcus caseolyticus						
Staphylococcus chromogenes	×					× *(Staphylococcus hyicus)*
Staphylococcus cohnii subsp. *cohnii*	×		×	×	×	×
Staphylococcus cohnii subsp. *urealyticum*	×			×	×	
Staphylococcus epidermidis	×		×	×	×	×
Staphylococcus equorum				×		×
Staphylococcus felis						
Staphylococcus gallinarum				×		×
Staphylococcus haemolyticus	×		×	×	×	×
Staphylococcus hominis subsp. *hominis*	×		×	×	×	×
Staphylococcus hominis subsp. *novobiosepticus*					×	
Staphylococcus hyicus	×		×	×	×	× *(Staphylococcus chromogenes)*
Staphylococcus intermedius			×	×	×	×
Staphylococcus kloosii				×		×
Staphylococcus lentus	×		×	×		×
Staphylococcus lugdunensis	×			×	×	×
Staphylococcus pasteuri						
Staphylococcus saccharolyticus						
Staphylococcus saprophyticus	×		×	×	×	×
Staphylococcus schleiferi	×			×	×	×
Staphylococcus sciuri	×		×	×	×	×
Staphylococcus simulans	×		×	×	×	×

Staphylococcus vitulinus				×		
Staphylococcus warneri	×		×	×	×	×
Staphylococcus xylosus	×		×	×	×	×
Streptococcaceae						
Abiotrophia adiacens		×				
Abiotrophis defectiva		×	×	×		
Aerococcus urinae			×	×		
Aerococcus viridans		×	×	×	×	×
Alloiococcus otitidis			×	×		
Dermacoccus nishinomiyaensis			×	×		
Enterococcus avium		×	×	×	×	×
Enterococcus casseliflavus			×	×	×	×
Enterococcus durans		×	×	×	× *(Enterococcus hirae)*	×
Enterococcus faecalis		×	×	×	×	×
Enterococcus faecium		×	×	×	×	×
Enterococcus gallinarum		×	×	×	×	
Enterococcus hirae				×	× *(Enterococcus durans)*	
Enterococcus malodoratus						
Enterococcus raffinosus					×	
Facklamia hominis						
Gemella haemolysans		×		×		
Gemella morbillorum		×	×	×		×
Globicatella sanguinis				×		
Granulicatella sp.						
Helcoccus kunzii				×		
Lactococcus species		×		×		
Leuconostoc species		×		×	×	
Pediococcus species				×	×	
Streptococcus acidominimus		×	×			
Streptococcus agalactiae		×	×	×	×	×
Streptococcus anginosus			×	×		
Streptococcus β-hemolytic non-A, non-B					×	
Streptococcus bovis		×	×		×	×
Streptococcus constellatus			×	×		
Streptococcus criceti						
Streptococcus cremoris/ thermophilus						
Streptococcus cristatus			×			
Streptococcus dysgalactiae/ subsp. *dysgalactiae*/subsp. *equisimilis equisimilis*		×	×	×		
Streptococcus equi subsp. *equi*		×	×	×		× *(Streptococcus equisimilis)*
Streptococcus equi subsp. *zooepidemicus*						×
Streptococcus equinus		×	×	×		×
Streptococcus gordonii			× *(Streptococcus sanguinis)*			

(continued)

Table 3.16–3 Database entries of the gram-positive organisms (human isolates) for bioMérieux and Dade MicroScan products[a] *(continued)*

Organism	API		VITEK GPI, version 10.01	VITEK 2 GP, version 4.01	MicroScan	
	Staph, version 4.0	20 Strep, version 6.0			Conventional Pos ID, LabPro version 1.6	Rapid Pos ID, LabPro version 1.6
Streptococcus groups E, G, L, P, and U			× Group G			
Streptococcus intermedius		×	×	×		
Streptococcus lactis/diacetylacis						
Streptococcus milleri group			×		×	×
Streptococcus mitis group		×	×	×	×	×
Streptococcus mutans		×	×	×	×	×
Streptococcus oralis		×	×	× *(Streptococcus mitis)*		
Streptococcus parasanguinis				×		
Streptococcus pneumoniae		×	×	×	×	×
Streptococcus porcinus		×		×		
Streptococcus pyogenes		×	×	×	×	×
Streptococcus salivarius		×	×	×	×	×
Streptococcus sanguinis			×	×		
Streptococcus sanguis		×	×		×	×
Streptococcus sobrinus				×		
Streptococcus uberis		×	×	×		
Streptococcus vestibularis				×		
Vagococcus fluvialis				×		
Weissella confusa						

[a] Reproduced from reference 2. Some products give a choice between two species; the alternate species is in parentheses.

[b] #, genus identification only.

Table 3.16–4 Database entries of the gram-positive organisms for BD Diagnostics, Remel, TREK, Biolog, and MIDI products[a]

Organism	BBL CRYSTAL		Remel RapID STR	Oxoid Microbact Staph 12S	TREK Sensititre GPID	Biolog GP2 version 6.11/6.12	MIDI version 5.0	BD Diagnostics Phoenix-100 GPID PMIc/II-100
	Gram-Pos ID	Rapid Gram-Pos ID						
Listeria species	× (four species)	× (three species)	× *(Listeria monocytogenes)*	*[b]	× (four species)	× (seven species)	× (five species)	× (five species)
Micrococcaceae								
Kocuria (Micrococcus) kristinae	#[c]	× *(Micrococcus)*			×	×	×	×
Kocuria (Micrococcus) rosea	#	× *(Micrococcus)*			×	×	×	×
Kocuria varians						×	×	×
Kytococcus sedentarius	#				×	×		×
Macrococcus (Staphylococcus) caseolyticus						×	×	×
Micrococcus luteus	#	×			×	×	×	×
Micrococcus lylae	#				×	×	×	×
Rothia dentocariosa	×					×	×	
Rothia mucilaginosus	× *(Stomatococcus)*	× *(Stomatococcus)*				×	×	×
Staphylococcus arlettae						×	×	
Staphylococcus aureus	×	×		×	×	×	×	×
Staphylococcus auricularis	×			×		×	×	×
Staphylococcus capitis subsp. *capitis*	×			×	×	×	×	×
Staphylococcus capitis subsp. *ureolyticus*	×			×				×
Staphylococcus caprae	×			×		×		×
Staphylococcus carnosus	×			×		×	×	×
Staphylococcus chromogenes				×		×	×	×
Staphylococcus cohnii subsp. *cohnii*	×			×		×	×	×
Staphylococcus cohnii subsp. *urealyticum*	×			×				×
Staphylococcus epidermidis	×	×		×	×	×	×	×
Staphylococcus equorum	×					×	×	×
Staphylococcus felis	×					×		×
Staphylococcus gallinarum	×	×				×	×	×
Staphylococcus haemolyticus	×	×		×	×	×	×	×
Staphylococcus hominis subsp. *hominis*	×	×		×	×	×	×	×
Staphylococcus hominis subsp. *novobiosepticus*						×		×
Staphylococcus hyicus				×	×	×	×	×
Staphylococcus intermedius	×	×		×	×	×	×	×
Staphylococcus kloosii	×					×	×	×
Staphylococcus lentus	×	×		×		×	×	×

(continued)

Table 3.16–4 Database entries of the gram-positive organisms for BD Diagnostics, Remel, TREK, Biolog, and MIDI products[a] *(continued)*

Organism	BBL CRYSTAL		Remel RapID STR	Oxoid Microbact Staph 12S	TREK Sensititre GPID	Biolog GP2 version 6.11/6.12	MIDI version 5.0	BD Diagnostics Phoenix-100 GPID PMIc/II-100
	Gram-Pos ID	Rapid Gram-Pos ID						
Staphylococcus lugdunensis	×	×		×		×	×	×
Staphylococcus pasteuri	×					×		×
Staphylococcus saccharolyticus	×	×						×
Staphylococcus saprophyticus	×	×		×	×	×	×	×
Staphylococcus schleiferi	× (two subspecies)					×	×	× (two subspecies)
Staphylococcus sciuri	×	×		×		×	×	×
Staphylococcus simulans	×	×		×		×	×	×
Staphylococcus vitulinus	×							×
Staphylococcus warneri	×	×		×	×	×	×	×
Staphylococcus xylosus	×	×		×	×	×	×	×
Streptococcaceae								
Abiotrophia species								
Aerococcus urinae	×					×		×
Aerococcus viridans	×	×	#		×	×	×	×
Alloiococcus otitidis	×					×		×
Dermacoccus nishinomiyaensis						×		×
Enterococcus avium	×	×	×		×	×	×	×
Enterococcus casseliflavus	× *(Enterococcus gallinarum)*	× *(Enterococcus gallinarum)*	× *(Enterococcus mundtii)*			×	×	×
Enterococcus cecorum						×	×	
Enterococcus columbae						×	×	
Enterococcus durans	×	×	× *(Enterococcus hirae)*		×	×	×	×
Enterococcus faecalis	×	×	×		×	×	×	×
Enterococcus faecium	×	×	×		×	×	×	×
Enterococcus gallinarum	× *(Enterococcus casseliflavus)*	× *(Enterococcus casseliflavus)*	×			×	×	×
Enterococcus hirae	×		× *(Enterococcus durans)*			×	×	×
Enterococcus malodoratus			×			×	×	
Enterococcus mundtii						×	×	
Enterococcus raffinosus	×	×	×			×		×
Enterococcus solitarius	×					×		
Gemella haemolysans	×	×			×	× *(Gemella morbillorum)*	×	×
Gemella morbillorum	×	×	×			× *(Gemella haemolysans)*		×
Globicatella sanguinis	×					×		×
Helcoccus kunzii	×					×		×
Lactococcus species	× (five species)	× (four species)				× (seven species)		× (six species)
Leuconostoc species	× (four species)	× (three species)	× (three species)			× (eight species)		× (five species)
Pediococcus species	× (three species)	× (three species)	× (two species)			×(five species)	× (one species)	× (five species)

Streptococcus acidominimus	×	×	×		×		×
Streptococcus agalactiae	×	×	×	×	×	×	×
Streptococcus anginosus	×	×	×	×	×	×	×
Streptococcus bovis	× (bovis I and II)	× (bovis I and II)	×	×	×	×	
Streptococcus constellatus	×	×	×	×	×		×
Streptococcus cricetus	×	×			×		
Streptococcus cristatus	×				×		×
Streptococcus crista	× (included in *Streptococcus sanguinis* group)	×					×
Streptococcus dysgalactiae/ subsp. *dysgalactiae*/subsp. *equisimilis*				×	×		×
Streptococcus equi subsp. *equi*/ subsp. *zooepidermicus*	×	×	Group C streptococcus	×	×	×	×
Streptococcus equinus	×	×	×		×	×	×
Streptococcus gordonii	×	×	× *(Streptococcus sanguinis)*		×	×	×
Streptococcus intermedius	×	×	×		×	×	×
Streptococcus milleri group	×	×		×			
Streptococcus mitis	×	×	×	×	×	×	×
Streptococcus mutans	×	×	×	×	× *(Streptococcus ratti)*	×	×
Streptococcus oralis	×	×			×	×	×
Streptococcus parasanguinis	×				×	×	×
Streptococcus pneumoniae	×	×		×	×	×	×
Streptococcus porcinus	×				×	×	×
Streptococcus pyogenes	×	×	×	×	×	×	×
Streptococcus salivarius	×	×	× *Streptococcus vestibularis)*	×	×	×	×
Streptococcus sanguinis					×		×
Streptococcus sanguinis	×	×	× *(Streptococcus gordonii)*	×		×	
Streptococcus sobrinus	×	×			×	×	×
Streptococcus uberis	×	×		×	×	×	×
Streptococcus vestibularis	×	×	× *(Streptococcus salivarius)*		×	×	×
Weisella confusa			×				

[a] Reproduced from reference 2. Some products give a choice between two species; the alternate species is indicated in parentheses.

[b] Oxoid has a similar product for *Listeria* identification called the Listeria Identification System 12 S. It identifies six species.

[c] #, genus-level identification only.

REFERENCES

1. **Carroll, K. C., and M. P. Weinstein.** 2007. Manual and automated systems for detection and identification of microorganisms, p. 192–211. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
2. **Funke, G., and K. A. Bernard.** 2003. Coryneform gram-positive rods, p. 480. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, DC.
3. **Rennie, R. P., C. Brosnikoff, L. Turnbull, L. B. Reller, S. Mirrett, W. Janda, K. Ristow, and A. Krilcich.** 2008. Multicenter evaluation of the Vitek 2 anaerobe and *Corynebacterium* identification card. *J. Clin. Microbiol.* **46:**2646–2651.

APPENDIX 3.16–1

Preparation of McFarland Turbidity Standards

I. PRINCIPLE

A chemically induced precipitation reaction between barium chloride and sulfuric acid to form barium sulfate ($BaSO_4$) can be used to approximate the turbidity of a bacterial suspension (3). Alternatively, a latex particle suspension can be purchased as an optical equivalent to the chemical standard (Remel, Inc.; Hardy Diagnostics), or a nephelometer can be used directly on the bacterial suspension. Varying $BaSO_4$ turbidities are referred to as McFarland turbidity standards and range between 0.5 and 10 (3).

II. REAGENTS AND SUPPLIES

A. The following reagents are prepared in sterile distilled water (2).
   1. Solution A: 0.048 mol of $BaCl_2$ per liter (1.175% [wt/vol] $BaCl_2 \cdot 2H_2O$)
   2. Solution B: 0.18 mol of H_2SO_4, chemically pure grade, per liter (1% [vol/vol])

B. Supplies
   1. Nine thoroughly cleaned test tubes that hold 10 ml of reagent
   2. Test tubes with screw caps that have the same diameter as broth or saline tubes that are used to prepare bacterial suspensions
   3. Parafilm, liquid paraffin, or airtight tape, such as duct tape.
   4. White card with contrasting black lines (available from Remel, Inc., and Hardy Diagnostics).

III. QUALITY CONTROL

As a control of the ability to prepare appropriate turbidity standards, a colony count of the bacterial suspension can be determined. Generally the suspension will have to be diluted 1:100 or 1:10 several times in broth and vortexed until the expected concentration of the suspension is 10^5/ml (*see* chart below for corresponding cell density). Such a suspension will yield 100 to 300 colonies on a plate when inoculated with a 0.001-ml loop. Inoculate a second plate with a 0.01-ml loop in case there is no growth on the first plate. Spread with a bent rod for greatest accuracy.

APPENDIX 3.16–1 *(continued)*

Include QC information on reagent container and in QC records.

IV. PROCEDURE

A. Procedure for preparation of standards (1)

1. Label each test tube from 0.5 to 8.
2. Dispense the appropriate aliquot of solution A *into* the appropriate aliquot of solution B with constant agitation to maintain a suspension, using the chart below.

Barium sulfate turbidity standard	Vol (ml) of:		Turbidity equivalent to cell density of *Escherichia coli* broth culture (10^8/ml)
	Solution A, 0.048 mol of $BaCl_2$/liter	Solution B, 0.18 mol of H_2SO_4/liter	
0.5	0.05	9.95	1.5
1	0.1	9.9	3
2	0.2	9.8	6
3	0.3	9.7	9
4	0.4	9.6	12
5	0.5	9.5	15
6	0.6	9.4	18
7	0.7	9.3	21
8	0.8	9.2	24

3. Verify the correct density of the 0.5 turbidity standard by using a spectrophotometer with a 1-cm light path and matched cuvette to determine the absorbance. The absorbance at 625 nm should be 0.08 to 0.10 for the 0.5 McFarland standard (2).
4. Transfer the barium sulfate suspensions in aliquots into screw-cap tubes of the same size as those used in growing or diluting the bacterial inoculum. Label each tube with the appropriate turbidity equivalent.
5. Tighten tubes and seal with airtight tape, melted paraffin, or Parafilm, and store in the dark at room temperature.
6. Vigorously agitate the barium sulfate turbidity standard on a mechanical vortex mixer before each use, and inspect for a uniformly turbid appearance. If large particles appear, the standard should be replaced. Latex particle suspensions should be mixed by inverting gently, not on a vortex mixer.
7. Verify the density of the 0.5 standard monthly and replace the standards if the density falls outside of the acceptable limits (2).

B. Procedure for use of standards

1. Vigorously agitate the chosen standard on a mechanical vortex mixer before use.
2. Obtain a screw-cap tube with the same diameter as the standard containing either broth, saline, or sterile water, as appropriate for the use of the bacterial suspension.
3. While working in a biological safety cabinet prepare a suspension of the bacteria matching the turbidity of the standard as closely as possible. Recap tube. Vortex suspension.
4. Using adequate light, hold the standard next to the culture tube and display them both against a white card with contrasting black lines.
5. Add culture (if suspension is too light) or additional broth (if suspension is too heavy) to achieve a turbidity optically comparable to the standard.

It is imperative that these cultures be handled in a biological safety cabinet.

References

1. **Baron, E. J., L. Peterson, and S. M. Finegold.** 1994. *Bailey and Scott's Diagnostic Microbiology*, 9th ed., p. 170–171. Mosby, St. Louis, MO.
2. **Clinical and Laboratory Standards Institute.** 2006. *Performance Standards for Antimicrobial Disk Susceptibility Tests*, 9th ed. Approved standard M2-A9. Clinical and Laboratory Standards Institute, Wayne, PA.
3. **McFarland, J.** 1907. Nephelometer: an instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. *JAMA* **49:**1176–1178.

3.17.1 Acetamide Utilization Test

I. PRINCIPLE

Acetamide agar is used to test an organism's ability to utilize acetamide by deamidation. The medium contains acetamide as the sole carbon source and inorganic ammonium salts as the sole source of nitrogen. Growth is indicative of a positive test for acetamide utilization. When the bacterium metabolizes acetamide by the enzymatic action of an acylamidase, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns the bromthymol blue indicator in the medium from green to blue, indicative of a positive test. Assimilation of acetamide will result in a yellow color and should not by mistaken for a positive result (2). In general, deamidation is limited to only a few organisms. This medium is recommended for differentiating *Pseudomonas aeruginosa* from other non-glucose-fermenting, gram-negative rods.

II. MICROORGANISMS TESTED

A. Isolated colonies of non-glucose-fermenting, gram-negative rods that are suggestive of *P. aeruginosa* and produce a fluorescent pigment

B. Unusual non-glucose-fermenting, gram-negative rods, as part of the identification

III. MEDIA, REAGENTS, AND SUPPLIES

A. Medium

1. Agar slant containing the following per liter

sodium chloride	5.0 g
magnesium sulfate	0.2 g
ammonium phosphate, monobasic	1.0 g
potassium phosphate, dibasic	1.0 g
acetamide	10.0 g
agar	15.0 g
bromthymol blue	0.08 g

2. The final pH is 6.8.

3. Store at 2 to 8°C.

◩ **NOTE:** Tablets (Key Scientific) are also available. Tablet is dissolved in 1 ml of distilled water and boiled to destroy any vegetative bacteria present. It is then heavily inoculated and incubated at 35°C for up to 6 days. A color change from yellow to purple is a positive reaction.

B. Supplies/equipment

1. Sterile inoculating loops or sticks

2. Incubator at 35°C

IV. QUALITY CONTROL

A. Perform QC on each new lot or shipment of media prior to putting it into use.

B. Inspect agar for evidence of prior freezing, contamination, cracks, dehydration, and bubbles prior to storage and before use. Discard any tubes that are blue.

C. Organisms

1. *P. aeruginosa* ATCC 27853—acetamide positive (growth; blue color)

2. *Escherichia coli* ATCC 25922—acetamide negative (no growth; green color)

V. PROCEDURE

A. Streak the slant back and forth with inoculum picked from the center of a well-isolated colony.
B. *Place cap loosely on tube.*
C. Incubate aerobically at 35 to 37°C for up to 4 days.
D. Observe a color change from green to blue along the slant.

VI. INTERPRETATION

A. A positive test is growth and change from green to intense blue color along the slant.
B. A negative test is no growth, no color change, and slant remains green.

VII. REPORTING RESULTS

A. Acetamide deamination is most actively accomplished by *Delftia* (*Comamonas*) *acidovorans, P. aeruginosa,* and *Alcaligenes faecalis* (3).
B. Of the fluorescent pseudomonads, *P. aeruginosa* is positive for acetamide. Unless the isolate is from a normally sterile site, the combination of fluorescent pigment, acetamide deamination, and oxidase positivity is sufficient to identify *P. aeruginosa* (1, 3).

VIII. LIMITATIONS

A. Growth on the slant without an accompanying color change may indicate a positive test. However, if the agar does not turn blue with further incubation, the test should be repeated with less inoculum.
B. A negative test does not rule out an identification of *P. aeruginosa,* and 6% of *Pseudomonas fluorescens* organisms have been reported to give a positive reaction (1, 3). Other fluorescent *Pseudomonas* spp. do not give positive reactions.
C. Other tests should be performed, such as growth at 42°C, to separate *P. aeruginosa* from the other fluorescent pseudomonads if the isolate is from a blood culture, since the patient could have received blood products contaminated with *P. fluorescens,* which can grow at refrigeration temperatures (1).
D. Tests with equivocal results should be repeated.
E. Do not stab the slant, since the test requires an aerobic environment.
F. Do not inoculate from broth cultures, due to carryover of media.
G. To avoid false-positive reactions, use a light inoculum to prevent carryover of substances from previous media.

REFERENCES

1. **Blondel-Hill, E., D. H. Henry, and D. P. Speert.** 2007. *Pseudomonas,* p. 734–748. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M.A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
2. **MacFaddin, J. F.** 1985. *Biochemical Tests for Identification of Medical Bacteria,* p. 15–17. Williams and Wilkins, Baltimore, MD.
3. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria,* 2nd ed., p. 5. Williams & Wilkins, Baltimore, MD.

3.17.2 Acetate Utilization Test

I. PRINCIPLE

Acetate agar is used to test an organism's ability to utilize acetate. The medium contains sodium acetate as the sole carbon source and inorganic ammonium salts as the sole source of nitrogen. Growth is indicative of a positive test for acetate utilization. When the bacteria metabolize acetate, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns the bromthymol blue indicator in the medium from green to blue (2). This medium is recommended in differentiating *Shigella* spp. from *Escherichia coli* (1). Approximately 84% of *E. coli* strains utilize acetate, whereas the majority of *Shigella* species are incapable of acetate utilization.

II. MICROORGANISMS TESTED

A. Non-lactose-fermenting, gram-negative rods that are oxidase negative, nonmotile, and anaerogenic, which are likely to be either *E. coli* or *Shigella*

B. Nonfermentative, gram-negative rods

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media

1. Sodium acetate agar slants contain the following ingredients per liter of solution.

sodium chloride	5.0 g
magnesium sulfate	0.1 g
ammonium phosphate, monobasic	1.0 g
potassium phosphate, dibasic	1.0 g
sodium acetate	2.0 g
agar	20.0 g
bromthymol blue	0.08 g

2. The final pH is 6.7.

3. Vendors include Hardy Diagnostics; Remel, Inc.; and BD Diagnostic Systems.

B. Supplies

1. Sterile inoculating loops or sticks

2. Sterile pipette

3. Incubator at 35°C

4. Sterile saline

IV. QUALITY CONTROL

A. Perform QC on new lots of media prior to putting them into use.

B. Inspect agar for evidence of prior freezing, contamination, cracks, dehydration, and bubbles prior to storage and before use. Discard any tubes that are blue.

C. Organisms

1. *E. coli* ATCC 25922—acetate positive (growth; blue color)

2. *Shigella flexneri* ATCC 12022—acetate negative (no growth or trace of growth)

V. PROCEDURE

Observe standard precautions.

A. Using an 18- to 24-h culture from a noninhibitory culture plate, prepare a turbid saline suspension. Inoculate the slant with 1 drop of the suspension.

B. Alternatively, streak the slant back and forth with a light inoculum picked from the center of a well-isolated colony.

C. *Place cap loosely on tube.*

D. Incubate aerobically at 35 to 37°C for up to 5 days for *Enterobacteriaceae;* incubate at 30°C for nonfermenting, gram-negative rods for up to 7 days.

E. Observe a color change from green to blue along the slant.

VI. INTERPRETATION

A. A positive test is growth and change from green to intense blue color along the slant.

B. A negative test is no growth, no color change, and slant remains green.

VII. REPORTING RESULTS

A. Most *E. coli* organisms are positive for acetate utilization; most *Shigella* organisms are negative.

B. Some *S. flexneri* organisms are acetate positive. Lysine decarboxylase, citrate, and serologic tests (e.g., Alkalescens-Dispar [AD] antisera for *E. coli*) are also helpful to rule out *Shigella*, if any of these results are positive.

VIII. LIMITATIONS

A. Growth on the slant without an accompanying color change may indicate a positive test. However, if the agar does not turn blue on further incubation, the test should be repeated with less inoculum.

B. Tests with equivocal results should be repeated.

C. Do not stab the slant, since the test requires an aerobic environment.

D. Do not inoculate from broth cultures, due to carryover of media.

E. To avoid false-positive reactions, use a light inoculum to prevent carryover of substances from previous media.

REFERENCES

1. **Trabulsi, L. R., and W. H. Ewing.** 1962. Sodium acetate medium for differentiation of *Shigella* and *Escherichia* cultures. *Public Health Lab.* **20:**137–140.
2. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria,* 2nd ed., p. 6–7. Williams & Wilkins, Baltimore, MD.

3.17.3 ALA (δ-Aminolevulinic Acid) Test for Porphyrin Synthesis

I. PRINCIPLE

The δ-aminolevulinic acid (ALA) test is a rapid test used to determine the growth requirement for hemin (X factor) in the identification of *Haemophilus* spp. (1). Organisms which do not require hemin can produce porphobilinogen from ALA by the enzyme porphobilinogen synthase. The porphobilinogen is then converted to protoporphyrin IV, the immediate precursor of the heme moiety, in a series of steps. The presence of porphyrins is detected by the emission of red-orange fluorescence under UV light (360 nm). This method is more rapid than the X factor strip and is superior because it avoids erroneous results due to carryover (2). In the tube test, if no fluorescence is observed, indole reagent can be added to detect porphobilinogen, the precursor to porphyrin (3), which is considered a positive test for porphobilinogen synthase.

ALA has also been described for detecting cytochromes in catalase-negative, gram-positive cocci (4). A positive test for porphobilinogen in a gram-positive coccus indicates that the organism is a member of the family *Micrococcaceae*, even if it is catalase negative. This test is useful for identification of organisms that have the colony morphology of staphylococci but are catalase negative.

II. MICROORGANISMS TESTED

A. Tiny gram-negative rods or coccobacilli growing only on CHOC with the typical *Haemophilus* colonial morphology (e.g., large size colony in 24 h) and which do not grow on BAP. They do grow on BAP by satelliting around *Staphylococcus* spp.

B. Gram-positive cocci that have the colonial appearance of staphylococci but are catalase negative

III. REAGENTS AND SUPPLIES

Include QC information on reagent container and in QC records.

A. ALA reagent (3)

1. Purchase disks (BD Diagnostic Systems; Hardy Diagnostics; Remel, Inc.). Store at 4°C *or*

2. Prepare by combining the following.

a. 33.5 mg of ALA (Sigma) (stored at −20°C)

b. 19.72 mg of $MgSO_4 \cdot 7H_2O$

c. 100 ml of 0.1 M Sorensen's phosphate buffer, pH 6.9

(1) 3.15 g of $NaH_2PO_4 \cdot H_2O$ and 7.4 g of $Na_2HPO_4 \cdot 7H_2O$ in 500 ml of deionized water

(2) Check pH; adjust with either 0.1 M HCl or 0.1 M NaOH.

(3) Store at 2 to 8°C. Reagent is stable for 6 months.

d. Reagent should appear colorless. Do not use if reagent is tan to brown or particulate matter is present.

e. Dispense in 0.5-ml aliquots into 13- by 100-mm tubes. Cover tightly and store at −20°C.

f. Label rack of tubes with preparation date, expiration date (9 months from preparation), storage requirements, and preparer's initials.

B. Kovács' indole reagent

1. Prepare in isoamyl alcohol for porphobilinogen test.

2. See Appendix 3.17.23–1.

C. Other supplies

1. Filter paper or test tubes

2. Petri dish

3. Sterile wooden sticks, plastic or wire inoculating loops

4. Long-wave UV light (360 nm or Wood's lamp)

5. Incubator at 35°C

IV. QUALITY CONTROL

A. Perform QC on each new lot or shipment of disks or reagent prior to putting it into use.

B. Test positive control with use to verify length of incubation and color reaction (optional).

C. Organisms

1. *Haemophilus influenzae* ATCC 10211—ALA negative; no red fluorescence

2. *Haemophilus parainfluenzae* ATCC 7901—ALA positive; red fluorescence

V. PROCEDURE

Observe standard precautions.

A. Inoculation

1. Filter paper method

a. Place piece of filter paper to fit flat in a petri dish.

b. With a pencil, make circles approximately 10 mm in diameter for each test, plus a circle for the positive control.

c. Inoculate center of each circle heavily with colony of test organism using a wooden stick or loop.

d. Slowly pour thawed ALA reagent over filter paper, covering all test circles.

2. Disk method

a. Place ALA-containing disks in bottom of petri dish, marking the petri dish near each disk with culture identifying number.

b. Add 0.04 ml (one small drop) of saline to each disk.

c. Inoculate center of each disk heavily with test organism using wooden sticks; repeat for each control organism. Colony should be visible on disk. Alternatively, touch disk to colony and then place in petri dish.

d. Place a filter paper saturated with water on lid of dish. Cover.

3. Tube method: add a heavy inoculum of organism to 0.5 ml of ALA reagent.

B. Incubation and observation

1. Incubate test in non-CO_2 incubator at 35°C for 2 h.

2. Observe reactions for red fluorescence under Wood's lamp in darkened room.

3. Reincubate for 2 to 4 h more if control is negative and reread.

4. If the *tube* test is negative, incubate for 18 to 24 h, add 0.5 ml of Kovács' reagent to the tube, and shake vigorously. Observe for immediate color change.

VI. INTERPRETATION

A. The presence of red fluorescence is a positive test, indicating that the organism does not require X factor or hemin and that the ALA has been utilized.

B. The lack of red fluorescence is a negative test, indicating that the organism requires X factor and that the ALA has not been utilized.

C. When the indole reagent is added, a red color in the *lower* aqueous phase indicates a positive test for porphobilinogen, which is also indicative that the organism does not require hemin for growth.

VII. REPORTING RESULTS

A. A negative test or no fluorescence for a gram-negative rod that grows as large colonies in 24 h on CHOC but not on BAP indicates *H. influenzae.*

B. A negative test or no fluorescence for a catalase-negative, oxidase-positive, gram-negative rod that takes several days to grow on CHOC, does not grow on BAP, even with a streak of staphylococci, and is sodium polyanethol sulfonate sensitive indicates *Haemophilus ducreyi.*

C. A positive test for fluorescence or the red color in the lower aqueous phase after adding indole reagent is consistent with *H. parainfluenzae, Haemophilus parahaemolyticus,* or *Haemophilus paraphrophilus.* The last organism is lactose

VII. REPORTING RESULTS *(continued)*

positive. Further biochemical tests are needed to separate (2). See Table 3.18.2–2.

D. A positive test for porphobilinogen in a gram-positive coccus indicates that the organism is a member of the family *Micrococcaceae*, even if it is catalase negative (4). *Aerococcus* and *Streptococcus* are porphobilinogen negative, and *Staphylococcus* and *Rothia mucilaginosa (Stomatococcus mucilaginosus)* are positive.

VIII. LIMITATIONS

A. Many organisms will give a positive reaction. If test is performed only on gram-negative rod colonies that grow well on CHOC in 24 h and not on BAP, results are for *Haemophilus* species.

B. The ALA test will not separate *H. influenzae* from *Haemophilus haemolyticus;* the latter is rare and not pathogenic. It will sometimes grow on BAP without the need for staphylococcal streak, if it is able to hemolyze the blood to supply it with V factor.

C. Read test only in a darkened room.

D. Make a heavy inoculum to avoid false-negative results.

E. Test only cultures less than 24 h old.

F. If test paper is not kept moist during the incubation, the reaction can be falsely negative.

G. *Francisella tularensis* is ALA negative but does not grow in 24 h on CHOC and is oxidase negative. It does not satellite around staphylococci on BAP.

REFERENCES

1. **Kilian, M.** 1974. A rapid method for the differentiation of *Haemophilus* strains—the porphyrin test. *Acta Pathol. Microbiol. Scand. Sect. B* **82:**835–842.
2. **Lund, M. S., and D. J. Blazevic.** 1977. Rapid speciation of *Haemophilus* with the porphyrin production test vs. the satellite test for X. *J. Clin. Microbiol.* **5:**142–144.
3. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria,* 2nd ed., p. 18. Williams & Wilkins, Baltimore, MD.
4. **Wong, J. D.** 1987. Porphyrin test as an alternative to benzidine test for detecting cytochromes in catalase-negative gram-positive cocci. *J. Clin. Microbiol.* **25:**2006–2007.

3.17.4 Antimicrobial Disk Tests for Identification (Especially of Staphylococci)

I. PRINCIPLE

Identification of coagulase-negative staphylococci to the species level is often not clinically useful (14). The use of the pyrrolidonyl-β-naphthylamide (PYR) test in combination with the coagulase test, ornithine decarboxylase, and a number of disk susceptibility tests allows identification of clinically important species: *Staphylococcus saprophyticus* in urinary tract and genital infections, *S. lugdunensis* in blood and joint cultures, and *S. haemolyticus,* which is important only because it can be intermediately susceptible to vancomycin. For repeatedly isolated coagulase-negative staphylococci from blood and clinically significant or postoperative specimens, antimicrobial susceptibility profiles are required. Nosocomial strains may need to be typed by pulse-field gel electrophoresis to determine the spread of clonal strains in outbreak situations.

For urinary tract specimens, for which a report of either *S. saprophyticus* or coagulase-negative staphylococci is sufficient, only the novobiocin disk need be tested, along with testing for any antimicrobials that are requested by caregivers (2, 11). *S. saprophyticus* is resistant to novobiocin (1, 7, 10); a few other staphylococcal species are also resistant to novobiocin, but they are rarely found in clinical specimens, especially in the urinary tract. Evaluations of the novobiocin disk method on both Mueller-Hinton (MH) agar (1, 7) and BAP (10, 12) have been published. Generally, zone sizes are larger for novobiocin on MH agar, and a 16-mm breakpoint has been recommended. Hébert et al. (10) tested 1,000 clinical isolates of coagulase-negative staphylococci on BAP and found that a lower zone size (<12 mm) for resistance increased the specificity of the test.

The bacitracin 0.04-U disk, previously considered useful for identification of *Streptococcus pyogenes,* is a very sensitive assay to separate staphylococci from *Rothia mucilaginosa* (formerly *Stomatococcus mucilaginosus* [3]) and *Micrococcus* (4, 10, 12). This separation is generally performed restrictively, usually on strains with pigment or sticky colony morphology from significant anatomic sites. Bacitracin differentiation may also be useful for penicillin-susceptible strains, since the great majority of *Micrococcus* and *Rothia* organisms are susceptible to penicillin and most coagulase-negative staphylococci are not (13). Alternatively, the furazolidone disk test (4), from BD Diagnostic Systems, or the modified oxidase test using a 6% solution of tetramethylphenylenediamine in dimethyl sulfoxide (5) sold as the Microdase test by Remel, Inc., can be used to separate these genera (*see* procedure 3.17.39). These tests are not covered in this procedure, since the bacitracin disk is easily obtained and does not require additional QC organism stocks.

The polymyxin B disks (or colistin disks) are useful to separate species of staphylococci and are also very useful in the identification of *Neisseria* and gram-negative rods (procedure 3.18.2).

II. MICROORGANISMS TESTED

A. Novobiocin

For gram-positive cocci in clusters which are catalase positive and coagulase negative isolated from urine specimens, usually from sexually active young women

B. Bacitracin

For penicillin-susceptible or "sticky" colonies of gram-positive cocci in clusters which are catalase positive and coagulase negative from invasive-site specimens to separate staphylococci from micrococci. Do not test lemon-yellow colonies, because they are presumed to be *Micrococcus.*

C. Polymyxin B

For differentiation of *R. mucilaginosa* from *Micrococcus* spp. and for separation of *S. aureus* and *S. intermedius* or characterization of other species of staphylococci, especially *S. epidermidis,* in unusual circumstances. This disk is also

II. MICROORGANISMS TESTED *(continued)*

used as part of the identification of *Neisseria*, *Vibrio*, and non-glucose-fermenting, gram-negative rods, especially from cystic fibrosis patients.

D. Penicillin and vancomycin

For screening gram-positive cocci in clusters from positive blood cultures for confirmation of Gram stain, for induction for the beta-lactamase test, for detection of unusual resistance, and for *Pediococcus* and *Leuconostoc* identification

III. MEDIA, REAGENTS, AND SUPPLIES

A. Disks

Store a small supply at 4°C; store stock at −20°C.

1. Novobiocin, 5 μg
2. Bacitracin, 0.04 U
3. Penicillin, 10 U
4. Vancomycin, 30 μg
5. Polymyxin B, 300 U (colistin [10-μg] disk can be substituted)

B. Media

1. BAP *or*
2. MH agar

C. Supplies

1. Swabs
2. Broth for inoculum

IV. QUALITY CONTROL

A. Perform QC of disks weekly or with each use for tests that are performed less often than weekly. Test on the medium that is used for test performance. For disks used for identification only (bacitracin, novobiocin), testing need not be performed weekly, but test each new lot or shipment of disks with both a susceptible and resistant strain prior to using for identification tests. Testing should be repeated monthly with a susceptible control organism for disks that are stored at 4°C rather than −20°C.

B. Strains, media, and zone size requirements

Antimicrobial agent	Test organism	Zone size (mm) with 6-mm disk
Novobiocin, 5 μg, on BAP or MH agar	*Staphylococcus aureus* ATCC 25923	≥22
	Staphylococcus saprophyticus ATCC 15305	≤15
Bacitracin, 0.04 U, on BAP	*Streptococcus pyogenes* ATCC 19615	≥12
	Staphylococcus aureus ATCC 25923	6
Polymyxin B, 300 U, on MH agar	*Pseudomonas aeruginosa* ATCC 27853	17–21 (6)
	Escherichia coli ATCC 25922	17–20 (6), 12–16 (BD package insert)
Colistin, 10 μg, on MH agar	*Pseudomonas aeruginosa* ATCC 27853	15–19 (6)
	Escherichia coli ATCC 25922	16–20 (6), 11–15 (BD package insert)
Penicillin, 10 U, on MH agar	*Staphylococcus aureus* ATCC 25923	26–37
Vancomycin, 30 μg, on MH agar	*Staphylococcus aureus* ATCC 25923	17–21

V. PROCEDURE

Observe standard precautions.

A. Hébert method (9, 10, 12) using BAP

1. Prepare no. 1 McFarland suspension of organism from overnight growth.
2. Inoculate a section of a BAP in one direction; area of inoculation should allow a separation of 10 mm between each disk to be placed on the inoculum.
3. After allowing 10 min for drying, place the disks on agar.
4. Tap disk with sterile stick to ensure adherence.
5. Incubate for *24 h* at 35°C in non-CO_2 incubator.

B. MH agar method (1, 7)

1. If other disk susceptibility testing is being performed to report susceptibility to antimicrobial agents on a urinary isolate, use MH agar for the novobiocin test (1, 7).
2. Prepare a no. 0.5 McFarland suspension of the organism and inoculate MH agar in three directions as for a disk susceptibility test (2).
3. Place novobiocin and other susceptibility test disks on agar.
4. Incubate for *18 h* at 35°C in a non-CO_2 incubator.

C. For positive blood cultures

1. Place a drop from the positive culture onto BAP and spread with a loop over one-fourth of the plate.
2. Place penicillin and vancomycin disks on this area of the plate.
3. Use interpretations for MH agar only for identification purposes, not for susceptibility reporting.

VI. INTERPRETATION

A. Novobiocin

1. Hébert method using BAP (10)
   - **a.** Zone of $<$12 mm is resistant.
   - **b.** Zone of $\geq$12 mm is susceptible.
2. NCCLS method using MH agar (1, 2, 7)
   - **a.** Zone of $\leq$16 mm is resistant.
   - **b.** Zone of $>$16 mm is susceptible.
3. Resistance corresponds to an MIC of $\geq$1.6 μg/ml.

B. Bacitracin, 0.04 U (4, 10, 12), on either BAP or MH agar

1. Zone of 6 mm is resistant.
2. Zone of $\geq$10 mm is susceptible.
3. Repeat tests with values between 7 and 10 mm; these are probably susceptible.

C. Polymyxin B (10) on either BAP or MH agar

1. Zone of $<$10 mm is resistant.
2. Zone of $\geq$10 mm is susceptible.

   ◩ **NOTE:** If colistin is substituted for polymyxin B, the zone sizes may be smaller for susceptible strains; for gram-negative rods, any zone is considered susceptible (M. York, personal observation).

D. Penicillin (2)

1. Zone of $\leq$28 mm is resistant.
2. Zone of $\geq$29 mm is susceptible.

E. Vancomycin (2)

1. Zone of $<$15 mm is resistant.
2. Zone of $\geq$15 mm is susceptible.

VII. REPORTING RESULTS

A. For gram-positive cocci in clusters which are catalase positive and coagulase negative
  1. Novobioicin-resistant strains
    a. In a urine culture, report as *S. saprophyticus* (11).
    b. From other body sites, *do not use this result* alone for species identification. Generally refer to the organism as "coagulase-negative staphylococci."
  2. If an isolate is penicillin susceptible and from a significant body site, use the bacitracin 0.04-U disk to separate coagulase-negative staphylococci (resistant) from *Micrococcus* (susceptible) and *Rothia* (susceptible) (4, 10, 12). The latter also adheres to the agar.
  3. Most staphylococcal species are susceptible to polymyxin B, but *S. aureus, S. lugdunensis,* and *S. epidermidis* (97.2% are resistant) are notable exceptions (10). Use this resistance as part of the identification of *S. epidermidis* and to separate *S. aureus* from *S. intermedius. Rothia* is also polymyxin B resistant. See Table 3.18.1–1.

B. Any gram-positive coccus that is resistant to vancomycin is either *Enterococcus, Pediococcus, Leuconostoc,* or possibly a vancomycin-resistant staphylococcus, which must be further tested to confirm the result (*see* section 5 and procedure 3.18.1).

C. Refer to procedure 3.18.2 for use of the polymyxin B disk in the identification of gram-negative rods.

VIII. LIMITATIONS

A. Strains of staphylococci, other than *S. saprophyticus,* that are resistant to novobiocin include *S. xylosus, S. kloosi,* and *S. cohnii.* Any strain may become resistant to novobiocin, including *S. aureus;* reporting *S. saprophyticus* should be limited to urinary tract isolates unless further tests for identification to the species level are performed.

B. While Goldstein et al. (7) recommend using $<$16 mm as the breakpoint for resistance to novobiocin for BAP and MH agar, their data and those of others demonstrate greater specificity without loss of sensitivity by using the $<$12-mm breakpoint for BAP (7, 10).

C. Harrington and Gaydos (8) reported a rapid novobiocin test that is completed in 5 h.

D. While *Micrococcus* and *R. mucilaginosa* can be resistant to penicillin, this is a very rare event (12, 13).

E. Staphylococci should show no zone of inhibition around the bacitracin 0.04-U disk on BAP (10). Zone sizes of $>$7 mm but less than the 10-mm breakpoint may be obtained for *Micrococcus* if incubation is not a full 24 h or MH agar is used (10).

F. Direct tests from blood cultures are not standardized but can work well to provide preliminary reports and guide subsequent identifications and more standardized antimicrobial susceptibility testing.

REFERENCES

1. **Almeida, R. J., and J. H. Jorgensen.** 1982. Use of Mueller-Hinton agar to determine novobiocin susceptibility of coagulase-negative staphylococci. *J. Clin. Microbiol.* **16:**1155–1156.
2. **Clinical and Laboratory Standards Institute.** 2006. *Performance Standards for Antimicrobial Disk Susceptibility Tests*, 9th ed. Approved standard M2-A9. Clinical and Laboratory Standards Institute, Wayne, PA.
3. **Collins, M. D., R. A. Hutson, V. Båverud, and E. Falsen.** 2000. Characterization of a *Rothia*-like organism from a mouse: description of *Rothia nasimurium* sp. nov. and reclassification of *Stomatococcus mucilaginosus* as *Rothia mucilaginosa* comb. nov. *Int. J. Syst. Evol. Microbiol.* **50:**1247–1251.
4. **Falk, D., and S. J. Guering.** 1983. Differentiation of *Staphylococcus* and *Micrococcus* spp. with the Taxo A bacitracin disk. *J. Clin. Microbiol.* **18:**719–721.
5. **Faller, A., and K. H. Schleifer.** 1981. Modified oxidase and benzidine tests for separation of staphylococci from micrococci. *J. Clin. Microbiol.* **13:**1031–1035.
6. **Gales, A. C., A. O. Reis, and R. N. Jones.** 2001. Contemporary assessment of antimicrobial susceptibility testing methods for polymyxin B and colistin: review of available interpretative criteria and quality control guidelines. *J. Clin. Microbiol.* **39:**183–190.
7. **Goldstein, J., R. Schulman, E. Kelley, G. McKinley, and J. Fung.** 1983. Effect of different media on determination of novobiocin resistance for differentiation of coagulase-negative staphylococci. *J. Clin. Microbiol.* **18:**592–595.
8. **Harrington, B. J., and J. M. Gaydos.** 1984. Five-hour novobiocin test for differentiation of coagulase-negative staphylococci. *J. Clin. Microbiol.* **19:**279–280.
9. **Hébert, G. A.** 1990. Hemolysins and other characteristics that help differentiate and biotype *Staphylococcus lugdunensis* and *Staphylococcus schleiferi*. *J. Clin. Microbiol.* **28:**2425–2431.
10. **Hébert, G. A., C. G. Crowder, G. A. Hancock, W. R. Jarvis, and C. Thornsberry.** 1988. Characteristics of coagulase-negative staphylococci that help differentiate these species and other members of the family *Micrococcaceae*. *J. Clin. Microbiol.* **26:**1939–1949.
11. **Meers, P. D., W. Whyte, and G. Sandys.** 1975. Coagulase-negative staphylococci and micrococci in urinary tract infections. *J. Clin. Pathol.* **28:**270–273.
12. **Mitchell, P. S., B. J. Huston, R. N. Jones, L. Holcomb, amd F. P. Koontz.** 1990. *Stomatococcus mucilaginosus* bacteremias. Typical case presentations, simplified diagnostic criteria, and a literature review. *Diagn. Microbiol. Infect. Dis.* **13:**521–525.
13. **von Eiff, C., M. Herrmann, and G. Peters.** 1995. Antimicrobial susceptibilities of *Stomatococcus mucilaginosus* and of *Micrococcus* spp. *Antimicrob. Agents Chemother.* **39:**268–270.
14. **Weinstein, M. P., S. Mirrett, L. Van Pelt, M. McKinnon, B. L. Zimmer, W. Kloos, and L. B. Reller.** 1998. Clinical importance of identifying coagulase-negative staphylococci isolated from blood cultures: evaluation of MicroScan Rapid and Dried Overnight gram-positive panels versus a conventional reference method. *J. Clin. Microbiol.* **36:**2089–2092.

3.17.5 Bile-Esculin and Esculin Tests

I. PRINCIPLE

The basis of the esculin test is the hydrolysis of esculin (a glucoside) into glucose and esculetin by a microorganism that has a constitutive (noninducible) β-glucosidase or esculinase enzyme. When esculetin is produced by the hydrolysis of esculin, it reacts with an iron salt in the medium to form a phenolic iron complex which produces a dark brown or black color (3). Alternatively, esculin is a fluorescent compound and its hydrolysis can be observed by a loss of fluorescence. If bile is added to the medium, the microorganism must be able to grow in its presence in order to hydrolyze esculin. The 40% bile (equivalent to 4% oxgall) in bile-esculin medium inhibits most strains of streptococci, other than *Streptococcus bovis*, but does not inhibit enterococci or *Listeria* (1, 2).

II. MICROORGANISMS TESTED

A. Gram-positive cocci in chains, which are catalase negative and morphologically identified as presumptive *S. bovis*

B. Isolates of alpha- or gamma-hemolytic, gram-positive cocci as part of differentiation of enterococci from other pyrrolidonyl-β-naphthylamide (PYR)-positive organisms

C. Non-spore-forming, hemolytic, gram-positive rods that are catalase positive and morphologically identified as presumptive *Listeria*

D. Positive blood cultures with gram-positive cocci in chains or gram-positive rods, to rapidly (4 h) identify enterococci and *Listeria*

E. Esculin without bile for the identification of oxidase-positive aerobic gram-negative rods, including *Aeromonas* spp. and yellow-pigmented non-glucose-fermenting rods (4)

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media

Store at 2 to 8°C.

1. Bile-esculin agar slants with iron(III) citrate. Agar plate media, such as Enterococcosel agar (BD Diagnostic Systems), have a similar formulation.

2. Bile-esculin-azide agar or broth with iron(III) citrate and azide. Azide will inhibit most gram-negative bacteria.

3. Peptose-yeast-esculin broth (usually in anaerobic atmosphere)

◩ **NOTE:** This medium can be decanted into small volumes for use on aerobic and microaerobic organisms.

4. Esculin agar (0.1% esculin in heart infusion basal medium) without bile or azide but with iron(III) citrate (Hardy Diagnostics)

◩ **NOTE:** Color Spot (Remel, Inc.) and Wee-Tab (Key Scientific) incorporate PYR and esculin tests on filter paper cards in a rapid test. BactiCard Strep (Remel, Inc.) and StrepQuick (Hardy Diagnostics) contain PYR, leucine aminopeptidase (LAP), and a rapid esculin test. Enterococcus/Group A Screen (Remel, Inc.) is a rapid tube test for esculin and PYR.

B. Reagents and supplies

1. Long-wave (360 nm) UV light

2. 1% ferric [iron(III)] ammonium citrate if iron(III) is not incorporated into the medium

IV. QUALITY CONTROL

A. Perform QC on new lots or shipments of media and ferric ammonium citrate reagent prior to putting them into use.
B. Inspect agar for freezing, contamination, cracks, dehydration, and bubbles prior to storage and before use.
C. Examine uninoculated broth for fluorescence.
D. Organisms
  1. *Enterococcus faecalis* ATCC 29212—bile-esculin and esculin positive
  2. *Escherichia coli* ATCC 25923—bile-esculin and esculin negative
  3. *Streptococcus pyogenes* ATCC 19615—bile-esculin and esculin negative

V. PROCEDURE

Observe standard precautions.

A. Tube test
  1. Inoculate slant or broth with a single colony. For enterococcus and *S. bovis* identification, use only 40% bile and inoculate with a 10-µl calibrated loopful of a 0.5 McFarland standard suspension prepared in sterile water (1).
  2. *Place cap loosely on tube.*
  3. Incubate for 24 h at 35°C (or up to 7 days for slow-growing gram-negative rods and anaerobes) and observe color change. For enterococci and *S. bovis,* read only at 18 to 24 h.
  4. For esculin broth without iron(III) citrate, observe daily for loss of fluorescence. Add 2 or 3 drops of 1.0% ferric ammonium citrate to the esculin tube if no fluorescence is observed. Observe for a color change. Aliquot broth for daily testing with indicator, if desired.
  5. When evaluating loss of fluorescence, use an uninoculated control tube for comparison.

B. Disk test
  1. Moisten disk with a single drop of distilled or deionized water. Do not saturate.
  2. Using a sterile loop, pick two or three well-isolated colonies from an overnight (18- to 24-h) culture.
  3. Allow to react at room temperature for 10 min.
  4. Observe the disk for the development of a dark brown or black color.

VI. INTERPRETATION

A. A positive tube test in medium containing ferric ammonium citrate is demonstrated by blackening of the medium.
B. A negative tube test is indicated by lack of color change. The medium will fluoresce under UV light (366 nm).
C. For esculin broth without iron(III) citrate, a positive test is demonstrated either by blackening of the broth after addition of the ferric [iron(III)] reagent or by the loss of fluorescence of the medium.
D. A negative test result also occurs in bile-esculin medium if the organism cannot grow in the presence of bile, regardless of the ability to hydrolyze esculin.
E. A positive disk test is the development of a dark brown or black color.
F. A negative disk test remains colorless.

VII. REPORTING RESULTS

A. *Listeria monocytogenes* is a bile-esculin-positive, beta-hemolytic, CAMP-positive, catalase-positive, motile, nonpigmented, small gram-positive rod.
B. Esculin- or bile-esculin-positive, catalase-negative, gram-positive cocci in *chains* may be *Enterococcus.* Confirmation includes large-diameter (1-mm) colonies that are PYR and LAP positive. *Lactococcus* organisms are not easily separated from enterococci.

VII. REPORTING RESULTS *(continued)*

C. Esculin- or bile-esculin-negative, gamma- or alpha-hemolytic, gram-positive cocci in *chains* that are bile insoluble (procedure 3.17.6) and catalase negative and grow well on BAP are presumptively identified as viridans group streptococci.

D. If the inoculum is controlled and the medium contains 40% bile, PYR-negative streptococci (catalase-negative, gram-positive cocci in chains) can be presumptively identified as *S. bovis* with a 97% sensitivity by a positive bile-esculin test (1).

E. Positive esculin reactions can separate the species of yellow, non-glucose-fermenting, gram-negative rods (Tables 3.18.2–6 and 3.18.2–7) and *Aeromonas* species.

VIII. LIMITATIONS

A. If the inoculum is too great or the concentration of bile is less than 40%, viridans group streptococci other than *S. bovis* can give a positive reaction on bile-esculin agar (1, 2). Esculin tests without bile do not separate *S. bovis* (previously referred to as group D streptococci, not enterococci) from other viridans group streptococci.

B. H_2S, which is produced by several organisms during metabolism, also reacts with iron to produce a black complex, which interferes with the interpretation of the esculin hydrolysis test. Therefore, for gram-negative rods, check tubes showing darkening after the addition of the reagent under UV light; intact esculin fluoresces white-blue, whereas hydrolyzed esculin has lost its fluorescence.

C. Some microorganisms, such as *E. coli*, have an inducible β-glucosidase and will react in this test only after prolonged incubation. Prolonged incubation should not be used if the test is being used to detect only constitutive β-glucosidase.

REFERENCES

1. **Chuard, C., and L. B. Reller.** 1998. Bile-esculin test for presumptive identification of enterococci and streptococci: effects of bile concentration, inoculation technique, and incubation time. *J. Clin. Microbiol.* **36:**1135–1136.
2. **Facklam, R. R.** 1973. Comparison of several laboratory media for presumptive identification of enterococci and group D streptococci. *Appl. Microbiol.* **26:**138–145.
3. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria,* 3rd ed., p. 8–26. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.
4. **Sneath, P. H. A.** 1956. Cultural and biochemical characteristics of the genus *Chromobacterium. J. Gen. Microbiol.* **15:**70–98.

3.17.6 Bile Solubility Test

I. PRINCIPLE

The bile solubility test is used to differentiate *Streptococcus pneumoniae* from alpha-hemolytic *Streptococcus* spp. It may be performed using a cell suspension on a slide or in a tube or by applying the reagent directly to the colony.

The bile solubility test is based on the observation that pneumococcal cells lyse when sodium desoxycholate (bile salts) is applied to the colony under specific conditions of time and temperature, but other streptococci do not lyse (6). The pneumococcus has an intracellular autolytic enzyme, an amidase, that causes the organism to undergo rapid autolysis when cultivated on artificial medium. The bile salts alter the surface tension of the medium and cause cell membrane rearrangement. The working mechanism of the test is not clearly understood; however, one theory is that the bile salts facilitate lysis of pneumococcal cells by activating the autolytic enzyme (3).

II. MICROORGANISMS TESTED

A. Any alpha-hemolytic, catalase-negative, gram-positive cocci in chains, having the characteristic central depression (flattened center) or mucoid colony morphology suggestive of *S. pneumoniae*

B. Any gram-positive cocci in lancet-shaped pairs from a positive blood culture

III. REAGENTS AND SUPPLIES

Include QC information on reagent container and in QC records.

A. Reagents

1. Bile salts

a. Purchase or prepare 10% bile salt solution

sodium desoxycholate (Sigma) …… 10 g
deionized water ……100 ml

b. Dispense in small amounts to minimize contamination. Shelf life is 270 days.

c. Store at 15 to 30°C. Storage of the reagent at cool temperatures can cause it to thicken.

2. 0.85% NaCl (sterilized)

3. Broth culture medium (e.g., BHI)

B. Supplies

1. Loops

2. Test tubes or slide

3. Pasteur pipettes

IV. QUALITY CONTROL

A. Test each new lot of reagent with known positive and negative controls before putting it into use.

B. Do not use if bile reagent is not a clear and very light amber.

C. Organisms

1. *S. pneumoniae* ATCC 49619—positive (bile soluble)

2. *Enterococcus faecalis* ATCC 29212—negative (bile insoluble)

V. PROCEDURE

Observe standard precautions.

A. Test tube method

1. Dispense 0.5 ml of sterile saline or suitable broth into a small test tube.
2. Prepare a heavy suspension of the organism in the saline (equivalent to no. 1 McFarland standard). Shake or vortex to form a uniform suspension.
3. Divide the suspension into two tubes, one labeled "TEST" and the other labeled "CONTROL."
4. Dispense 5 drops of bile reagent into the tube marked "TEST." Add 5 drops of saline to the tube marked "CONTROL." Gently mix each tube.
5. Incubate the tubes for 3 h at 35°C, checking hourly for clearing, or examine each tube by Gram stain or methylene blue wet mount for lysis of cells at 15 min.

B. Direct plate method

1. Place a drop of bile spot reagent near a suspected 18- to 24-h colony; gently roll the drop over several representative colonies by tilting the plate. Take care not to dislodge the colonies.

   ◩ **NOTE:** Do not touch the agar surface with the tip of the dropper of bile reagent.
2. Keep the plate right side up and incubate at 35°C for 15 to 30 min or until the drop has evaporated. Placing the plate on a heat block is a substitute for use of an incubator.
3. Observe for flattening of the colony. Be sure the colony did not simply float away.

C. Direct slide blood culture test (5)

1. Add 1 drop of blood culture broth to 1 drop of bile reagent on a glass slide and allow to dry.
2. As a control, add 1 drop of broth blood culture to 1 drop of *water* and allow to dry.
3. Gram stain and examine for cocci.

VI. INTERPRETATION

A. Test tube method

Bile solubility is demonstrated as a clearing or loss of turbidity, relative to the "CONTROL" tube, within 3 h or lysis of cells observed microscopically.

B. Direct slide blood culture method

If all the cocci in the smear are completely lysed and the control smear shows intact bacteria, the organism is bile soluble.

C. Direct plate method

1. Bile solubility is demonstrated as a disintegration or flattening of the colony within 30 min, leaving an area of alpha-hemolysis where the colonies were located.
2. Insolubility is demonstrated when there is no change in the integrity of the colony within 30 min.

VII. REPORTING RESULTS

A. If either the spot or tube test demonstrates bile solubility of an alpha-hemolytic colony from a catalase-negative, lancet-shaped, gram-positive coccus, definitively report as *Streptococcus pneumoniae* (1).

B. If the test does not demonstrate bile solubility, the organism is likely a viridans group streptococcus, but a percentage of bile-resistant organisms may still be *S. pneumoniae;* further testing is indicated from typical pneumococcal colonies (4).

VIII. LIMITATIONS

A. Some *S. pneumoniae* organisms will not lyse in the presence of bile, possibly due to the loss of virulence factor or capsule. If lysis is not present, the isolate may still be *S. pneumoniae.* Therefore, colonies resembling *S. pneumoniae* which are not bile soluble should be further identified using another method, such as optochin susceptibility and/or DNA probe (4).

B. Use bile solubility only to differentiate *S. pneumoniae* from other alpha-hemolytic streptococci.

C. In one study comparison with the DNA probe and Quellung test, using 529 presumed viridans group streptococci, the tube test had a 96% specificity (23 false positives) and the colony test had a 99.4% specificity (4). For greatest specificity, tube test-positive, spot test-negative strains should be confirmed with an optochin disk test or DNA probe.

D. The colony test has a sensitivity of 99% and the tube test has a sensitivity of 99 to 100% for encapsulated strains of *S. pneumoniae* (2, 4). However, Mundy et al. (4) showed that the colony test was not very sensitive (three positive results) in the detection of 33 unencapsulated strains that were detected by DNA probe. The tube test detected 19 of the 33 unencapsulated strains positive by probe. All 33 had some zone of inhibition of optochin, although most were in the intermediate category. Whether DNA probe-positive, bile-negative strains are important pathogens or represent false-positive DNA probe tests is not known.

E. Normal autolysis of *S. pneumoniae* may be inhibited by a high concentration of bile salts. Evaporation may cause the reagent to become more concentrated, thus affecting the test.

F. The bile solubility test is not reliable with old cultures that have autolysed.

G. When performing the bile solubility tube test using saline or unbuffered broth, it is essential to adjust the pH to neutral before adding the reagent, in order to avoid false-negative reactions.

H. When testing using the plate method, care must be taken not to dislodge the colony being tested, thus leading to false-positive results. If the direct plate is difficult to interpret, the test should be repeated using the tube or slide method.

I. Storage of the reagent at cool temperatures can cause it to thicken. Warm the reagent bottle in a 37°C incubator to liquefy the reagent before use.

REFERENCES

1. **Clinical and Laboratory Standards Institute.** 2008. *Abbreviated Identification of Bacteria and Yeast,* 2nd ed. Approved guideline M35-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
2. **Kellogg, J. A., D. A. Bankert, C. J. Elder, J. L. Gibbs, and M. C. Smith.** 2001. Identification of *Streptococcus pneumoniae* revisited. *J. Clin. Microbiol.* **39:**3373–3375.
3. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria,* 3rd ed., p. 27–34. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.
4. **Mundy, L. S., E. N. Janoff, K. E. Schwebke, C. J. Shanholtzer, and K. E. Willard.** 1998. Ambiguity in the identification of *Streptococcus pneumoniae.* Optochin, bile solubility, quellung, and the AccuProbe DNA probe tests. *Am. J. Clin. Pathol.* **109:**55–61.
5. **Murray, P. R.** 1979. Modification of the bile solubility test for rapid identification of *Streptococcus pneumoniae. J. Clin. Microbiol.* **9:**290–291.
6. **Neufeld, F.** 1900. Über eine spezifische bakteriolytische Wirkung der Galle. *Z. Hyg. Infektionskr.* **34:**454–464.

3.17.7 Butyrate Esterase Test

I. PRINCIPLE

The butyrate test is a rapid test for the detection of the enzyme butyrate esterase. When used in conjunction with characteristic morphology on BAP, typical Gram stain, and a positive oxidase test, the butyrate test is useful for the definitive identification of *Moraxella catarrhalis.*

The value of tributyrin hydrolysis for differentiating *M. catarrhalis* from *Neisseria* spp. was first reported by Berger in 1962 (1). Subsequently, several authors have reported the usefulness of butyrate esterase in differentiating *M. catarrhalis,* using various substrates. Both bromochloro-indolyl butyrate (IB) (2, 3) and 4-methylumbelliferyl butyrate (MUB) (4, 5, 6, 7) can serve as substrates. Hydrolysis of the IB substrate by the butyrate esterase releases indoxyl, which in the presence of oxygen spontaneously forms indigo, a chromogenic compound which appears blue to blue-violet. Hydrolysis of the MUB substrate produces a fluorescent compound visible under UV light.

II. MICROORGANISMS TESTED

Gram-negative, oxidase-positive diplococci growing on BAP as white colonies that remain together when lifted with a loop or wire

III. REAGENTS AND SUPPLIES

Include QC information on reagent container and in QC records.

A. Disks

1. Disks impregnated with IB or MUB
2. Store at 2 to 8°C. Protect from light.
3. Vendors include Hardy Diagnostics; Remel, Inc.; and Key Scientific.

◩ **NOTE:** The BactiCard *Neisseria* (Remel, Inc.) includes the IB substrate as one test in the kit.

B. MUB tube test

1. MUB stock solution

   MUB (Sigma) ……100 mg
   dimethyl sulfoxide
   (Sigma) …… 10 ml
   Triton X-100 ……100 ml

2. 0.1 M citrate buffer, 90 ml; final pH, 5.0
   a. 0.1 M citric acid
   b. 0.05 M $Na_2HPO_4 \cdot 12H_2O$
3. Dissolve the MUB powder in dimethyl sulfoxide–Triton X-100 solution.
4. Prepare a 1:10 dilution of the stock solution in the citrate buffer.
5. Dispense 250-µl aliquots into sterile snap-cap tubes.
6. The substrate is stable for at least 1 month.
7. Clearly label the substrate, indicating preparation and expiration dates.
8. Record the expiration date in a work record.
9. Store frozen at −20°C or below. Protect from light.

C. Supplies/equipment

1. Sterile wooden applicator sticks or bacteriologic loops
2. Distilled water
3. Petri dish, slide, or tube
4. Long-wave (360 nm) UV light (Wood's lamp) (MUB reagent only)

IV. QUALITY CONTROL

A. Discard disks if they do not appear white with no visible color.

B. Perform QC on each new lot and shipment of disks or MUB reagent prior to putting them into use.

C. Organisms

1. *M. catarrhalis* ATCC 25240—butyrate positive

2. *Neisseria gonorrhoeae* ATCC 43069 or *Neisseria lactamica* ATCC 23970—butyrate negative

V. PROCEDURE

Observe standard precautions.

A. Disk method

1. Remove disk from vial and place on a clean glass slide or petri dish.

2. Add 1 drop of distilled or deionized water to moisten the disk.

3. Obtain a heavy, visible inoculum with a sterile wooden applicator stick or loop from a 24- to 72-h culture and rub it onto the disk.

4. Incubate at room temperature (15 to 30°C) for up to 5 min.

5. *Incubation for slightly longer periods may yield false-positive results. Do not* read after 5 min.

B. Tube method

1. Thaw tubes at room temperature.

2. Inoculate substrate using several colonies to obtain a turbid suspension.

3. Incubate at room temperature (15 to 30°C) for up to 5 min. *Incubation for slightly longer periods may yield false-positive results.*

4. Observe fluorescence using long-wave UV light in a darkroom.

VI. INTERPRETATION

A. A positive test results in a blue to blue-violet color (IB substrate) or fluorescence (MUB substrate) within 5 min, indicating the hydrolysis of IB or MUB, respectively, by butyrate esterase.

B. A negative test is indicated by no color change.

VII. REPORTING RESULTS

A. Report as *Moraxella catarrhalis* if an oxidase-positive, gram-negative diplococcus meets the following criteria.

1. Grows on BAP as colonies that remain together when sampled *and*

2. Is butyrate positive

B. Test butyrate-negative colonies that are suggestive of *M. catarrhalis* by the above-listed criteria for DNase production. Report DNase-positive colonies as *Moraxella catarrhalis.*

VIII. LIMITATIONS

A. Do not incubate test beyond 5 min, to avoid false-positive reactions.

B. Many strains of other *Moraxella* species, as well as *Eikenella* and *Acinetobacter,* may give a positive or weakly positive reaction (2, 6). The organism must be an oxidase-positive, gram-negative *diplo*coccus with typical morphology to be accurately identified as *M. catarrhalis.*

C. Unrelated organisms such as staphylococci and pseudomonads may also give positive results.

D. False-negative tests may result from using too small an inoculum. If the organism is suspected but the test is negative, repeat with a large inoculum or test for DNase production.

REFERENCES

1. **Berger, U. V.** 1962. Ueber die Spaltung von Tributyrin durch *Neisseria. Arch. Hyg. Bakteriol.* **146:**388–391.
2. **Dealler, S. F., M. Abbott, M. J. Croughan, and P. M. Hawkey.** 1989. Identification of *Branhamella catarrhalis* in 2.5 min with an indoxyl butyrate strip test. *J. Clin. Microbiol.* **27:**1390–1391.
3. **Janda, W. M., and P. Ruther.** 1989. B.CAT Confirm, a rapid test for confirmation of *Branhamella catarrhalis. J. Clin. Microbiol.* **27:**1130–1131.
4. **Louie, M., E. G. Ongsansoy, and K. R. Forward.** 1990. Rapid identification of *Branhamella catarrhalis.* A comparison of five rapid methods. *Diagn. Microbiol. Infect. Dis.* **13:**205–208.
5. **Perez, J. L., A. Pulido, F. Pantozzi, and R. Martin.** 1990. Butyrate esterase (4-methylumbelliferyl butyrate) spot test, a simple method for immediate identification of *Moraxella* (*Branhamella*) *catarrhalis* [corrected]. *J. Clin. Microbiol.* **28:**2347–2348.
6. **Speeleveld, E., J. M. Fosséprés, B. Gordts, and H. W. Van Landuyt.** 1994. Comparison of three rapid methods, tributyrine, 4-methylumbelliferyl butyrate, and indoxyl acetate, for rapid identification of *Moraxella catarrhalis. J. Clin. Microbiol.* **32:**1362–1363.
7. **Vaneechoutte, M., G. Verschraegen, G. Claeys, and P. Flamen.** 1988. Rapid identification of *Branhamella catarrhalis* with 4-methylumbelliferyl butyrate. *J. Clin. Microbiol.* **26:**1227–1228.

3.17.8 CAMP Factor Tests (Standard and Rapid) and the Reverse CAMP Test

I. PRINCIPLE

Streptococcus agalactiae produce a thermostable, extracellular, diffusible protein that acts synergistically with the beta-lysin produced by *Staphylococcus aureus* to produce a zone of enhanced lysis of sheep or bovine erythrocytes (1, 6). The protein was named CAMP factor for the initials of the authors of the manuscript that first described the phenomenon (3). The standard CAMP test relies on the elaboration of two toxins during growth to form a typical arrowhead or flame-shaped clearing at the juncture of the two organisms when they are placed perpendicular to each other (Fig. 3.17.8–1). The rapid test utilizes an extract of staphylococcal beta-lysin that acts directly with the CAMP factor previously diffused in the medium around the *S. agalactiae* colony. A positive CAMP reaction is indicated by an enhanced hemolysis within 30 min of adding the CAMP factor reagent (7, 11). The test is useful in the identification of both *S. agalactiae* and many gram-positive rods, including *Listeria monocytogenes.*

A reverse CAMP reaction is a reaction whereby hemolysis by the beta-hemolysin of staphylococci is inhibited through the production of phospholipase C or D by some organisms (e.g., *S. agalactiae*, *Listeria*, *Corynebacterium* spp., and *Clostridium perfringens*). An arrow of no hemolysis is formed at the junction of the organism being tested with the staphylococci if the reverse CAMP test is positive (Fig. 3.17.8–1) (2, 8).

II. MICROORGANISMS TESTED

A. Gram-positive cocci in pairs and chains that are catalase negative with medium-sized, smooth, whitish colonies with a small halo of beta-hemolysis. Nonhemolytic isolates also may be tested, since the CAMP factor can still be present.

B. Gram-positive rods, as part of their identification

C. Gram-positive cocci in clusters, as part of the identification of staphylococci to the species level, if *Staphylococcus intermedius* AB148 is used as the strain that supplies the hemolysin (9)

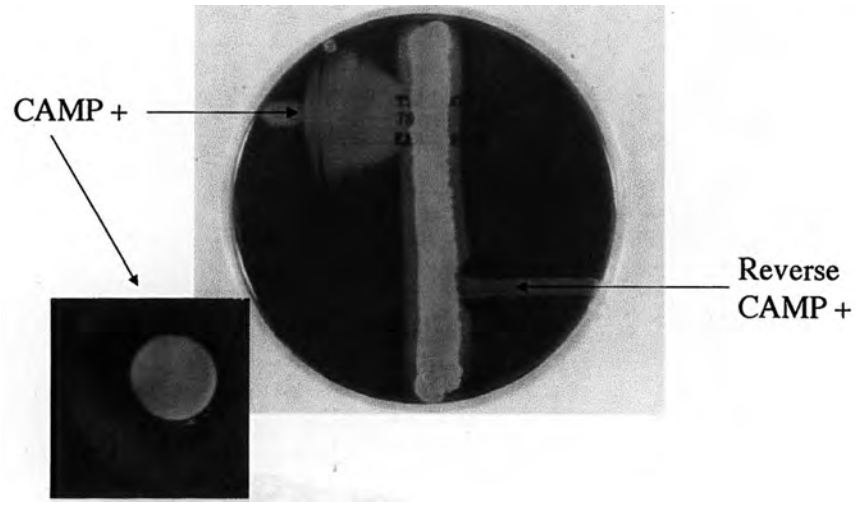


Figure 3.17.8–1 Demonstration of positive CAMP and reverse CAMP test, using *S. aureus* streak and CAMP disk.

III. MEDIA, REAGENTS, AND SUPPLIES

Include QC information on reagent container and in QC records.

A. BAP

B. Beta-lysin reagent

1. Culture of *S. aureus* ATCC 25923

2. Commercial reagents

a. Disks containing beta-lysin of *S. aureus* (catalog no. SS697; Remel, Inc.); store at 4°C (Fig. 3.17.8–1).

b. Spot CAMP liquid reagent (catalog no. Z206; Hardy Diagnostics)

3. Preparation of spot CAMP reagent (5)

a. Inoculate two 5-ml tubes of BHI with a swab of growth from a fresh subculture of *S. aureus* ATCC 25923.

b. Incubate overnight (shaking is optional) at 35°C in air.

c. Working with gloves in a laminar-flow safety cabinet, combine the two broth suspensions and filter sterilize them using a 0.45-μm-pore-size cellulose-acetate filter.

d. Aliquot the filter-sterilized broth into small tubes of about 1 ml.

e. Label with the name of reagent (CAMP), date of preparation, and expiration date of 6 months from preparation.

f. Freeze at −20°C or lower.

g. Store defrosted reagent at 4°C and use within 2 weeks. Do not refreeze.

IV. QUALITY CONTROL

A. Test each lot of rapid beta-lysin reagent with a positively and negatively reacting organism in the same manner as patient isolates are tested prior to putting the reagent into use.

B. Test each lot or shipment of commercial beta-lysin reagent or disks with a positively and negatively reacting organism in the same manner as patient isolates are tested prior to putting the reagent into use.

C. Test each lot or shipment of BAP with a positively reacting organism. This can be performed on the same plate as the test organism when the test is performed, by streaking the positive control in a line parallel to the test organism. Alternatively, if BAP is purchased from a commercial medium vendor, omit testing if vendor supplies documentation that BAP has tested acceptably for the CAMP reaction (4).

D. Organisms

1. *S. agalactiae* ATCC 12386—positive

2. *Streptococcus pyogenes* ATCC 19615—negative

3. Periodically use an in-house laboratory strain of *Arcanobacterium haemolyticum* to demonstrate the reverse CAMP test for training purposes.

V. PROCEDURE

Observe standard precautions.

A. Standard method

1. Streak *S. aureus* ATCC 25923 in a straight line across the center of the plate.

2. Streak the unknown microorganism in the same manner perpendicular to the staphylococcus, but avoid touching the previously streaked area.

3. Streak the positive control organism parallel to and approximately 1 in. from the unknown organism.

4. Label the location of each streak on the back of the plate.

5. Incubate the plate overnight at 35°C in a CO_2 incubator.

B. Disk method

1. Place disk on warmed BAP.

2. Streak microorganism 2 to 3 mm from the edge of the disk.

3. Incubate the plate overnight at 35°C in a CO_2 incubator.

C. Spot rapid method

1. Place 1 drop or a 10-μl loopful of reagent next to a presumptive *S. agalactiae* colony growing on BAP. Do not worry if the liquid touches or even engulfs the colony.

V. PROCEDURE *(continued)*

2. Incubate the plate right side up, to prevent the spot CAMP reagent from running over the plate's surface, for 20 min at 35°C.
3. Examine with transmitted light for a zone of enhanced hemolysis next to the colony.
4. Reincubate for up to 30 min if reaction is initially negative. Use a hand lens if necessary for examining the plate.
5. Refrigeration may enhance reaction after incubation.

VI. INTERPRETATION

A. A positive result in the standard assay is the formation of a distinct arrowhead of hemolysis at the intersection of the staphylococcus and test organism streaks (Fig. 3.17.8–1).

B. A positive reverse CAMP or phospholipase D is indicated by a distinct arrow of no hemolysis at the intersection of the two hemolytic organisms (Fig. 3.17.8–1).

C. In the disk test, a positive result is indicated by a distinct crescent- or arc-shaped zone of complete hemolysis at the intersection of the disk of beta-lysin and the isolate (Fig. 3.17.8–1).

D. In the rapid spot test, the presence of clear enhanced hemolysis only where the diffused hemolysis overlaps is a positive result.

E. Lack of enhanced hemolysis near the colony being tested is a negative test.

VII. REPORTING RESULTS

A. A streptococcus which gives a positive CAMP test and is morphologically and biochemically consistent (catalase-negative, gram-positive cocci in pairs and chains) is reported as *Streptococcus agalactiae*.

B. The following gram-positive rods are CAMP test positive: *Rhodococcus equi, L. monocytogenes, Propionibacterium avidum/granulosum, Actinomyces neuii, Turicella otitidis, Corynebacterium glucuronolyticum, Corynebacterium coyeae, Corynebacterium imitans,* and some strains of *Corynebacterium striatum* and *Corynebacterium afermentans* group.

C. *Corynebacterium pseudotuberculosis, Corynebacterium ulcerans,* and *A. haemolyticum* are reverse-CAMP positive.

D. *C. perfringens* is reverse CAMP or phospholipase D positive.

VIII. LIMITATIONS

A. Increased nonspecific hemolysis at the intersections (a "matchstick" effect) may be seen with other streptococci, but only group B streptococci will produce a definite arrowhead. Verify typical group B streptococcus, colony morphology, and hemolysis.

B. The test has a 98% sensitivity in detecting *S. agalactiae* (10). Isolates with a negative CAMP test could still be *S. agalactiae* and require further testing.

C. *S. pyogenes* can give a reaction that may be interpreted as positive. When there is a question, *S. pyogenes* is pyrrolidonyl-β-naphthylamide (PYR) positive but *S. agalactiae* is PYR negative.

D. The CAMP test separates *L. monocytogenes,* the human pathogen, from most other *Listeria* species.

E. If the agar is too thin or hemolyzed, the reaction may be very weak.

REFERENCES

1. **Bernheimer, A. W., R. Linder, and L. S. Avigad.** 1979. Nature and mechanism of action of the CAMP protein of group B streptococci. *Infect. Immun.* **23:**838–844.
2. **Buchanan, A.** 1982. Clinical laboratory evaluation of a reverse CAMP test for presumptive identification of *Clostridium perfringens*. *J. Clin. Microbiol.* **16:**761–762.
3. **Christie, R., N. E. Atkins, and E. Munch-Petersen.** 1944. A note on lytic phenomenon shown by group B streptococci. *Aust. J. Exp. Biol. Med. Sci.* **22:**197–200.
4. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Clinical and Laboratory Standards Institute.** 2008. *Abbreviated Identification of Bacteria and Yeast*, 2nd ed. Approved guideline M35-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
6. **Darling, C. L.** 1975. Standardization and evaluation of the CAMP reaction for the prompt, presumptive identification of *Streptococcus agalactiae*. *J. Clin. Microbiol.* **1:**171.
7. **DiPersio, J. R., J. E. Barrett, and R. L. Kaplan.** 1985. Evaluation of the spot-CAMP test for the rapid presumptive identification of group B streptococci. *Am. J. Clin. Pathol.* **84:**216–219.
8. **Hansen, M. V., and L. P. Elliott.** 1980. New presumptive identification test for *Clostridium perfringens*: reverse CAMP test. *J. Clin. Microbiol.* **12:**617–619.
9. **Hébert, G. A., C. G. Crowder, G. A. Hancock, W. R. Jarvis, and C. Thornsberry.** 1988. Characteristics of coagulase-negative staphylococci that help differentiate these species and other members of the family *Micrococcaceae*. *J. Clin. Microbiol.* **26:**1939–1949.
10. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 35–56. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.
11. **Ratner, H. B., L. S. Weeks, and C. W. Stratton.** 1986. Evaluation of spot CAMP test for identification of group B streptococci. *J. Clin. Microbiol.* **24:**296–297.

3.17.9 Carbohydrate Utilization Tests

I. PRINCIPLE

The ability of a microorganism to utilize carbohydrates can be key to its identification. There are a wide array of media available, using different pH indicators and nutrients. Usually the medium contains a protein source, pH indicator, and a 1% concentration of carbohydrate. The 1% concentration is optimal for most reactions and decreases the possibility of reversal reactions. Reversion occurs when the carbohydrate is depleted, thereby resulting in the masking of acid by-products by alkaline by-products of protein product utilization. It is very important that the correct base and indicator are used in testing so that the results will match the biochemical reactions expected from identification tables.

To detect fermentation with acid production of various sugars by gram-positive microorganisms, the medium used generally contains bromcresol purple (4), which turns from purple to yellow at pH 5.2. For gram-negative rods that ferment glucose, the preferred medium contains Andrade's indicator, acid fuchsin. The medium is straw colored and turns fuchsia-red when acid is produced. Andrade's indicator is very difficult to make correctly and must be aged for 6 months to produce the correct color (11). Commercial identification systems often use neutral red or phenol red, which turns from red to yellow with a drop in pH. Results using these indicators compare favorably with results with Andrade's indicator or the bromcresol purple base, and published table reactions generally apply whether media with Andrade's indicator, bromcresol purple, or phenol red or commercial systems are used for enterococcus, gram-positive rods, and gram-negative rods (reference 9, p. 426, 480, and 648, respectively). Since staphylococcus tables were created using the bromcresol purple indicator, results with commercial systems may be slightly different from the reactions in the tables (reference 9, p. 394).

Bacteria can be divided by their ability to degrade carbohydrates anaerobically (ferment) or aerobically (oxidize). Fermentation is a metabolic process in which an organic substrate serves as the final hydrogen acceptor rather than oxygen. Agar-based media are recommended for this determination, since the oxidative reaction can be observed at the top of the tube and fermentation can be observed in the bottom of the tube. The best medium to use for the determination of whether a gram-negative rod is an oxidizer or fermenter is either triple sugar iron agar (TSI) or Kligler's iron agar (KIA) (11). Fermenters will turn the butt yellow, while oxidizers and nonutilizers produce no change or alkalization of the butt in the tube. Once the organism is determined to be a fermenting rod, either Andrade's or neutral or phenol red base broths are used to determine which other sugars are fermented. Once an organism is determined to be non-glucose-fermenting, oxidative-fermentative (OF) medium is used to establish whether an organism can oxidize glucose or other sugars or does not produce acid from sugar fermentation. This medium contains bromthymol blue, which turns yellow when acid is produced from glucose (5). King (8) developed another medium with phenol red as the indicator, but it is not commercially available. OF basal medium is used to detect utilization of other sugars by non-glucose-fermenting rods. The recommended medium to determine whether a gram-positive rod is an oxidizer or fermenting rod is reported to be semisolid cystline Trypticase agar (CTA) with glucose, rather than KIA, TSI, or OF medium (reference 9, p. 479).

To complicate testing, some gram-negative organisms (generally those that do not grow on MAC) will not react in the KIA or TSI media, OF medium, or even Andrade's broth, because these bacteria are too fastidious for growth. In such cases rapid or conventional CTA media with phenol red indicator is the choice (6, 10) to detect the ability to utilize carbohydrates. The indicator turns from red to yellow at pH 6.8. The rapid medium does not require the growth of the organism to detect acid production but relies on preformed enzymes for the reactions. These microorganisms include *Neisseria; Haemophilus, Kingella, Capnocytophaga, Actinobacillus;* and lipid-requiring gram-positive rods (reference 9, p. 593, 609, and 479, respectively). Another alternative that is often used for demonstration of fermentation of fastidious gram-negative rods and gram-positive rods is to add a few drops of sterile rabbit serum to the Andrade's broth to allow the organism to grow. However, maltose cannot be tested since serum can split maltose (reference 9, p. 609). Carbohydrate reactions for *Neisseria* are problematic due to growth inhibition and poor oxidative metabolism; enzymatic tests are preferred (*see* Table 3.18.2–1 and reference 9, p. 593).

The production of gas from the fermentation of sugars can be determined by one of several methods. In agar-based tubes inoculated with gram-negative rods, disruption of the agar is evidence of gas production. If a Durham tube is inverted into broth, gas is apparent if the broth in the tube is displaced with air. When examining gram-positive bacteria, gas pro-

duction may be discerned with the use of melted paraffin overlay. The "hot loop" method is useful for fastidious gram-negative rods, such as *Haemophilus aphrophilus*, where the production is key to the identification (7). In this method, a red-hot inoculating needle is plunged near the side of the glass tube of a 48- to 72-h culture of the organism in THIO in a biological safety cabinet (11). Gas bubbles appear along the line of insertion to indicate positive gas from glucose. Occasionally organisms ferment lactose better than glucose and will demonstrate gas from lactose in the Durham tube when no gas is seen in the glucose tube.

II. MICROORGANISMS TESTED

A. Gram-positive rods and fermentative gram-negative rods
- **1.** Andrade's "Enteric fermentation medium" is preferred. Add rabbit serum for fastidious gram-negative rods.
- **2.** Heart infusion purple broth base medium can be used if the organism is not fastidious.
- **3.** Rapid sugar fermentation media and CTA are used for fastidious rods.

B. Gram-positive cocci (staphylococci, streptococci, enterococci)
- **1.** Generally these tests are present in commercial kits, and laboratories rarely stock sugar tests for identification.
- **2.** Use heart infusion purple broth base media for conventional testing (4).

◩ **NOTE:** *Leuconostoc* produces copious amounts of gas from glucose in MRS broth (procedure 3.17.32) as evidenced by displacement of a melted Vaspar, liquid paraffin or petroleum jelly plug (reference 9, p. 440). However, leucine aminopeptidase (LAP) (procedure 3.17.26) and arginine dehydrolase (procedure 3.17.15) testing can substitute for this test to separate *Leuconostoc* from *Weissella confusa* and *Pediococcus* (*see* Fig. 3.18.1–3).

C. Glucose-oxidizing gram-negative rods, which are generally oxidase positive and grow on MAC, as part of the identification process
- **1.** OF basal medium is used for testing.
- **2.** See TSI and KIA procedure (procedure 3.17.25) to determine if the organism is a glucose-fermenting organism rather than a glucose oxidizer before using the OF basal medium.

D. Identification of *Neisseria* species
- **1.** Phenol red-based media, such as rapid sugars and CTA
- **2.** The addition of ascitic fluid to CTA is recommended but not necessary.

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media
- **1.** Heart infusion purple broth
  - **a.** BHI, peptone, yeast extract, NaCl
  - **b.** Bromcresol purple, pH 7.4
  - **c.** 1% Carbohydrates, filter sterilized and added after sterilizing the broth

  ◩ **NOTE:** This medium was developed for streptococci. A plate medium with gelatin rather than agar and another protein source was developed for staphylococci and enteric bacteria.
- **2.** Enteric fermentation broth medium (1, 3)
  - **a.** Peptone, meat extract, and NaCl
  - **b.** Andrade's indicator, pH 7.2
  - **c.** 1% Carbohydrates, filter sterilized and added after sterilizing the broth
- **3.** CTA with or without ascitic fluid (11)
  - **a.** Cystine, Trypticase peptone, inorganic salts, and agar (low concentration for semisolid agar) available in dehydrated powder (BBL, BD Diagnostic Systems)
  - **b.** Phenol red, pH 7.3
  - **c.** The addition of ascitic fluid (or serum or yeast extract) is recommended for testing *Neisseria* species.
  - **d.** 1% Carbohydrates, filter sterilized and added after sterilizing and cooling the agar to 50°C
  - **e.** This medium is modified by adding 2% carbohydrates to CTA in a small-volume tube (Remel, Inc.) for rapid fermentation.

III. MEDIA, REAGENTS, AND SUPPLIES *(continued)*

Include QC information on reagent container and in QC records.

4. Rapid sugar broth medium

a. Formula for buffer-salt solution (6, 11)

K_2HPO_4 (anhydrous) ..0.04 g
KH_2PO_4 .....0.01 g
KCl .....0.8 g
1% phenol red, aqueous .....0.4 ml
distilled water .....100 ml
Adjust pH to 7.0. Filter sterilize and store at 4°C.

b. Prepare 20% carbohydrate solution in either distilled water or peptone broth (per liter: peptone, 10 g; meat extract, 3 g; NaCl, 5 g) and filter sterilize.

c. Add 1 drop (0.05 ml) of carbohydrate to 2 drops (0.1 ml) of buffer. Final concentration of sugars after adding 1 drop (0.05 ml) of culture will be 5%.

5. OF basal medium

a. Tryptone, inorganic salts, agar

◩ **NOTE:** Hugh and Leifson's formula (5) employs a low peptone/carbohydrate ratio and a minimal amount of agar (semisolid).

b. Bromthymol blue, pH 7.1 (color of medium is green)

c. 1% Carbohydrates, filter sterilized and added after sterilizing and cooling the agar to 50°C.

B. Other products

1. Carbohydrate oxidation tablets for glucose oxidizers (Key Scientific Products)

2. Carbohydrate fermentation, Wee-Tab (Key Scientific Products) for staphylococci, streptococci, and enteric rods, but not for *Neisseria* spp.

3. Commercial kits (2, 9; procedure 3.16)

4. Ten-millimeter disks with carbohydrate are available and can be added to the phenol red broth or semisolid basal medium or OF medium without carbohydrate after inoculation (BD Diagnostic Systems).

5. Aerobic low-peptone (ALP) medium (Hardy Diagnostics) for the identification of nonfermenting, gram-negative bacilli. ALP medium can be used to detect acidification of carbohydrates or alkalization of organic salts and amides.

C. Supplies

1. Rabbit serum (catalog no. 16120-099; Invitrogen Life Technologies, Carlsbad, CA). Heat for 1 h at 60°C prior to use.

2. Sterile tubes

3. Wire or disposable inoculating needles or loops

4. 35°C heat block or incubator without increased CO_2

IV. QUALITY CONTROL

A. Perform QC using a positively and negatively reacting organism on each new lot and shipment of media prior to putting them into use.

B. Inspect agar for evidence of prior freezing, contamination, cracks, dehydration, and bubbles prior to storage and before use. Discard any tubes with bubbles in the agar. Discard tubes that are not the appropriate color.

C. Check rabbit serum for sterility by inoculation onto CHOC.

D. Since carbohydrates are filter sterilized, check media and carbohydrate solutions for contamination when received or prepared.

E. Organisms

Because the number and type of organism used for QC are extensive, refer to the QC section of the manual (section 14, Table 14.2–2).

V. PROCEDURE

A. Warm medium to room temperature.

B. Inoculation

1. Purple broth and enteric fermentation broth medium

a. Before inoculating broth with Durham tube, check for trapped air bubbles. If necessary, invert tube until air bubbles are gone.

b. Inoculate (carbohydrate of choice) with isolated colonies from an 18- to 24-h pure culture of the organism.

c. Inoculate colony directly with a loop or wire or prepare a broth or saline suspension and inoculate with 1 drop per tube if a battery of carbohydrates is being tested.

V. PROCEDURE *(continued)*

d. Add 1 drop of rabbit serum for fastidious gram-negative rods or corynebacteria.

e. Incubate for up to 7 days at 35°C in a non-CO_2 incubator with caps loose.

2. CTA

a. Inoculation

(1) Standard media: with a loop, inoculate media heavily, mixing throughout the entire top 5 to 10 mm.

(2) Rapid test media: fill a 3-mm loop with organisms and vigorously inoculate below the surface of the agar.

(3) Inoculate a control tube without carbohydrate for comparison.

b. *Tighten caps.*

c. Incubate for 2 to 4 h or up to 24 h for rapid media and up to 7 days for standard media at 35°C in a non-CO_2 incubator or heat block (preferred).

3. Rapid sugar broth medium

a. Prepare a very heavy suspension of organism in buffer-salt solution (equivalent to no. 2 McFarland turbidity standard).

b. Inoculate 1 drop (0.05 ml) to each tube containing 2 drops (0.1 ml) of buffer-salt and 1 drop (0.05 ml) of 20% carbohydrate. Alternatively, inoculate the combined carbohydrate buffer-salt solution with a loopful of the suspected organism.

c. Inoculate 1 drop of distilled water and 1 drop of organism suspension to 2 drops of buffered salt as a control tube.

d. *Tighten caps.*

e. Incubate for 2 h or up to 4 h at 35°C in a heat block.

4. OF basal medium

a. With a needle, pick up a large amount of organism from an agar plate or slant and stab into the medium four or five times to a depth of 2.5 cm.

b. Inoculate a control tube without carbohydrate for comparison.

c. *Cap the tubes loosely* and incubate for 24 h and up to 7 days at 30°C in a non-CO_2 incubator or heat block (preferred).

◩ **NOTE:** Inoculation of a tube overlaid with oil, petroleum jelly, or Vaspar (advocated for gas production of *Leuconostoc*) is not recommended to differentiate fermentation from oxidation among gram-negative rods. Rather, use KIA, TSI, or glucose broth medium with serum in conjunction with OF medium to determine if the organism is a glucose-fermenting rod (11).

5. Carbohydrate oxidation tablets (COT) for glucose oxidizers

a. Add each tablet to 1 ml of distilled water in a small test tube.

b. Heat in a boiling water bath for 10 min. Cool before using.

c. For multiple carbohydrate tests, inoculate with a heavy bacterial suspension made in 1 to 2 ml of sterile distilled water. Transfer 1 or 2 drops of this suspension to each tube with a sterile pipette. For a single test, inoculate the tube with a loopful of the organism being tested without making a suspension.

d. *Cap the tubes loosely* and incubate at 37°C for 24 h and up to 4 days.

6. Wee-Tabs

a. Add one tablet to a small tube and add 0.25 ml of distilled water.

b. Inoculate with a loopful of the organism being tested. If doing multiple tests, make a heavy suspension of the organism into 1 ml of water and add a drop of this suspension to each tube.

c. Add oil overlay if desired.

d. Incubate at 35°C for 6 to 24 h.

◩ **NOTE:** Do not use Wee-Tabs for *Neisseria* or any organism that will not grow in high salt.

C. Examine for color change compared to control tubes without carbohydrate.

VI. INTERPRETATION

A. Purple broth
- **1.** Acid (positive) reaction: yellow (pH 5.2)
- **2.** Alkaline (negative) reaction: purple
- **3.** Gas production: bubbles in Durham tube

B. Andrade's enteric fermentation broth
- **1.** Acid (positive) reaction: pink to red (pH 5.0)
- **2.** Alkaline (negative) reaction: straw to tan (no change)
- **3.** Gas production: bubbles in Durham tube

C. CTA, rapid fermentation broth or agar, COT, carbohydrate fermentation Wee-Tabs
- **1.** Acid (positive) reaction: yellow (pH 6.8)
- **2.** Alkaline (negative) reaction: red or no change compared to control

D. OF medium
- **1.** Acid (positive) reaction: yellow (pH 6.0). Fermenters generally turn the entire tube yellow in 18 h, whereas oxidizers turn the top half of the tube yellow.
- **2.** Weak acid reaction: yellow-green to green compared to the control, which is more alkaline or blue-green
- **3.** Negative or nonoxidizer: inoculated control and carbohydrate tube are the same color.

VII. REPORTING RESULTS

A. Record each carbohydrate result separately. Indicate whether gas is produced or not. When using OF medium, also note whether the organism is an oxidizer, fermenter, or nonoxidizer.

B. *Acinetobacter* may be a nonoxidizer or an oxidizer.

C. *Neisseria, Pseudomonas, Stenotrophomonas,* and *Burkholderia* are glucose oxidizers.

D. *Enterobacteriaceae* are glucose fermenters.

E. Arabinose fermentation is important in the separation of *Enterococcus faecium* (positive) from *Enterococcus faecalis* (negative).

F. *Neisseria* species are separated by the utilization of glucose, maltose, lactose, and sucrose.

G. *Alcaligenes xylosoxidans* subsp. *xylosoxidans* oxidizes xylose but may not demonstrate oxidation of glucose.

VIII. LIMITATIONS

A. CTA basal medium without sugars is excellent for the maintenance of stock cultures at room temperature for several months (10).

B. Glucose oxidizers do not ferment other sugars.

C. If an organism appears to be a nonoxidizer in OF medium, it may be a fermenter that is not able to grow in OF medium. Repeat the test using fermentation media and rabbit serum to determine if the organism is a fermenter.

D. Inoculation of the basal medium without carbohydrate is important in the determination of true utilization of the sugar.

E. The Durham tube is usually placed in the glucose tube only, but it can be helpful in the lactose tube.

F. Even a small bubble indicates gas production.

G. Andrade's medium is not appropriate for testing *Neisseria* or streptococci.

REFERENCES

1. **Andrade, E.** 1906. Influence of glycerine in differentiating certain bacteria. *J. Med. Res.* **14:**551–556.
2. **Evangelista, A. T., A. L. Truant, and P. Bourbeau.** 2001. Rapid systems and instruments for the identification of bacteria, p. 22–49. *In* A. L. Truant (ed.), *Manual of Commercial Methods in Microbiology*. ASM Press, Washington, DC.
3. **Ewing, W. B., and B. R. Davis.** 1970. *Media and Tests for Differentiation of* Enterobacteriaceae. Center for Disease Control, Atlanta, GA.
4. **Facklam, R. R., and J. A. Elliott.** 1995. Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clin. Microbiol. Rev.* **8:**479–495.
5. **Hugh, R., and E. Leifson.** 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram-negative bacteria. *J. Bacteriol.* **66:**24–26.
6. **Kellogg, D. S., Jr., and E. M. Turner.** 1973. Rapid fermentation confirmation of *Neisseria gonorrhoeae. Appl. Microbiol.* **25:**550–552.
7. **King, E. O., and H. W. Tatum.** 1962. *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus. J. Infect. Dis.* **111:**85–94.
8. **King, E. O.** 1967. *Identification of Unusual Pathogenic Gram-Negative Bacteria.* Center for Disease Control, Atlanta, GA.
9. **Murray, P. R., E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.).** 2007. *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
10. **Vera, H. D.** 1948. A simple medium for identification and maintenance of the gonococcus and other bacteria. *J. Bacteriol.* **55:**531–536.
11. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed., p. 2, 7–19. Williams & Wilkins, Baltimore, MD.

3.17.10 Catalase Test

I. PRINCIPLE

Bacteria that synthesize the enzyme catalase hydrolyze hydrogen peroxide into water and gaseous oxygen, which results in the liberation of gas bubbles. The test is useful in the initial characterization of most bacteria (1, 2).

II. MICROORGANISMS TESTED

A. Young (18 h old, if possible) colonies of bacteria growing on agar media, preferably BAP or CHOC

B. For anaerobes, expose colonies to air for 30 min prior to testing.

C. See section 7 for catalase testing of mycobacteria.

III. REAGENTS AND SUPPLIES

A. Hydrogen peroxide reagent

1. 30% For *Neisseria* (4)

Caution: *30% H_2O_2 is extremely caustic to skin. If contact occurs, wash immediately with 70% ethyl alcohol, not water.*

2. 15% For anaerobes

3. 3% For other bacteria (purchase or dilute 30% 1:10 in deionized water prior to use)

◩ **NOTE:** 30% Reagent can be used for all tests, but it is more hazardous.

4. Store at 2 to 8°C.

B. Supplies

1. Glass slide

2. Sterile wooden sticks or plastic or platinum loops or wires

IV. QUALITY CONTROL

A. Perform QC on each new lot or shipment of reagent prior to putting it into use.

B. Organisms

1. *Staphylococcus aureus* ATCC 25923—catalase positive

2. *Streptococcus pyogenes* ATCC 19615—catalase negative

V. PROCEDURE

A. Touch the center of an 18- to 24-h, well-isolated colony to a clean glass slide.
  1. Be sure colony is visible to the naked eye on slide.
  2. If colony is from BAP, use care not to pick up blood.
B. Place 1 drop of peroxide reagent on slide and observe *immediately* for effervescence.
  1. Use a magnifying lens if necessary.
  2. Hold over dark background to enhance bubbles.
C. Discard slide into sharps container.

VI. INTERPRETATION

A. A positive test shows immediate appearance of bubbles.
B. A weak reaction has one or two bubbles.
C. A negative test shows no bubbles or a few bubbles after 20 s.

VII. REPORTING RESULTS

A. The catalase test separates staphylococci (positive) from streptococci and enterococci (negative).
B. *Bacillus* spp. are catalase positive, and *Clostridium* spp. are catalase negative.
C. The test is useful to separate among the fastidious gram-negative rods.
D. *Neisseria gonorrhoeae* produces an enhanced elaboration of bubbles not seen with other members of the genus due to superoxol (4).

VIII. LIMITATIONS

A. RBCs contain catalase. To avoid false-positive results, do not pick up blood agar with colony. If colony does not easily pick up or grow well, repeat the test from CHOC, which does not interfere with the assay.
B. Do not test from Mueller-Hinton agar (2).
C. Selecting colonies with some metal bacteriological loop materials will yield false-positive results; platinum loops do not yield false-positive results.
D. Because the enzyme is present in viable cultures only, do not test colonies that are older than 24 h. Older cultures may give false-negative results.
E. Do not reverse the order of adding the reagent to the colony; false-negative results can occur.
F. Do not mix the reagent and the colony.
G. Some strains of *S. aureus* may appear catalase negative by this method. See the aminolevulinic acid (ALA) test (procedure 3.17.3) for further testing to identify these problematic organisms.
H. To confirm the lack of catalase for *Gardnerella vaginalis*, Reimer and Reller (3) recommend streaking a CHOC plate as for disk susceptibility testing and adding a dot of viridans group streptococci (*Streptococcus sanguis* ATCC 35557). A clear zone of inhibition around a dot of viridans group streptococci confirms the lack of catalase.

REFERENCES

1. **Levin, M., and D. Q. Anderson.** 1932. Two new species of bacteria causing mustiness in eggs. *J. Bacteriol.* **23:**337–347.
2. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 78–97. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.
3. **Reimer, L. G., and L. B. Reller.** 1985. Use of a sodium polyanetholesulfate disk for the identification of *Gardnerella vaginalis. J. Clin. Microbiol.* **21:**146–149.
4. **Saginur, R., B. Clecner, J. Portnoy, and J. Mendelson.** 1982. Superoxol (catalase) test for identification of *Neisseria gonorrhoeae. J. Clin. Microbiol.* **15:**475–477.

3.17.11 Cetrimide Test

I. PRINCIPLE

Cetrimide is a quaternary ammonium, cationic detergent that is toxic to most bacteria, except *Pseudomonas aeruginosa* and a few other bacteria (1, 2). When cetrimide is in contact with bacteria, nitrogen and phosphorus are released from the bacterial cell. Organisms, other than *P. aeruginosa* and a few other pseudomonads, are unable to withstand this germicidal activity. Growth is observed on the inoculated slant of agar medium containing cetrimide for a positive result. Magnesium chloride and potassium sulfate in the medium enhance the production of pyocyanin and pyoverdin (fluorescein) by *P. aeruginosa.*

II. MICROORGANISMS TESTED

Isolated colonies of non-glucose-fermentative, gram-negative rods that are suggestive of *P. aeruginosa*

III. MEDIA, REAGENTS, AND SUPPLIES

A. Medium

1. Agar slants containing the following ingredients per liter of deionized water

pancreatic digest of gelatin	20.0 g
K_2SO_4	10.0 g
$MgCl_2$	1.4 g
cetyltrimethylammonium bromide	0.3 g
agar	13.6 g
glycerin	10.0 ml

2. Four milliliters of a 22.5% solution of cetrimide (0.9 g of hexadecyltrimethylammonium) can substitute for the cetyltrimethylammonium bromide.

3. Final pH, 7.2

4. Store at 2 to 8°C.

B. Supplies

1. Sterile inoculating loops or sticks

2. Wood's or UV light (360 nm) or short-wavelength (254-nm) UV light (preferred)

IV. QUALITY CONTROL

A. Perform QC on each new lot or shipment of media prior to putting it into use.

B. Inspect agar for evidence of freezing, contamination, cracks, dehydration, and bubbles prior to storage and before use.

C. Organisms

1. *P. aeruginosa* ATCC 27853—growth; yellow-green to blue pigment

2. *Escherichia coli* ATCC 25922—inhibited

V. PROCEDURE

A. Streak the slant back and forth with inoculum picked from the center of a well-isolated colony.

B. *Place cap loosely on tube.*

C. Incubate aerobically at 35 to 37°C for up to 7 days.

D. Observe for growth and pigment.

V. PROCEDURE *(continued)*

E. If no pigment is visible, examine growth under UV light for the presence of fluorescein.

F. If negative for pigment at 24 h, incubate additional days at 25°C in the dark to enhance pigment production.

VI. INTERPRETATION

A. Positive: growth. Optionally a yellow-green (fluorescein) to dark blue-green (pyocyanin) color may be observed.

B. Negative: no growth

VII. REPORTING RESULTS

A. *P. aeruginosa* is definitively identified if an oxidase-positive, gram-negative rod grows on cetrimide agar and produces a blue-green (pyocyanin) pigment.

B. *Pseudomonas fluorescens* and *Pseudomonas putida* may also grow and may produce a fluorescent pigment on this medium (2) but are separated from *P. aeruginosa* because they do not grow at 42°C.

VIII. LIMITATIONS

A. Growth on this medium alone is not sufficient for identification of *P. aeruginosa* to the species level, since other non-glucose-fermenting species (e.g., *Achromobacter xylosoxidans* subsp. *xylosoxidans* and *Alcaligenes faecalis*) may grow. Pigment must also be present.

B. Lack of growth on cetrimide agar does not rule out an identification of *P. aeruginosa.*

REFERENCES

1. **Lowburg, E. J. L.** 1955. The use of cetrimide product in a selective medium for *Pseudomonas pyocyanea. J. Clin. Pathol.* **8:**47–48.
2. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria,* 2nd ed., p. 6. Williams & Wilkins, Baltimore, MD.

3.17.12 Citrate Utilization Test (Simmons)

I. PRINCIPLE

Citrate agar is used to test an organism's ability to utilize citrate as a source of energy. The medium contains citrate as the sole carbon source and inorganic ammonium salts as the sole source of nitrogen (3). Growth is indicative of utilization of citrate, an intermediate metabolite in the Krebs cycle. When the bacteria metabolize citrate, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns the bromthymol blue indicator in the medium from green to blue above pH 7.6 (2). This medium is recommended as part of differentiating among the species of *Enterobacteriaceae.* With the exception of a few species, *Salmonella, Edwardsiella, Citrobacter, Klebsiella, Enterobacter, Serratia,* and *Providencia* usually give a positive reaction and *Escherichia, Shigella, Morganella,* and *Yersinia* give a negative reaction. *Proteus* is citrate variable.

II. MICROORGANISMS TESTED

Enterobacteriaceae, as part of the identification to the species level

III. MEDIA, REAGENTS, AND SUPPLIES

A. Medium

1. Agar slants containing citrate, ammonium salts, buffer, and bromthymol blue
2. Store at 2 to 8°C.
3. Final pH, 6.8

B. Supplies

1. Sterile inoculating loops or sticks
2. Incubator at 35°C

IV. QUALITY CONTROL

A. Perform QC on each new lot or shipment of media prior to putting it into use.

◩ **NOTE:** The Clinical and Laboratory Standards Institute (formerly NCCLS) has proposed elimination of user QC for citrate agar purchased from commercial sources. Consult with current regulatory agencies prior to discontinuation of user QC (1).

B. Inspect agar for evidence of freezing, contamination, cracks, dehydration, and bubbles prior to storage and before use. Discard any tubes that are blue.

C. Organism

1. *Klebsiella pneumoniae* ATCC 13883—citrate positive (growth; blue color)
2. *Escherichia coli* ATCC 25922—citrate negative (no growth or trace of growth)

V. PROCEDURE

A. Streak the slant back and forth with a light inoculum picked from the center of a well-isolated colony.

B. *Place cap loosely on tube.*

C. Incubate aerobically at 35 to 37°C for up to 4 days.

D. Observe a color change from green to blue along the slant.

VI. INTERPRETATION

A. A positive test is growth with color change from green to intense blue along the slant.
B. A negative test is no growth and no color change; slant remains green.

VII. REPORTING RESULTS

A. *E. coli* is citrate negative.
B. Many other *Enterobacteriaceae* are citrate positive.

VIII. LIMITATIONS

A. Luxuriant growth on the slant without an accompanying color change may indicate a positive test. However, if the agar does not turn blue on further incubation, the test should be repeated with less inoculum.
B. Tests with equivocal results should be repeated.
C. Do not stab the slant, since the test requires an aerobic environment.
D. Do not inoculate from broth cultures, due to carryover of media.
E. To avoid false-positive reactions, use a light inoculum to prevent carryover of substances from previous media.
F. The reactions of this medium alone are not sufficient for identification to the species level.

REFERENCES

1. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
2. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria,* 3rd ed., p. 98–104. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.
3. **Simmons, J. S.** 1926. A culture medium for differentiating organisms of typhoid-colon-aerogenes groups and for isolation of certain fungi. *J. Infect. Dis.* **39:**209–214.

3.17.13 Coagulase Test—Protein A/ Clumping Factor Agglutination Method

I. PRINCIPLE

Staphylococcus aureus is separated from other species of staphylococci by the presence of coagulase, which is demonstrated in the 4- to 24-h tube test to detect free coagulase. Clumping factor, termed bound coagulase, can be detected rapidly in the slide test, but this test requires several colonies and lacks sensitivity. *S. aureus* produces another substance in its cell wall, protein A, which binds to the FC moiety of human immunoglobulin G (IgG) (7). If latex or erythrocyte particles are coated with IgG and with human fibrinogen, a staphylococcus will agglutinate if either clumping factor or protein A is present in the bacterial cell wall (1–5, 11). Other species, such as *Staphylococcus lugdunensis* and *Staphylococcus schleiferi* subsp. *schleiferi*, produce clumping factor and can be positive in the assay. These species are important in serious infections and require that positive agglutination results be confirmed with other tests or observations. Still other staphylococci possess protein A, including *Staphylococcus saprophyticus*, so the test should be performed restrictively on urinary isolates.

More recently, it was discovered that some *S. aureus* organisms, especially methicillin-resistant *S. aureus* (MRSA), can mask the cell wall with capsular polysaccharides (6, 10) and prevent the reaction between the IgG or fibrinogen. Newer kits have added antibodies to the latex or erythrocytes, which bind to the specific bacterial surface antigens that have been reported to interfere with the test. These new reagents are more sensitive but have reduced the specificity further (9, 12, 13). In the United States these clones of MRSA, having the specific surface antigens, are less common. Thus, most laboratories choose to use tests with higher specificity and use the tube coagulase test when it is important to detect MRSA (13).

Despite all the problems with agglutination tests, they are very useful in rapid identification of staphylococci and in increasing the sensitivity of the identification of *S. aureus*, since there are also problems of sensitivity with the tube and slide coagulase tests (procedure 3.17.14).

II. MICROORGANISMS TESTED

A. Colonies of gram-positive cocci in clusters which are catalase positive, as part of the identification of *S. aureus*

B. From urine cultures, use test with caution on nonhemolytic colonies that are presumptive for staphylococci, since *S. saprophyticus* may give a false-positive result.

C. Only test colonies that are less than 24 h old and are not from mannitol salt agar.

III. REAGENTS AND SUPPLIES

A. Reagents

1. Products

- **a.** Murex Staphaurex and Staphaurex Plus (Wellcome Diagnostics; Remel, Inc.)
- **b.** Prolex Staph kit (Pro-Lab Diagnostics, Austin, TX)
- **c.** StaphTex (Hardy Diagnostics)
- **d.** Bacti Staph (Remel, Inc.)
- **e.** Staphyloslide (BD Microbiology Systems)
  This is a hemaglutination test rather than a latex test.
- **f.** Slidex Staph kit and Slidex Staph Plus (bioMérieux Vitek, Hazelwood, MO)

◩ **NOTE:** "Plus" indicates that the additional antibodies to surface antigens have been used to coat the particles.

2. Test suspension

- **a.** Store at 2 to 8°C in an upright position.
- **b.** Do not freeze. Protect reagents from bright light.

III. REAGENTS AND SUPPLIES *(continued)*

c. Do not *vortex* latex reagents.
d. Store at workbench on cold pack during working hours and return to 4°C for overnight storage.

3. Some kits have a negative control latex of unsensitized particles to detect autoagglutination.

B. Supplies

1. Loops or sterile sticks
2. Black coated cards (supplied by vendors for serologic agglutination tests); white cards for erythrocyte suspensions

IV. QUALITY CONTROL

A. Inspect suspension for granularity each time the test is performed, as it is dropped onto the test card. If there is evidence of autoagglutination, do not use the suspension.
B. At a minimum, perform QC on each new lot or shipment of reagent prior to putting it into use.
C. Organisms
1. *Staphylococcus aureus* ATCC 25923—agglutination positive
2. *Staphylococcus epidermidis* ATCC 12228 or ATCC 14990—agglutination negative

V. PROCEDURE

◩ **NOTE:** Verify any differences between this general procedure and the package insert prior to using test.

A. Warm the reagents to room temperature.
B. Invert the reagent bottle several times to obtain an even suspension.
C. Dispense a drop of the test suspension into a circle on the reaction card for each culture to be tested.
D. From a plate that is <24 h old, touch two to four colonies with the flat end of a stick.
E. Emulsify the sample of culture in the suspension and spread over a 10-mm area of the circle.
F. If an autoagglutination control is included in the kit, repeat the above steps with the control reagent.
G. Rotate the card gently for up to, *but no longer than*, 20 s and examine for agglutination. Do not use a magnifying lens.
H. Dispose of the card in infectious-waste container.

VI. INTERPRETATION

A. A positive result is indicated by the development of an agglutination pattern showing rapid strong clumping of the particles with *clearing* of the background. Most positive results are instantaneous.
B. A negative result is indicated when the particles do not agglutinate and the appearance remains substantially unchanged throughout the test.
C. If the control test shows clumping or the test shows clumping but the background remains unchanged, autoagglutination has likely occurred. Perform a tube coagulase test.

VII. REPORTING RESULTS

A. Only test gram-positive cocci in clusters which are catalase positive. Report as *Staphylococcus aureus* if the test is positive and the isolate is clearly beta-hemolytic.

B. If the test is positive and the colony is nonhemolytic and from a urine specimen or a normally sterile site, confirm with tube coagulase. In a urine specimen, this may be *S. saprophyticus.* In a blood culture, it may be *S. lugdunensis* or another coagulase-negative staphylococcus.

C. Report negative tests from catalase-positive, gram-positive cocci in clusters as "coagulase-negative staphylococci," but confirm with a tube test if the isolate is from a normally sterile body site, especially if it is methicillin resistant.

VIII. LIMITATIONS

A. The advantage of agglutination assays is that they are very sensitive and rapid in separating *S. aureus* from coagulase-negative staphylococci. False-positive reactions do occur with strains of *Staphylococcus capitis, S. saprophyticus,* and *Staphylococcus warneri,* since these species possess protein A (2). *S. lugdunensis* and *S. schleiferi* can also give a positive result, since they possess clumping factor. Positive tests must be confirmed with the tube test for nonhemolytic strains from urine specimens or for all isolates from sites where identification to the species level is critical for clinical reasons (e.g., blood cultures).

B. Although *S. lugdunensis* and *S. schleiferi* produce clumping factor, the reaction is less efficient in the latex test and cannot be relied upon to detect these species. Use other methods, such as pyrrolidonyl-β-naphthylamide (PYR), to detect these strains in invasive sites.

C. A false-negative test, especially for MRSA with capsular antigens, can result. For isolates from significant sites where MRSA is important, e.g., blood and joint isolates, performing both the agglutination and the tube coagulase test will detect all strains of *S. aureus.*

D. *Staphylococcus intermedius* and *Staphylococcus hyicus* may agglutinate the latex reagent, but it is clinically not important to separate these animal pathogens from *S. aureus,* because they are rarely found in humans (less than 0.1% in one study [8]) and they are considered as pathogenic as *S. aureus.*

E. Do not perform testing from growth on mannitol salt agar.

REFERENCES

1. **Baker, J. S., M. A. Bormann, and D. H. Boudreau.** 1985. Evaluation of various rapid agglutination methods for the identification of *Staphylococcus aureus. J. Clin. Microbiol.* **21:**726–729.
2. **Berke, A., and R. C. Tilton.** 1986. Evaluation of rapid coagulase methods for the identification of *Staphylococcus aureus. J. Clin. Microbiol.* **23:**916–919.
3. **Dickson, J. I., and R. R. Marples.** 1986. Coagulase production by strains of *Staphylococcus aureus* of differing resistance characters: a comparison of two traditional methods with a latex agglutination system detecting both clumping factor and protein A. *J. Clin. Pathol.* **39:**371–375.
4. **Essers, L., and K. Radebold.** 1980. Rapid and reliable identification of *Staphylococcus aureus* by a latex agglutination test. *J. Clin. Microbiol.* **12:**641–643.
5. **Lairscey, R., and G. E. Buck.** 1987. Performance of four slide agglutination methods for identification of *Staphylococcus aureus* when testing methicillin-resistant staphylococci. *J. Clin. Microbiol.* **25:**181–182.
6. **Lally, R., and B. Woolfrey.** 1984. Clumping factor defective MRSA. *Eur. J. Clin. Microbiol.* **3:**151–152.
7. **Langone, J. J.** 1982. Protein A of *Staphylococcus aureus* and related immunoglobulin receptors produced by streptococci and pneumonococci. *Adv. Immunol.* **32:**157–252.
8. **Mahoudeau, I., X. Delabranche, G. Prevost, H. Monteil, and Y. Piemont.** 1997. Frequency of isolation of *Staphylococcus intermedius* from humans. *J. Clin. Microbiol.* **35:**2153–2154.
9. **Personne, P., M. Bes, G. Lina, F. Vandenesch, Y. Brun, and J. Etienne.** 1997. Comparative performances of six agglutination kits assessed by using typical and atypical strains of *Staphylococcus aureus. J. Clin. Microbiol.* **35:**1138–1140.
10. **Ruane, P. J., M. A. Morgan, D. M. Citron, and M. E. Mulligan.** 1986. Failure of rapid agglutination methods to detect oxacillin-resistant *Staphylococcus aureus. J. Clin. Microbiol.* **24:**490–492.

REFERENCES *(continued)*

11. **Smith, S. M., and C. Berezny.** 1986. Comparative evaluation of identification systems for testing methicillin-resistant strains of *Staphylococcus aureus. J. Clin. Microbiol.* **24:**173–176.
12. **Smole, S. C., E. Aronson, A. Durbin, S. M. Brecher, and R. D. Arbeit.** 1998. Sensitivity and specificity of an improved rapid latex agglutination test for identification of methicillin-sensitive and -resistant *Staphylococcus aureus* isolates. *J. Clin. Microbiol.* **36:**1109–1112.
13. **Wilkerson, M., S. McAllister, J. M. Miller, B. J. Heiter, and P. P. Bourbeau.** 1997. Comparison of five agglutination tests for identification of *Staphylococcus aureus. J. Clin. Microbiol.* **35:**148–151.

3.17.14 Coagulase Test—Rabbit Plasma Method

I. PRINCIPLE

Staphylococcus aureus, the most pathogenic of the staphylococci, is separated from other species by the presence of coagulase. Coagulase is a thermostable thrombin-like substance that activates fibrinogen to form fibrin, resulting in a fibrin clot (2, 4). This is demonstrated in the test tube by the formation of a clot when plasma is inoculated with the staphylococcus. The substance is known as free coagulase, since it is liberated by the cell. In most, but not all, *S. aureus* organisms, fibrinogen binding cell surface receptor is also present in cell wall, called "bound coagulase" or "clumping factor." Clumping factor is demonstrated by the ability of the organism to act directly on the fibrinogen in the plasma to clump it in a slide assay. The test for clumping factor is rapid but requires several colonies, and as stated above, the factor is not present in all *S. aureus* organisms. In addition, clumping factor can be masked by cell surface capsular polysaccharides and can be present in other species, namely, *Staphylococcus lugdunensis* and *Staphylococcus schleiferi* (7). Negative slide tests must be followed with a confirmatory tube test.

Coagulase is also present in highly pathogenic species of staphylococci from animals: *Staphylococcus intermedius* from dogs, *Staphylococcus delphini* from dolphins, *Staphylococcus lutrae* from otters, and *Staphylococcus hyicus* from pigs. It is generally not cost-effective to separate these species in most human specimens, because infections with them either are very rare or present with morbidity similar to that of *S. aureus* (5).

In addition, the coagulase clot can be destroyed by *S. aureus* fibrinolysin or staphylokinase, a plasmid-carried enzyme which is more active at 35°C than at 25°C (2, 6, 8). Despite these caveats, algorithms can easily be implemented in the laboratory to rapidly identify *S. aureus* with a high degree of accuracy.

II. MICROORGANISMS TESTED

A. Colonies of gram-positive cocci in clusters which are catalase positive, as part of the identification of *S. aureus*

B. Positive blood cultures containing gram-positive cocci in clusters for rapid detection of *S. aureus*

III. REAGENTS AND SUPPLIES

Include QC information on reagent container and in QC records.

A. Plasma

◩ **NOTE:** Do not use human plasma for the test, as it is less sensitive and potentially infectious with human pathogenic viruses (6, 8).

1. Dehydrated and reconstituted rabbit plasma in EDTA
   - **a.** Rehydrate according to manufacturer's instructions.
   - **b.** Dispense 0.5 ml into sterile 12- by 75-mm tubes.
   - **c.** Reagent expires after 1 month if stored at −20°C or 5 days if stored at 2 to 8°C.

2. Frozen rabbit plasma with EDTA (Coagulase Cryovial; Hardy Diagnostics)

3. 5% $CaCl_2$ (optional)

B. Supplies

1. Loops or sterile sticks

2. Glass or plastic tubes

3. Glass slides or black coated cards (supplied by vendors for serologic agglutination tests)

IV. QUALITY CONTROL

A. Do not use plasma that has not been stored refrigerated or frozen or that appears turbid.

B. Perform QC of coagulase plasma on new lots prior to putting them into use.

C. Organisms

1. *S. aureus* ATCC 25923—coagulase positive

2. *Staphylococcus epidermidis* ATCC 12228 or ATCC 14990—coagulase negative

V. PROCEDURE

A. Slide test

◩ **NOTE:** To avoid misidentifications, only perform this test on classic-looking white to yellow, creamy, opaque, *hemolytic* colonies of gram-positive cocci in clusters that are catalase positive. Hemolysis should only be observed on fresh BAP at ≤18 h.

1. Add 10 μl of deionized water to slide or black card.

2. Emulsify several colonies into the water to obtain a smooth milk-colored suspension.

◩ **NOTE:** If clumps occur and the organism does not suspend in the water, the slide test cannot be performed. The organism is inherently sticky and is said to autoagglutinate. Perform the tube test for free coagulase instead.

3. Add 1 to 3 μl of rabbit plasma and observe for clumping immediately, not to exceed 10 s.

4. Dispose of the card in an infectious-waste container or the slide in a sharps container.

B. Tube test

1. Bring tube of plasma to 25°C.

2. Inoculate with one colony of staphylococcus growing on *noninhibitory medium* or 2 drops of blood from a positive blood culture.

3. Incubate at 35°C without CO_2 for *up to* 4 h and observe for clot formation hourly. Do not agitate the tube during observations; rather, gently tilt to observe the clot.

4. Incubate for an additional 20 h at 25°C for late clot formation (2).

◩ **NOTE:** Do not leave the test at 35°C for more than 4 h, since *S. aureus* fibrinolysin can lyse the clot. If 4 h of incubation at 35°C is inconvenient for staffing, the test is most sensitive when incubated at 25°C for the entire time, but the clot may take longer to form (1). Alternatively, if the tube is left at 35°C, add a drop or two of 5% $CaCl_2$ at 24 h. If a clot forms, the isolate is coagulase negative; if a clot does not form, fibrinolysin has lysed a previously formed clot and this result confirms the isolate as coagulase positive (H. D. Isenberg, unpublished data).

VI. INTERPRETATION

A. Slide test

1. A positive test is the demonstration of agglutination of the bacterial cells after the plasma is added.

2. A negative test is demonstrated by the lack of agglutination.

B. Tube test

1. A positive test meets one of the following criteria.

a. Complete clot formation or any degree of clot formation before 24 h

b. No clot formation after addition of 1 or 2 drops of 5% $CaCl_2$ to a tube without a clot at 24 h

VI. INTERPRETATION
(continued)

2. A negative test meets one of the following criteria.
- **a.** A lack of clot formation at 24 h at 25°C
- **b.** No clot after 24 h at 35°C, but after addition of 1 or 2 drops of 5% $CaCl_2$ to the tube, a clot forms.

◩ A flocculent or fibrous precipitate is not a true clot and is recorded as a negative test.

VII. REPORTING RESULTS

A. Report as *Staphylococcus aureus* if the tube test is positive and the organism is catalase positive and a gram-positive coccus in clusters.

B. For gram-positive cocci in clusters from a positive blood culture that are coagulase positive, report as "Probable *Staphylococcus aureus;* confirmation to follow" until the colony can be observed and the catalase test is performed.

C. For a negative tube test from catalase-positive, gram-positive cocci in clusters that have creamy white colonies, report as "coagulase-negative staphylococci."

D. A positive slide test is reported as *S. aureus;* however, the test should be confirmed with a tube test from nonhemolytic or only slightly hemolytic colonies from sterile sites, such as blood, to separate *S. aureus* from *S. lugdunensis* and *S. schleiferi.*

E. A negative slide test is not valid and should be followed with a tube test for confirmation.

VIII. LIMITATIONS

A. Methicillin-resistant *S. aureus* can be deficient in bound coagulase, which results in a negative slide test (3).

B. *S. intermedius* and *S. hyicus* may be positive in the tube test; these species are generally found only in dogs and pigs, respectively, but are as infectious as *S. aureus* when they infect humans. Both form nonhemolytic colonies on fresh (≤18-h) plates and are Voges-Proskauer negative, which separates them from *S. aureus. S. intermedius* is also pyrrolidonyl-β-naphthylamide (PYR) positive (Table 3.18.1–1).

C. *S. lugdunensis* and *S. schleiferi* produce slide coagulase, but the reaction is more efficient if human plasma is used rather than rabbit plasma. They can be separated from *S. aureus* by their strongly positive PYR reaction and from *S. intermedius* by a negative tube coagulase test (*see* Table 3.18.1–1).

D. Do not use citrated blood, as false-positive results can occur.

E. Coagulase testing cannot be performed from growth on mannitol salt agar.

REFERENCES

1. **Baker, J. S., M. A. Bormann, and D. H. Boudreau.** 1985. Evaluation of various rapid agglutination methods for the identification of *Staphylococcus aureus. J. Clin. Microbiol.* **21:**726–729.
2. **Cowan, S. T.** 1938. The classification of staphylococci by precipitation and biological reactions. *J. Pathol. Bacteriol.* **46:**31–45.
3. **Lally, R., and B. Woolfrey.** 1984. Clumping factor defective MRSA. *Eur. J. Clin. Microbiol.* **3:**151–152.
4. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria,* 3rd ed., p. 105–119. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.
5. **Mahoudeau, I., X. Delabranche, G. Prevost, H. Monteil, and Y. Piemont.** 1997. Frequency of isolation of *Staphylococcus intermedius* from humans. *J. Clin. Microbiol.* **35:**2153–2154.
6. **Orth, D. S., L. R. Chung, and A. W. Anderson.** 1971. Comparison of animal sera for suitability in coagulase testing. *Appl. Microbiol.* **21:**420–425.
7. **Patel, R., K. E. Piper, M. S. Rouse, J. R. Uhl, F. R. Cockerill III, and J. M. Steckelberg.** 2000. Frequency of isolation of *Staphylococcus lugdunensis* among staphylococcal isolates causing endocarditis: a 20-year experience. *J. Clin. Microbiol.* **38:**4262–4263.
8. **Yrios, J. W.** 1977. Comparison of rabbit and pig plasma in the tube coagulase test. *J. Clin. Microbiol.* **5:**221–224.

3.17.15 Decarboxylase-Dihydrolase Tests

I. PRINCIPLE

Arginine, lysine, and ornithine decarboxylase media are used to detect an organism's ability to decarboxylate or hydrolyze an amino acid, forming an amine that produces an alkaline pH. The basal medium is usually Møller's formula (7) and contains meat peptones and beef extract, which supply nitrogenous nutrients to support bacterial growth. Glucose is the fermentable carbohydrate. The pH indicators are bromcresol purple and cresol red. Pyridoxal is an enzyme cofactor which enhances decarboxylase activity. Arginine, lysine, and ornithine are amino acids that are singly added to basal medium to detect the production of enzymes which decarboxylate or hydrolyze these substrates. When an organism in the medium ferments glucose, acids are produced which lower the pH, resulting in a color change from purple to yellow. If decarboxylation or hydrolysis of the amino acid occurs in response to the acid pH, alkaline end products (amines) are formed which result in the medium reverting to its original color (purple). When the organism does not ferment glucose, the medium does not turn yellow; the test can still be performed, but it is important to include a control without amino acids for comparison.

Decarboxylation of lysine yields cadaverine, decarboxylation of ornithine yields putrescine, and decarboxylation of arginine yields agmatine, which is hydrolyzed by a dihydrolase to form putrescine. In another reaction arginine dihydrolase converts arginine to citrulline, which is converted to ornithine and then to putrescine. Since decarboxylation is an anaerobic reaction, the contents of each tube must be sealed with oil or paraffin.

II. MICROORGANISMS TESTED

A. Enteric gram-negative rods and *Vibrio*, *Plesiomonas*, and *Aeromonas* for identification to the species level
B. Probable *Stenotrophomonas* and *Burkholderia* (lysine and arginine)
C. Fluorescent *Pseudomonas* (arginine)
D. Coagulase-negative staphylococci (ornithine)
E. Viridans group streptococci (arginine)
F. Miscellaneous non-glucose-fermenting, gram-negative rods (arginine)
G. Spreading indole-negative *Proteus* (ornithine)

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media

1. Peptone, beef extract, glucose, pyridoxal with bromcresol purple and cresol red indicators, and amino acids as listed below
  a. Møller's decarboxylase base (no amino acids)
  b. Møller's arginine decarboxylase (1% arginine)
  c. Møller's lysine decarboxylase (1% lysine)
  d. Møller's ornithine decarboxylase (1% ornithine)

2. Motility-indole-ornithine medium (MIO) (*see* motility tests [procedure 3.17.31] for inoculation method)

3. Tablets (Key Scientific Products) Refer to http://www.keyscientific.com for procedure.

III. MEDIA, REAGENTS, AND SUPPLIES *(continued)*

4. Lysine iron agar
Used as a screen for stool pathogens to detect lysine decarboxylase-positive (purple color in butt) *Salmonella;* however, the medium is also helpful to distinguish *Salmonella* from *Proteus* and *Providencia,* which deaminate lysine and produce a red slant.

B. Reagents
1. Mineral oil
2. Vaspar, liquid paraffin, or petroleum jelly, maintained at 56°C in liquid form

C. Other supplies
1. Sterile sticks or inoculating loops
2. Incubator at 35°C

IV. QUALITY CONTROL

A. Examine media for lack of turbidity and purple color. Discard if not purple.
B. Test new lot or shipment of medium with a positive and negative control prior to putting it into use. Use the staphylococcus controls for validating use with staphylococci.
C. Organisms

Test organism	Control tube	Result[a]		
		Arginine	Lysine	Ornithine
Klebsiella pneumoniae ATCC 13883	Yellow	−	+	−
Enterobacter cloacae ATCC 23355	Yellow	+	−	+
Staphylococcus aureus ATCC 25923	Yellow	NA	NA	−
Staphylococcus lugdunensis ATCC 700328	Yellow	NA	NA	+

[a] −, yellow; +, purple; NA, not applicable.

V. PROCEDURE

A. Inoculate each broth being tested with one or two colonies from an 18- to 24-h culture.
1. For non-glucose-fermenting, gram-negative rods
a. Include the control tube
b. Inoculate the tubes *heavily* (≥no. 8 McFarland turbidity standard [7]; *see* Appendix 3.16–1).
2. For *Enterobacteriaceae,* the control tube is not needed, since all strains ferment glucose.
3. For gram-positive cocci, the control tube is usually not needed.
B. Overlay the inoculated tubes with approximately 1 ml of mineral oil or a 4-mm plug of melted petroleum jelly, Vaspar, or paraffin, being careful to cover broth layer entirely without introducing air.
C. Tighten the caps on the tubes.
D. Incubate aerobically at 35°C for at least 18 h and up to 7 days, and observe daily for purple color.

◩ **NOTE:** MIO is stabbed with a wire; oil overlay is not necessary if reactions are read only at 18 h. Incubation and color interpretations are the same as for broth media. Refer to http://www.keyscientific.com for procedure for tablets.

VI. INTERPRETATION

A. A positive test is turbid purple to faded-out yellow-purple color (alkaline).
B. A negative test is bright clear yellow color (acid) or no change (nonfermenting rods).
C. Control tube must remain its original color or turn yellow. Turbidity must be seen. An alkaline or purple color in the control invalidates the test. Compare questionable results to the control tube.

VII. REPORTING RESULTS

A. *Staphylococcus lugdunensis* is the only staphylococcus that is pyrrolidonyl-β-naphthylamide (PYR) and ornithine positive (5).

B. *Leuconostoc* is arginine negative, and *Weissella confusa* is arginine positive. Both are PYR-negative, vancomycin-resistant, gram-positive cocci with rod-like forms.

C. Arginine is useful in the identification of *Enterococcus* to the species level; *Enterococcus avium* is arginine negative, but *Enterococcus faecalis* and *Enterococcus faecium* are arginine positive (2).

D. *Stenotrophomonas maltophilia* and *Burkholderia cepacia* are among the few non-glucose-fermenting, gram-negative rods that are lysine positive.

E. Among the polymyxin B-resistant nonfermenters, *Burkholderia mallei* and *Burkholderia pseudomallei* are arginine positive.

F. *Streptococcus anginosus* and *Streptococcus sanguis* are the viridans group streptococci that are arginine positive (1).

G. Reactions for *Enterobacteriaceae* are found in references 3 and 4.

H. *Plesiomonas* is positive for lysine, arginine, *and* ornithine, which separates it from *Vibrio* and *Aeromonas,* whose results are variable with each species but are not positive for all three decarboxylases (*see* Table 3.18.2–8).

I. Other reactions for the non-glucose-fermenting, gram-negative rods are found in the tables in Weyant et al. (8) and in the tables in procedure 3.18.2.

VIII. LIMITATIONS

A. Mineral oil or a similar barrier to gas release must be applied to the surface of each inoculated broth medium. Oil overlay decreases the possibility of an alkaline shift occurring in the medium due to oxidation.

B. Test interpretation should *not* be made prior to 18 to 24 h of incubation. Earlier interpretation may lead to erroneous results. Glucose fermentation occurs within the first 10 to 12 h of incubation. Fermentation produces an acidic environment which results in a yellow color development. The production of decarboxylase enzymes will not be induced until the acidic state has been established.

C. Non-glucose-fermenting microorganisms may display weak decarboxylase activity, thereby resulting in an insufficient production of amines necessary to convert the pH indicator system. Some nonfermenters, however, will produce sufficient amines to result in a deeper purple color than in an uninoculated tube of basal medium.

D. A gray color may indicate reduction of the indicator, rather than production of alkaline end products. To aid in reading the reaction, add additional bromcresol purple.

E. If two layers of different colors appear, shake the tube gently before interpreting the reaction (6).

F. Nonfermenting bacteria that are arginine positive must be lysine and ornithine negative (6).

REFERENCES

1. **Coykendall, A.** 1989. Classification and identification of the viridans streptococci. *Clin. Microbiol. Rev.* **2:**315–328.
2. **Facklam, R. R., and M. D. Collins.** 1989. Identification of *Enterococcus* species by a conventional test scheme. *J. Clin. Microbiol.* **27:**731–734.
3. **Farmer, J. J., III, B. R. Davis, F. W. Hickman-Brenner, A. McWhorter, G. P. Huntley-Carter, M. A. Asbury, C. Riddle, H. G. Wathen-Grady, C. Elias, G. R. Fanning, A. G. Steigerwalt, C. M. O'Hara, G. K. Morris, P. B. Smith, and D. J. Brenner.** 1985. Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. *J. Clin. Microbiol.* **21:**46–76.
4. **Farmer, J. J., III, K. D. Boatwright, and J. M. Janda.** 2007. *Enterobacteriaceae:* introduction and identification, p. 649–669. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
5. **Hébert, G. A.** 1990. Hemolysins and other characteristics that help differentiate and biotype *Staphylococcus lugdunensis* and *Staphylococcus schleiferi. J. Clin. Microbiol.* **28:**2425–2431.
6. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria,* 3rd ed., p. 120–135. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.
7. **Møller, V.** 1955. Simplified tests for some amino acid decarboxylases and for the arginine dihydrolase system. *Acta Pathol. Microbiol. Scand.* **36:**158–172.
8. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria,* 2nd ed., p. 7–8. Williams & Wilkins, Baltimore, MD.

3.17.16 DNase Test-Rapid Thermonuclease Test

I. PRINCIPLE

DNases are enzymes that hydrolyze DNA and release free nucleotides and phosphate. The DNases produced by bacteria are extracellular endonucleases that cleave DNA, yielding a high concentration of oligonucleotides. There are several media used to detect these enzymes, using no indicators (2) or various indicators (toluidine blue [5, 7] or methyl green [MG] [6]) to detect the hydrolysis of DNA. The first medium was developed by Jeffries et al. (2), with no indicator. The hydrolysis of DNA was observed by a clearing of the agar after addition of HCl (the oligonucleotides dissolve in acid but DNA salts are insoluble). When MG indicator is added, DNA combines with the MG to produce a green color. When the DNA is hydrolyzed, the complex is released and the free MG is colorless at pH 7.5 (4). When toluidine blue O (TBO) is added, a complex is formed with the DNA, which changes structure when DNA is hydrolyzed, resulting in a bright pink color (4). The media with dyes can inhibit some microbial growth. Using a heavy inoculum prevents this problem and makes the test more rapid, as it detects preformed enzymes. *Staphylococcus aureus* possesses a heat-stable enzyme, a thermonuclease. To detect this enzyme, first the organisms are destroyed by heat and then the free DNase reacts with the medium.

II. MICROORGANISMS TESTED

A. Gram-negative rods that are presumptive for *Stenotrophomonas maltophilia* (positive) and are colistin or polymyxin B resistant to separate from *Burkholderia cepacia* (negative). MG agar is preferred.

B. Gram-negative diplococci that are presumptive *Moraxella catarrhalis* (positive). TBO agar is preferred.

C. Gram-positive cocci that are presumptive for *S. aureus* (positive) and are difficult to separate from other closely related species and have a questionable coagulase reaction. Use only TBO for staphylococcus heat-stable testing, since it is more sensitive in detection of preformed enzyme. Some staphylococci do not grow on media containing dyes.

D. *Enterobacteriaceae* to identify *Serratia* spp. (positive) and separate them from *Klebsiella* spp. and *Enterobacter* spp. (1, 5). *Serratia fonticola* is the only *Serratia* sp. that is negative for DNase.

E. Oxidase-positive, indole-positive, gram-negative rods to separate *Aeromonas* spp. and *Vibrio cholerae* (positive) from *Plesiomonas shigelloides* (negative) (3)

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media

1. TBO agar
   Medium is royal blue and provides better delineation of DNase activity (4).
2. MG agar
   Medium is green and supports the growth of both gram-positive and gram-negative bacteria.
3. BHI for staphylococci

B. Supplies/equipment

1. Sterile sticks, needles or inoculating loops
2. Pasteur pipettes or drinking straws
3. Boiling heat block
4. Incubators at 35 and 30°C

IV. QUALITY CONTROL

A. Inspect agar for freezing, contamination, cracks, and dehydration prior to storage and before use. Discard plates that are not blue (TBO) or green (MG).

B. Perform QC on each new lot or shipment of media prior to putting it into use. If plates are reused after incubation, add a positive and negative control to the plate each time the test is performed.

C. Organisms

1. Thermonuclease test

a. Positive control: *S. aureus* ATCC 25923—change to pink color (TBO)

b. Negative control: *Staphylococcus epidermidis* ATCC 12228—no color change

2. DNase test (choose one or more positive controls)

a. Positive controls

(1) *M. catarrhalis* ATCC 25240—change to pink color (TBO) or colorless (MG)

(2) *Serratia marcescens* ATCC 13880—change to pink color (TBO) or colorless (MG)

(3) *S. aureus* ATCC 25923—change to pink color (TBO) or colorless (MG)

b. Negative control: *Escherichia coli* ATCC 25922—no color change

V. PROCEDURES

A. DNase test method

1. After touching several colonies from an 18-h culture, inoculate a segment of the agar surface with a very visible, heaping amount of organism equivalent to an entire colony. Several colonies can be placed on a single plate. Use either circular (1/4-in. diameter) or line (1/2-in. length) method for inoculation. Do not streak the entire plate, as it will be difficult to see the reaction.

2. Incubate without added CO_2 for 24 h and up to 72 h at the following temperatures.

a. 35°C for staphylococci or *M. catarrhalis* (TBO agar is more sensitive and rapid, since organism does not grow well on media with dyes)

b. 25°C for enteric gram-negative rods and *Vibrionaceae*

c. 25 or 30°C for non-glucose-fermenting, gram-negative rods (MG agar is preferred)

◩ **NOTE:** DNases are generally most active at lower temperatures, and all testing may be performed at room temperature (e.g., 22 to 25°C) if the organism will grow at a lower temperature (4).

3. Examine for color change. Incubate a full 72 h before calling result negative.

B. Thermonuclease method

1. After touching several well-isolated staphylococcal colonies with a sterile needle, inoculate BHI.

2. Incubate at 35°C for 18 h. Growth from a positive blood culture can also be used.

3. Place broth in boiling heat block for 15 min.

4. Cool to room temperature.

5. Punch a hole in the TBO agar with the large end of a Pasteur glass pipette or drinking straw and remove the agar plug.

6. Fill the well with 2 drops of cooled broth culture.

7. Incubate at 35°C for 3 h, and observe for color change.

VI. INTERPRETATION

A. MG agar

1. Hold plates against a white background in indirect light.

2. Positive test: the development of a clear halo around the colony or the well in the agar

3. Negative test: no clear zone in the medium or around the well in the agar. Agar remains green.

B. TBO agar

1. *Use transmitted light to observe changes in color.*

2. Positive test: the development of a pink or red halo around the colony or the well in the agar

3. Negative test: no change in the royal blue color of the medium

VII. REPORTING RESULTS

A. Gram-negative rods that are non-glucose-fermenting and that are lysine and DNase positive but negative or weakly positive for oxidase are *S. maltophilia.*

B. Gram-negative diplococci that are oxidase positive and DNase positive are *M. catarrhalis.*

C. Gram-positive cocci in clusters that have a thermostable DNase are usually *S. aureus,* but some other staphylococci can give a positive test. *S. schleiferi* is DNase and pyrrolidonyl-β-naphthylamide (PYR) positive. See Table 3.18.1–1 for other tests to separate.

D. *Serratia* spp. produce a DNase, which separates nonpigmented strains from most other *Enterobacteriaceae* (1, 5).

E. Oxidase-positive, indole-positive, gram-negative rods that grow on MAC and are DNase negative are likely to be *P. shigelloides,* rather than the closely related *Aeromonas* and *Vibrio* spp., which are generally DNase positive.

VIII. LIMITATIONS

A. An inoculum that is too broad may result in complete decolorization of the media, due to the reduction of the dye. If this occurs, the test must be repeated.

B. MG medium is better for organisms, such as gram-negative rods, that first grow on the medium and then demonstrate a positive test.

C. For *Moraxella* and gram-positive cocci with TBO testing, a low inoculum can result in a false-negative test, since these organisms may not grow well on the medium.

REFERENCES

1. **Ewing, W. H.** 1986. *Edwards and Ewing's Identification of Enterobacteriaceae,* 4th ed. Elsevier Scientific Publishing Co., New York, NY.
2. **Jeffries, C. D., F. Holtman, and G. D. Guse.** 1957. Rapid method for determining the activity of microorganisms on nucleic acids. *J. Bacteriol.* **73:**590–591.
3. **Krieg, N. R., and J. G. Holt (ed.).** 1984. *Bergey's Manual of Systematic Bacteriology,* vol. 1, p. 484, 550. Williams & Wilkins, Baltimore, MD.
4. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria,* 3rd ed., p. 136–159. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.
5. **Schreier, J. B.** 1969. Modification of deoxyribonuclease test medium for rapid identification of *Serratia marcescens. Am. J. Clin. Pathol.* **51:**711–716.
6. **Smith, P. B., G. A. Hancock, and D. L. Rhoden.** 1969. Improved medium for detecting deoxyribonuclease-producing bacteria. *Appl. Microbiol.* **18:**991–993.
7. **Waller, J. R., S. L. Hodel, and R. N. Nuti.** 1985. Improvement of two toluidine blue O-mediated techniques for DNase detection. *J. Clin. Microbiol.* **21:**195–199.

3.17.17 Fluorescent-Pigment Agars for *Pseudomonas* Identification

I. PRINCIPLE

The kind of peptone in the basal medium markedly affects the production of pigment by bacteria (2). Two pigment-enhancing media were developed by King et al. (2). The pyocyanin or pyorubrin pigment of *Pseudomonas aeruginosa* is enhanced on P agar or "Tech" agar. The fluorescein or pyoverdin pigment of the fluorescent pseudomonads is enhanced on F agar or "Flo" agar. When the P agar is incubated at 42°C, the ability of *P. aeruginosa*, but not *Pseudomonas fluorescens* or *Pseudomonas putida*, to grow at that temperature is also determined. The peptone choices in F agar were found to enhance the production of fluorescein and inhibit the production of pyocyanin. Pseudomonas P agar contains a peptone containing less than 0.1% phosphorus to minimize the inhibitory effect on pyocyanin production. The incorporation of magnesium chloride and potassium sulfate stimulates the production of pyocyanin and pyorubrin.

II. MICROORGANISMS TESTED

Isolated colonies of oxidase-positive, indole-negative, non-glucose-fermentative, gram-negative rods growing on MAC and suggestive of *Pseudomonas* spp.

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media (3)

1. P agar slants (P agar [Remel, Inc.])

a. P agar

Bacto Peptone	20.0 g
magnesium chloride	1.4 g
potassium sulfate	10.0 g
agar	15 g
glycerol	10.0 ml
water, distilled	990 ml
pH	6.8 to 7.0

b. Cetrimide agar (procedure 3.17.11) contains all the essential ingredients to enhance both pyocyanin and fluorescein production, with 3% cetrimide to inhibit organisms other than *P. aeruginosa* (Pseudosel [BD Diagnostic Systems, Hardy Diagnostics])

2. F agar slants (F agar [Remel, Inc.])

a. F agar

Proteose Peptone no. 3	20.0 g
magnesium sulfate (hydrated)	1.5 g
dipotassium phosphate	1.5 g
agar	15 g
glycerol	10.0 ml
water, distilled	990 ml
pH	6.8 to 7.0

b. Fluorescence-denitrification (FN), fluorescence indole denitrification (FIN), and fluorescence lactose denitrification (FLN) media are similar to F agar but have added nitrate, tryptophan, and lactose, respectively, with a pH indicator to allow for additional testing (FIN and FLN; Hardy Diagnostics).

B. Supplies

1. Sterile inoculating loops or sticks

2. Wood's or long-wave (360 nm) UV light or short-wavelength (254 nm) UV light (preferred)

3. 35°C incubator or heat block

4. 42°C incubator or heat block

IV. QUALITY CONTROL

A. Perform QC on each new lot or shipment of media prior to putting it into use.
B. Inspect agar for freezing, contamination, cracks, dehydration, and bubbles prior to storage and before use.
C. Organisms
  1. P agar
    a. *P. aeruginosa* ATCC 27853—positive (blue-green pigment)
    b. *P. fluorescens* ATCC 13525—negative (no blue-green pigment)
  2. F agar
    a. *P. fluorescens* ATCC 13525—positive (fluorescent pigment)
    b. *Escherichia coli* ATCC 25922—negative (no fluorescent pigment)

V. PROCEDURE

A. Streak each slant back and forth with inoculum picked from the center of a well-isolated colony. Also stab the deep of FN, FIN, and FLN media.
B. *Place cap loosely on tube.*
C. Incubate aerobically at 35°C for up to 7 days; alternatively, when using two agar media, incubate P agar at 42°C for up to 7 days.
D. Observe for growth and color visually and, if negative, under UV light for fluorescent pigment.
E. If negative at 24 h, incubate additional days in the dark at 25°C.

VI. INTERPRETATION

A. Pseudomonas F agar, cetrimide, and FN, FIN, and FLN agars
  1. Positive: bright yellow-green color diffusing into the agar; fluorescent zone surrounding growth
  2. Negative: no yellow-green pigment (or no growth on cetrimide agar)
B. Pseudomonas P agar and cetrimide
  1. Positive: a blue-green color indicating pyocyanin production
  2. Negative: no pigment production (or no growth on cetrimide agar)
C. Pyorubin production is indicated by a pink, red, or red-brown color on any medium.
D. FN, FLN, and FIN agar for nitrate reduction (*see* nitrate test, procedure 3.17.35, for QC)
  1. Positive: slits in medium indicative of gas bubbles
  2. Negative: no slits in medium
E. FLN for lactose acidification (*see* procedure 14.2 for QC of medium for acid from lactose)
  1. Positive: yellow color change of the slant as a result of lactose acidification
  2. Negative: slant remains red

VII. REPORTING RESULTS

A. *P. aeruginosa* is definitively identified if it is oxidase positive and does one of the following.
  1. Produces a blue-green pigment
  2. Produces a red-brown pigment
  3. Produces a yellow-green pigment or fluorescent pigment and grows at 42°C
  ◩ **NOTE:** *P. aeruginosa* is nitrate positive and lactose negative.
B. *P. fluorescens* or *P. putida* is identified if there is a yellow-green pigment or fluorescent pigment and no growth at 42°C (3). See lecithinase or gelatin procedure (procedure 3.17.27 or 3.17.18, respectively) to separate.
C. *P. putida* is nitrate negative, and *P. fluorescens* is infrequently positive.

VIII. LIMITATIONS

A. If *P. aeruginosa* has a characteristic odor and is oxidase positive and indole negative, no further testing is indicated, except from cystic fibrosis (CF) patients (1).

B. Not all *P. aeruginosa* isolates produce pigments, especially those from CF patients. If the isolate is from a CF patient and lacks pigments, it is identified as *P. aeruginosa* if it meets all of the following criteria.

1. Is oxidase positive and indole negative

2. Has characteristic mucoid colony form and has the characteristic odor

3. Is polymyxin B susceptible

C. Mueller-Hinton agar when being used for disk susceptibility testing may substitute for F agar.

D. Not all fluorescent pseudomonads grow on cetrimide or produce pigments.

REFERENCES

1. **Clinical and Laboratory Standards Institute.** 2008. *Abbreviated Identification of Bacteria and Yeast,* 2nd ed. Approved guideline M35-A2. Clinical and Laboratory Standards Institute, Wayne, PA.

2. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.* **44:**301–307.

3. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria,* 2nd ed., p. 17–18. Williams & Wilkins, Baltimore, MD.

3.17.18 Gelatin Liquefaction

I. PRINCIPLE

Gelatinases are proteolytic enzymes that hydrolyze gelatin into polypeptides and individual amino acids. These enzymes destroy the structure of the gelatin, and it becomes liquid. As an alternative to a gelatin medium, X-ray film that has not been developed can be used because it is coated with gelatin. After exposure to gelatinase, the film will lose its coating and leave behind a clear photographic film. Kohn (2) has reported an excellent rapid test medium for detection of small amounts of preformed gelatinase without growth of the organism. Gelatin is complexed with charcoal and formalin, and only a small amount of gelatin is needed to release the charcoal particles. This method is used in the API 20E (bioMérieux, Inc.) and shows a reaction in 18 h rather than several days for a nutrient tube gelatin assay that requires growth of the organism and liquefaction of the medium. Gelatin testing is useful in many taxonomic identifications of both gram-negative rods (1) and gram-positive rods.

II. MICROORGANISMS TESTED

A. Gram-negative rods that require gelatin for identification, especially for the separation of the fluorescent *Pseudomonas: Pseudomonas putida* (negative) from *Pseudomonas fluorescens* (positive)

B. Gram-positive rods as needed for identification to the species level

III. MEDIA, REAGENTS, AND SUPPLIES

A. Nutrient gelatin with heart infusion or beef extract and peptone

1. Store at 2 to 8°C, at which it is in solid form.

2. At room temperature, it will liquefy.

B. Other options

1. X-ray film

a. Cut X-ray film that is exposed but not developed into strips having a width and length that fit into a test tube.

b. Prepare sterile tubes with 1 ml of sterile distilled water or saline.

2. Kohn gelatin charcoal medium (required reagents are listed below, and recipe for preparation is detailed in reference 3)

nutrient gelatin	15.0 g
powdered charcoal	3.0 to 5.0 g
distilled water	100 ml
TSB	3 to 4 ml per tube

◩ **NOTE:** Charcoal disks are soaked in 10% formalin (10 ml of 40% formaldehyde and 1 g of calcium carbonate in 90 ml of 0.85% NaCl), cut, and washed prior to sterilization. Then TSB is added to each disk.

C. Other supplies

1. Inoculating needle

2. Incubator at 22, 30, or 35°C, depending on the isolate

IV. QUALITY CONTROL

A. Inspect tubes for contamination, dehydration, and lack of liquefaction at refrigeration temperatures before storage and before use.
B. Inspect X-ray film for loss of gelatin coating.
C. Perform QC on each new lot or shipment of media prior to putting it into use. Use an uninoculated control with each use.
D. Organisms
  1. *Pseudomonas aeruginosa* ATCC 10145—liquefaction (positive)
  2. *Escherichia coli* ATCC 25922—no liquefaction (negative)

V. PROCEDURE

A. Incubation temperatures
  1. 22°C for *Enterobacteriaceae* and fluorescent *Pseudomonas* spp.
  2. 30°C for nonfermenting, gram-negative rods
  3. 35°C for other organisms and for X-ray and Kohn methods

B. Tube method
  1. The medium should be solidified at room temperature. After touching several well-isolated colonies with a sterile needle, stab directly down the center of the tube to approximately 10 mm from the bottom. Repeat to inoculate heavily.
  2. Incubate the test and an uninoculated control tube for 48 h.
  3. Gently remove the inoculated and uninoculated tubes from the incubator and refrigerate for at least 30 min or until the control tube solidifies. (Gelatin is a liquid at 28°C or higher.)
    a. Do not shake or invert the tubes prior to refrigeration.
    b. Gently invert to detect liquefaction by the test organism after 30 min of refrigeration.
  4. Reincubate a negative test for up to 2 weeks if indicated by the nature of the organism, and examine at regular intervals.

C. X-ray method
  1. After touching several colonies from an 18-h culture, inoculate 1 ml of sterile distilled water equivalent to a no. 2 McFarland standard.
  2. Place a gelatin strip or X-ray strip in the water and cap tube. Do not press the strip to the side of the tube such that it is not in contact with the water.
  3. Prepare a tube without inoculum as a control.
  4. Incubate for up to 48 h at 35°C.
  5. Examine at regular intervals for clearing of the strip.

D. Kohn method
  1. Inoculate gelatin-charcoal tube heavily, equivalent to a no. 2 McFarland standard, with growth of microorganism from an 18-h culture.
  2. Set up an uninoculated control tube.
  3. Incubate for 24 h or longer at 35°C.
  4. Shake and observe for particles dispersed throughout medium.

VI. INTERPRETATION

A. Tube method
  1. Positive test: at the end of the refrigeration period, the control tube will be resolidified and the test tube will remain liquid at least to the depth of the stab. (Some organisms only partially liquefy gelatin or liquefy just at the surface of the tube.)
  2. Negative test: at the end of the refrigeration period, the control tube and the test tube will be resolidified, even at the top of the tube.

VI. INTERPRETATION *(continued)*

B. X-ray film method

1. Positive test: green gelatin layer is removed on immersed portion of film, exposing transparent, bluish film.

2. Negative test: green gelatin layer remains undisturbed.

C. Kohn gelatin disks

1. Positive test: a visible black cloud observed after tube is shaken.

2. Negative test: no free charcoal particles in medium and tube remains as the uninoculated tube after shaking.

VII. REPORTING RESULTS

A. *Proteus* spp. are gelatinase positive.

B. *P. fluorescens* is gelatinase positive, but *P. putida* is gelatinase negative.

C. *Serratia* spp. are usually gelatinase positive.

VIII. LIMITATIONS

A. Gelatinase usually acts at the surface of the tube medium. Shaking the tube while it is warm may result in a false-negative interpretation.

B. Gelatin may vary in its gelling ability; therefore, incubate an uninoculated control with the test. The control must be refrigerated along with the test, prior to reading.

C. For the X-ray film method, the strips must be immersed in the liquid and not cling to the sides of the tube without immersion. Only part of the film must be immersed, so that a difference in reaction can be visualized.

D. Do not use photographic film.

E. False-positive results may occur with the X-ray film method in some media or saline on prolonged incubation, i.e., greater than 4 h. This can be detected in the uninoculated control tube.

REFERENCES

1. **Ewing, W. H.** 1986. *Edwards and Ewing's Identification of Enterobacteriaceae,* 4th ed. Elsevier Scientific Publishing Co., New York, NY.
2. **Kohn, J.** 1953. A preliminary report of a new gelatin liquefaction method. *J. Clin. Pathol.* **6:**249.
3. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria,* 3rd ed., p. 170–182. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.

3.17.19 Glucan and Polysaccharide Production

I. PRINCIPLE

Streptococci can produce glucans (extracellular polysaccharides) on agar containing sucrose. The two types of glucans, dextrans and levans, yield different consistencies. Characteristic colonies and type of glucans produced (e.g., slime, adherence) can be used to identify streptococci to the species level.

Some saprophytic *Neisseria* spp. can produce an amylosucrase enzyme that synthesizes an iodine-reacting polysaccharide from sucrose. The polysaccharide is detected by the production of a brown to black color when iodine is added to the agar medium.

II. MICROORGANISMS TESTED

A. Gram-positive cocci that are catalase negative
   1. To separate *Streptococcus bovis,* which produces copious amounts of watery slime, from other viridans group streptococci that are Voges-Proskauer positive and esculin positive
   2. To separate *Streptococcus mutans,* which is glucan positive, from other streptococci

B. To detect the production of iodine-reacting polysaccharide from sucrose to separate *Neisseria meningitidis, Neisseria gonorrhoeae,* and *Neisseria lactamica* from saprophytic strains of *Neisseria* (4)

III. MEDIUM, REAGENT, AND SUPPLIES

A. Medium
Heart infusion agar with 5% sucrose (2) (Remel, Inc.)

B. Reagent
0.2% Iodine with 0.4% potassium iodide freshly diluted 1:5 with distilled water

C. Supplies
   1. Sterile sticks or inoculating loops
   2. Incubators at 35°C

IV. QUALITY CONTROL

A. Inspect agar for freezing, contamination, cracks, and dehydration prior to storage and before use.

B. Perform QC on new lots of media prior to putting them into use.

C. Organisms
   1. *S. bovis* ATCC 33317—copious amounts of glucans (positive)
   2. *Enterococcus faecalis* ATCC 25922—no glucans produced (negative)
   3. *Neisseria polysaccharea* ATCC 43768—polysaccharides produced (positive)
   4. *N. gonorrhoeae* ATCC 43069—no polysaccharide production (negative)

V. PROCEDURE

A. After touching several colonies from an 18-h culture, inoculate plate, streaking in quadrants for isolation.
B. Incubate without added CO_2 for 24 to 48 h. The plate may be incubated with the lid up for streptococci, since glucans can fall onto the lid in the inverted position.
C. Observe colonies for glucan production.
D. For *Neisseria,* apply 2 drops of iodine solution and observe for dark reddish brown to black color that fades quickly. The color returns with the addition of more iodine.

VI. INTERPRETATION

A. Positive glucan test: formation of large mucoid, runny material external to the colonies (levans). In some cases, large opaque gumdrop-like refractile colonies that are adherent are present (2). Colonies may be white-dry-adherent and form a depression in the agar.
B. Negative glucan test: no mucoid or runny colonies and no adherent colonies
C. Positive amylosucrase test: brown to black color with addition of iodine
D. Negative amylosucrase test: no brown color with addition of iodine

VII. REPORTING RESULTS

A. *S. bovis* produces great amounts of watery glucans (levans), promoting very large colonies, which run together and can drip into the cover if the plate is inverted (1).
B. *Streptococcus salivarius* produces colonies that are opaque, gummy, and non-adherent (levans). They can be similar to those of *S. bovis* but are more gummy (1).
C. *Streptococcus sanguis* and *Streptococcus mitis* may produce glucans, which are hard and adherent, with the texture and consistency of polyethylene. The agar can be depressed. Less than half of the strains of these species produce glucans (1).
D. *S. mutans* produces puddles of glucans that even produce a droplet on the top of the colony (1).
E. A positive test for amylosucrase indicates that an oxidase-positive, gram-negative diplococcus is not *N. gonorrhoeae* or *N. meningitidis.*

VIII. LIMITATIONS

A. The identification of *S. bovis* is difficult, and the organism can be confused with *S. mutans* and *S. salivarius* (3). Glucan agar is helpful in the identification.
B. The API Rapid Strep and RapID 32 (bioMérieux, Inc.) do an excellent job of separating *S. bovis* from other streptococci.

REFERENCES

1. **Coykendall, A.** 1989. Classification and identification of the viridans streptococci. *Clin. Microbiol. Rev.* **2:**315–328.
2. **Facklam, R., and J. A. Elliott.** 1995. Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clin. Microbiol. Rev.* **8:**479–495.
3. **Ruoff, K., S. I. Miller, C. V. Garner, M. J. Ferraro, and S. B. Calderwood.** 1989. Bacteremia with *Streptococcus bovis* and *Streptococcus salivarius:* clinical correlates of more accurate identification of isolates. *J. Clin. Microbiol.* **27:**305–308.
4. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria,* 2nd ed., p. 5. Williams & Wilkins, Baltimore, MD.

3.17.20 Gram Reaction Enzymatic Test

I. PRINCIPLE

In the Gram stain, some gram-positive rods appear gram variable or gram negative, especially members of the genera *Bacillus, Erysipelothrix, Lactobacillus,* and *Listeria,* which may result in misclassification. Alternatively, some organisms that are gram negative may, at times, appear to be gram positive, since they have been known to resist the alcohol-acetone decolorization step in the Gram stain. Among these problem organisms are members of the *Neisseriaceae* family, which include *Moraxella catarrhalis, Neisseria, Kingella,* and *Acinetobacter* spp. Erroneous Gram stain reactions represent one of the most frequent causes of misidentification, which can result in a delay of appropriate therapy.

In 1976, Cerny reported using L-alanine-4-nitroanilide to detect the presence of cell wall aminopeptidase, which correlated with gram negativity (2). In their comparison study with the 3% KOH method, Carlone et al. yielded more correct Gram stain determinations with L-alanine-4-nitroanilide than with the 3% KOH method (1). Manafi and Kneifel in 1990 (3) evaluated various chromogenic and fluorogenic substrates for their ability to distinguish gram-positive from gram-negative bacteria. They preferred the fluorogenic compound L-alanine-7-amido-4-methylcoumarin for a rapid test due to the pronounced blue fluorescence of a positive reaction and increased test sensitivity.

II. MICROORGANISMS TESTED

Use a fresh colony, less than 48 h old, of an aerobic or facultative microorganism growing on any laboratory medium.

III. REAGENTS AND SUPPLIES

A. Disks

1. Impregnated with L-alanine-7-amido-4-methylcoumarin (Gram-Sure; Remel, Inc.), L-alanine-4-nitroanilide (LanaGram; Hardy Diagnostics), and L-alanine-*p*-nitroanilide (APNA; Key Scientific)
2. Store in original container at 2 to 8°C until used.
3. Minimize exposure to light.
4. Protect disks from moisture by removing only those disks necessary for testing from the vial. Promptly replace the cap and return the vial to 2 to 8°C.

B. Supplies

1. Inoculating loop, swab, or stick
2. Long-wave UV light

IV. QUALITY CONTROL

A. Do not use product if any of the following is true.

1. The color of the disk has changed (refer to manufacturer's package insert).
2. The color of the desiccant has changed to pink.
3. There are other signs of deterioration.

B. Perform QC on each new lot or shipment of reagent prior to putting it into use, with the following QC organisms.

1. *Escherichia coli* ATCC 25922—positive
2. *Staphylococcus aureus* ATCC 25923—negative

V. PROCEDURE

A. Add 0.25 ml of purified water to a 10- by 75-mm test tube.
B. Inoculate heavily with an isolated bacterial colony to produce a milky suspension.
C. Add one disk to the inoculated tube.
D. Incubate at room temperature (25°C) for 5 to 20 min.
E. Observe under long-wave UV light for the appearance of blue fluorescence (methylcoumarin substrate), or observe visually for the development of a pale yellow to bright yellow color (nitroanilide substrate).

VI. INTERPRETATION

A. A positive test (gram negative) shows blue fluorescence (methylcoumarin substrate) or pale to bright yellow color (nitroanilide substrate).
B. A negative test (gram positive) shows no fluorescence (methylcoumarin substrate) or no change in color (nitroanilide substrate).

VII. REPORTING RESULTS

A. Aerobic, gram-negative rods and coccobacilli demonstrate a bright blue fluorescence (methylcoumarin substrate) or a pale to bright yellow color change (nitroanilide substrate).
B. Gram-positive rods and coccobacilli demonstrate no fluorescence (methylcoumarin substrate) or no change in color (nitroanilide substrate).

VIII. LIMITATIONS

A. This test is designed to serve as an adjunct to (not a replacement for) the traditional Gram stain method.
B. Obligate anaerobic and gram-positive cocci may fail to give the expected results and should not be tested with this product. Such genera as *Campylobacter*, *Bacteroides*, and *Streptococcus* are likely to produce false results.
C. Do not use the nitroanilide substrate to test yellow colonies or colonies from media with dye.
D. Nitroanilide substrate requires up to 20 min. Negative test reactions (no color change) should be held for the full 20 min.

REFERENCES

1. **Carlone, G. M., M. J. Valadez, and M. J. Pickett.** 1982. Methods for distinguishing gram-positive from gram-negative bacteria. *J. Clin. Microbiol.* **16:**1157–1159.
2. **Cerny, G.** 1976. Method for the distinction of gram-negative from gram-positive bacteria. *Eur. J. Appl. Microbiol.* **33:**223–225.
3. **Manafi, M., and W. Kneifel.** 1990. Rapid methods for differentiating gram-positive from gram-negative aerobic and facultative anaerobic bacteria. *J. Appl. Bacteriol.* **69:**822–827.

3.17.21 Hippurate Hydrolysis Rapid Test

I. PRINCIPLE

The hippurate test is used in the identification of *Campylobacter jejuni*, *Listeria monocytogenes*, *Gardnerella vaginalis*, and *Streptococcus agalactiae*, by detecting the ability of the organism to hydrolyze sodium hippurate to benzoic acid and glycine by the action of the enzyme hippuricase.

The ability of bacterial species to hydrolyze hippurate was classically tested using ferric chloride indicator to detect benzoic acid. However, a 2-h rapid method, as opposed to the 48-h classical method, for detecting hippurate hydrolysis has since been developed (3). The rapid test employs ninhydrin as the indicator, which reacts with any protein or amino acid and, in this case, detects glycine. The rapid hippurate hydrolysis test has been shown to be as specific and as sensitive as the classical method, which detects the benzoic acid by-product.

II. MICROORGANISMS TESTED

A. Presumptive *L. monocytogenes*: tiny gram-positive rods that are catalase positive, motile at 25°C, and beta-hemolytic

B. Presumptive *S. agalactiae*: gram-positive cocci that are catalase negative, displaying a translucent colony with a characteristic narrow zone of beta-hemolysis

C. Presumptive *C. jejuni*: curved gram-negative rods that are oxidase and catalase positive and do not grow aerobically at 35°C (2, 4)

D. Presumptive *G. vaginalis*: catalase-negative, tiny gram-variable rods

III. MEDIA, REAGENTS, AND SUPPLIES

Include QC information on reagent container and in QC records.

A. Rapid hippurate tubes

1. Commercial hippurate reagents
- **a.** Tube containing 20 g of sodium hippurate per liter when rehydrated (Hardy Diagnostics)
- **b.** Disks or tablets to be reconstituted in a tube (BD Diagnostic Systems; Key Scientific; Oxoid, Inc.; Remel, Inc.)

2. Prepare 1.0% hippurate.
- **a.** Add 1 g of sodium hippurate (Sigma Chemical Co.) to 100 ml of distilled water.
- **b.** Dissolve completely by mixing well.
- **c.** Dispense in capped tubes in 0.2- to 0.4-ml amounts (e.g., Durham tubes work well).
- **d.** Freeze at −20°C. Shelf life is very long. Discard when QC fails.

◩ **NOTE:** Piot et al. (6) found that a pH of 6.4 was optimal to detect positive reactions for *G. vaginalis.*

B. Ninhydrin

1. If reagent is lyophilized, reconstitute by adding distilled water in the amount recommended by the manufacturer.

2. Ninhydrin preparation
- **a.** Mix 50 ml of acetone and 50 ml of 1-butanol thoroughly in a dark glass bottle.
- **b.** Add 3.5 g of ninhydrin; mix.

III. MEDIA, REAGENTS, AND SUPPLIES *(continued)*

c. Store at room temperature for up to 6 months.

◩ **NOTE:** Care must be taken when handling this solution not to spill reagent onto skin or clothing, as a blue color will develop that does not wash off but will wear off the skin in 24 to 48 h.

C. Supplies

1. Sterile wooden sticks or inoculating loops
2. Incubator at 35°C
3. Test tubes
4. Distilled water

IV. QUALITY CONTROL

A. Test each new lot and shipment of reagent with known positive and negative controls, and retest at least monthly thereafter. Discard all reagents and prepare new ones if the reagents do not pass QC.

B. Organisms

1. *S. agalactiae* ATCC 12386—hippurate positive
2. *Streptococcus pyogenes* ATCC 19615—hippurate negative

V. PROCEDURE

A. Prepare hippurate tube.

1. Add 0.2 ml (3 or 4 drops) of distilled water at a pH of 6.8 to 7.2 to reconstitute lyophilized tube test reagent.
2. Add 2 drops of distilled water to empty tube for disk or tablet tests.
3. Defrost one 0.4-ml tube per test for prepared reagent.

B. In the tube, make a heavy suspension (equivalent to no. 3 McFarland standard) from an 18- to 24-h culture. Use care not to pick up agar, which contains protein.

C. For disk or tablet tests, add reagent after inoculation of the tube with the culture.

D. Incubate the tube for 2 h at 35 to 37°C.

E. After the 2-h incubation period, add 2 drops of the ninhydrin solution to the hippurate reagent-organism mixture. Add an additional 2 drops if test has 0.4 ml of hippurate.

F. Reincubate at 35 to 37°C for 30 min. Observe the tubes at 10-min intervals for the appearance of a deep blue color, which is a positive test. The color change will usually appear within 10 to 15 min after the ninhydrin indicator solution has been added.

VI. INTERPRETATION

A. A positive reaction is indicated by the appearance of a deep blue color (about the color of crystal violet) within 30 min.

B. A negative reaction is indicated by a faint purple color or no color change.

VII. REPORTING RESULTS

A. *L. monocytogenes* organisms are tiny gram-positive rods that are catalase positive, motile at 25°C, beta-hemolytic, CAMP positive, and hippurate positive.

B. *S. agalactiae* organisms are catalase-negative, gram-positive cocci that are identified by having a characteristic narrow zone of beta-hemolysis and are hippurate positive.

C. *C. jejuni* organisms are curved, gram-negative rods that are identified by having a positive oxidase and catalase reaction, having no growth aerobically at 35°C, and being hippurate positive.

D. *G. vaginalis* organisms are catalase-negative, gram-variable rods that are hemolytic on human blood agar and are hippurate positive.

VII. REPORTING RESULTS *(continued)*

E. *Arcanobacterium haemolyticum* is hippurate negative and reverse-CAMP positive, while *Arcanobacterium pyogenes* is hippurate positive and reverse-CAMP negative. Both are hemolytic.

VIII. LIMITATIONS

A. Not all *S. agalactiae* organisms are beta-hemolytic. Viridans group streptococci can be hippurate positive; another test must be done on nonhemolytic colonies to confirm the identification.

B. A small number of enterococci are beta-hemolytic and may hydrolyze hippurate, but they are pyrrolidonyl-β-naphthylamide (PYR) positive.

C. A small percentage of *C. jejuni* organisms are hippurate negative and must be identified by other methods (5).

D. A negative test does not rule out the identification of *G. vaginalis*, since the biotypes that cause bacterial vaginosis can be hippurate negative (1).

E. False-positive results can occur if incubation with ninhydrin exceeds 30 min.

REFERENCES

1. **Aroutcheva, A. A., J. A. Simoes, K. Behbakht, and S. Faro.** 2001. *Gardnerella vaginalis* isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems. *Clin. Infect. Dis.* **33:**1022–1027.
2. **Harvy, S. M.** 1980. Hippurate hydrolysis by *Campylobacter fetus. J. Clin. Microbiol.* **11:**435–437.
3. **Hwang, M. N., and G. M. Ederer.** 1975. Rapid hippurate hydrolysis method for presumptive identification of group B streptococci. *J. Clin. Microbiol.* **1:**114–115.
4. **Morris, G. K., M. R. el Sherbeeny, C. M. Patton, H. Kodaka, G. L. Lombard, P. Edmonds, D. G. Hollis, and D. J. Brenner.** 1985. Comparison of four hippurate hydrolysis methods for identification of thermophilic *Campylobacter* species. *J. Clin. Microbiol.* **22:**714–718.
5. **Nicholson, M. A., and C. M. Patton.** 1995. Evaluation of disk method for hippurate hydrolysis by *Campylobacter* species. *J. Clin. Microbiol.* **33:**1341–1343.
6. **Piot, P., E. Van Dyck, P. A. Totten, and K. K. Holmes.** 1982. Identification of *Gardnerella (Haemophilus) vaginalis. J. Clin. Microbiol.* **15:**19–24.

3.17.22 Hydrogen Sulfide Production

I. PRINCIPLE

Certain organisms are capable of enzymatically liberating sulfur from inorganic sulfur as hydrogen sulfide (H_2S) compounds or from sulfur-containing amino acids through proteolysis of proteins. Detection of H_2S from inorganic sulfur compounds is a two-step process where the bacterium reacts with sodium thiosulfate in the medium to yield a sulfite and H_2S. The colorless H_2S gas released reacts with ferric ions or lead acetate to yield ferrous sulfide or lead sulfide, which are insoluble black precipitates. The most common sulfide indicators are ferrous sulfate, ferric citrate, ferric ammonium sulfate or citrate, peptonized iron, and lead acetate. The sensitivity of each indicator varies, and H_2S detected by one indicator may not be detected by another (3).

Lead acetate is the most sensitive reagent and should be used for organisms that produce trace amounts of H_2S. Since all members of the family *Enterobacteriaceae* are capable of producing various amounts of H_2S, the least sensitive approach (Kligler's iron agar [KIA] or triple sugar iron agar [TSI]) is best for routine identification of these organisms. Several plate media, such xylose-lysine-desoxycholate (XLD), Hektoen enteric agar, and salmonella-shigella agar (SS), are also capable of detecting H_2S production. While these media are used primarily as screening tests for fecal pathogens, the tube tests are also useful biochemical tests for H_2S production by *Erysipelothrix* and fastidious gram-negative rods, such as *Campylobacter.*

II. MICROORGANISMS TESTED

A. Organisms
- **1.** Gram-negative rods, to determine their ability to produce hydrogen sulfide
- **2.** Catalase-negative gram-positive rods, to detect H_2S production to separate lactobacilli from *Erysipelothrix*
- **3.** *Campylobacter*, to detect H_2S production (For use in testing campylobacters, media should be less than 1 week old or boiled and reslanted prior to use.)

B. Use as part of the battery to distinguish enteric pathogens (procedure 3.8.1).

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media
- **1.** KIA (refer to procedure 3.17.25 for details on preparation)
- **2.** TSI (refer to procedure 3.17.25 for details on preparation)
- **3.** Sulfide-indole-motility agar (SIM)
- **4.** XLD, Hektoen, or other primary culture media to select for fecal pathogens

B. Supplies
- **1.** Wire or disposable inoculating needles for tubed media and inoculating loops for plate media
- **2.** 35°C heat block for tubes or incubator without increased CO_2
- **3.** Optional: lead acetate paper

IV. QUALITY CONTROL

A. Perform QC on each new lot or shipment of tubed media prior to putting it into use.
- **1.** Refer to motility procedure (procedure 3.17.31) for QC of SIM and the KIA and TSI procedure (procedure 3.17.25) for QC of KIA and TSI.
- **2.** It is not necessary for users to QC Hektoen, XLD, and SS agar if it has been done by the manufacturer. Refer to Clinical and Laboratory Standards Institute (formerly NCCLS) document M22-A3 for more information (2).

IV. QUALITY CONTROL *(continued)*

B. Inspect agar for freezing, contamination, cracks, dehydration, and bubbles prior to storage and before use. Discard any tubes with bubbles in the agar. Discard TSI or KIA tubes that are not red or that lack a deep butt. The slant and butt should be equal in length.

V. PROCEDURE

A. Tube media

1. Warm medium to room temperature and examine for cracks. Do not use if cracks appear.
2. Using a sterile inoculating needle, touch the center of a well-isolated colony.
3. Stab to within 3 to 5 mm from the bottom of the tube.
4. Withdraw the needle.
5. For KIA or TSI, streak the entire surface of the agar slant.
6. Optional for fastidious organisms: add a strip of lead acetate paper to top of tube and hold in place with the cap of the tube so that it extends 1 in. into the tube.
7. *Place cap loosely on tube.* Do *not* tighten the cap to allow for release of gas in the tube.
8. Incubate aerobically at 35 to 37°C for 18 to 24 h.
9. Observe for black precipitate indicating hydrogen sulfide production.
10. If desired, extend incubation only to detect H_2S production. Campylobacters may take 3 days for production of H_2S.

B. Plate media

1. Streak plate so as to obtain isolated colonies.
2. Incubate aerobically at 35 to 37°C for 18 to 24 h.
3. Observe for blackened colonies.

VI. INTERPRETATION

A. Positive reactions

1. H_2S production in tube media: black color throughout the medium, a black ring at the junction of the butt and slant, or any black precipitate in the butt. Blackening usually begins at the line of inoculation.
2. H_2S production in plate media: black colonies surrounded by a brownish black zone or metallic sheen
3. Lead acetate paper: brownish black coloration of the paper strip

B. Negative reactions

1. H_2S production in tube media: no blackening in tube
2. H_2S production in plate media: no blackening and no metallic-sheen colonies
3. Lead acetate paper: no change in color of strip

VII. REPORTING RESULTS

A. If a catalase-negative, vancomycin-resistant, gram-positive rod is H_2S positive, report as "*Erysipelothrix rhusiopathiae.*"

B. If a campylobacter-like organism is H_2S positive, report as "Probable *Campylobacter hyointestinalis.*" See Table 3.8.2–4 for other tests to confirm the identification.

C. For evaluation of enteric pathogens, see procedure 3.8.1.

D. *Shewanella putrefaciens/algae* is a foul-smelling, oxidase-positive, gram-negative rod that is H_2S positive and does not produce acid on TSI or KIA (4).

VIII. LIMITATIONS

A. H_2S production may be inhibited on TSI for organisms that utilize sucrose and suppress the enzyme mechanism that results in production of H_2S (1).

B. SIM is more sensitive in the detection of H_2S than either TSI or KIA, because of its semisolid nature, its lack of interfering carbohydrates, and the use of peptonized iron as an indicator (3).

C. Lead acetate paper is 10 times more sensitive than other media (5).

D. Lead acetate is toxic to bacteria and may inhibit the growth of some bacteria. Do not allow the media to touch the strip.

REFERENCES

1. **Bulmash, J. M., and M. Fulton.** 1964. Discrepant tests for hydrogen sulfide. *J. Bacteriol.* **88:**1813.
2. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
3. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria,* 3rd ed., p. 205–220. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.
4. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria,* 2nd ed. Williams & Wilkins, Baltimore, MD.
5. **ZoBell, C. E., and C. B. Reltham.** 1934. A comparison of lead, bismuth, and iron as detectors of hydrogen sulphide produced by bacteria. *J. Bacteriol.* **28:**169–176.

3.17.23 Indole Test

I. PRINCIPLE

The ability of an organism to split indole from the amino acid tryptophan is due to the presence of tryptophanase. Indole, if present, combines with the aldehyde in the reagent to produce a pink to red-violet quinoidal compound (benzaldehyde reagent) or a blue to green color (cinnamaldehyde reagent). In the rapid spot test, indole is detected directly from a colony growing on a medium rich in tryptophan.

II. MICROORGANISMS TESTED

A. Fresh growth of a gram-negative rod on medium that does not contain dyes and contains tryptophan, e.g., BAP or CHOC
B. Anaerobic gram-positive rods
C. Anaerobic gram-negative rods

III. MEDIA, REAGENTS, AND SUPPLIES

A. Reagents
Obtain indole reagents commercially, or prepare in-house.
- **1.** Rapid spot indole: see Appendix 3.17.23–1.
  - **a.** Prepare either 5% *p*-dimethylaminobenzaldehyde or 1% paradimethylaminocinnamaldehyde in 10% (vol/vol) concentrated HCl (1, 6)
  - **b.** Do not use the spot benzaldehyde reagent for anaerobes.
- **2.** Tube indole
  - **a.** Kovács' method for aerobically growing organisms (3)
    - (1) Reagent: see Appendix 3.17.23–1.
    - (2) Medium: broth that contains tryptophan (containing peptone, tryptone, or casein). Semisolid agar, such as motility-indole-ornithine agar or sulfide-indole-motility agar, can be used.
  - **b.** Ehrlich's method for anaerobes and weak indole producers (2)
    - (1) Reagent: see Appendix 3.17.23–1.
    - (2) Medium: heart infusion or anaerobic medium with tryptophan
    - (3) Xylene
- **3.** Clearly label indole reagents, indicating preparation and expiration dates.
  - **a.** Record the expiration date in a work record (in-house preparation) or on a receipt record (commercial record).
  - **b.** Store indole reagents in a dark bottle at 4°C.

B. Supplies
- **1.** Sterile loop, swab, or stick for harvesting
- **2.** Filter paper (optional)

IV. QUALITY CONTROL

A. Do not use benzaldehyde reagents (including Ehrlich's and Kovács') if color is not pale yellow.

B. Perform QC

1. On each new lot or shipment of reagent prior to putting it into use

2. Although not required, QC in-house-prepared reagents weekly, as they can deteriorate, especially if not stored at 4°C. Discard if reactions become weak (4).

C. Organisms

1. *Escherichia coli* ATCC 25922—indole positive

2. *Pseudomonas aeruginosa* ATCC 27853—indole negative

3. For Ehrlich's reagent for use with anaerobic microorganisms

a. *Porphyromonas asaccharolytica* ATCC 25260—indole positive

b. *Bacteroides fragilis* ATCC 25285—indole negative

V. PROCEDURE

A. Rapid spot indole

Use one of the methods below.

1. Moisten filter paper with reagent. Using a wooden stick, rub portion of colony onto paper.

2. Sweep the colony onto a swab. Add drop of indole reagent to the colony swab.

3. Add reagent directly to the colony growing on the agar surface.

B. Tube test

1. Inoculate liquid tube medium or stab agar medium with colony.

2. Incubate for 18 to 24 h. If broth is used for indole production, decant a portion of the medium to a second tube before testing.

3. For Ehrlich's method

a. Add 0.5 ml of xylene to tube and invert to mix. Let settle.

b. Add 6 drops of Ehrlich's indole reagent down the side of the tube and observe color below the xylene layer.

4. For Kovács' method, add 3 drops of Kovács' reagent down the side of the tube and observe color change at meniscus.

5. If test is negative, repeat test after an additional 24 h of incubation, if desired.

VI. INTERPRETATION

A. The development of a brown-red to purple-red color (benzaldehyde reagents) or blue color (cinnamaldehyde reagent) within 20 s indicates the presence of indole.

B. A negative test is colorless or slightly yellow.

VII. REPORTING RESULTS

A. *E. coli* is indole positive, as are many other *Enterobacteriaceae, Vibrio, Aeromonas, Plesiomonas,* and *Pasteurella.*

B. Several fastidious gram-negative rods are indole positive, such as *Cardiobacterium hominis* and *Pasteurella bettyae.* See Table 3.18.2–3 for others.

C. *Propionibacterium acnes* is indole positive.

D. *Delftia (Comamonas) acidovorans* produces a pumpkin orange reaction from the production of anthranilic acid, rather than indole, from tryptophan with Kovács' reagent (5).

VIII. LIMITATIONS

A. Detectable indole will diffuse to colonies within 5 mm of a 2- to 3-mm colony, giving false-positive results.

B. Do not use media that contain dyes (e.g., EMB, MAC).

C. Growth medium must contain an adequate amount of tryptophan. Do not use Mueller-Hinton agar for test, because tryptophan is destroyed during the acid hydrolysis of casein.

D. Only the cinnamaldehyde reagent can be used for spot testing of anaerobic microorganisms. It is the more sensitive reagent, but it is less stable.

E. Do not use a plate with a nitrate disk to perform the indole test, as nitrate can interfere with the spot indole test by inducing false-negative results.

F. If the rapid indole test is negative, the isolate could be positive in the more sensitive tube test. Extraction with xylene is the most sensitive test. Xylene substitutes are less sensitive.

G. For fastidious gram-negative rods, such as *C. hominis*, a heavy inoculum and extraction are necessary.

REFERENCES

1. **Bale, M. J., S. M. McLaws, and J. Matsen.** 1984. The spot indole test for identification of swarming *Proteus*. *Am. J. Clin. Pathol.* **83:**87–90.
2. **Böhme, A.** 1905. Die Anwendung der Ehrlichschen Indolreaktion für bakteriologische Zwecke. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. I Abt. Orig.* **40:**129–133.
3. **Kovács, N.** 1928. Eine vereinfachte Methode zum Nachweis der Indolbildung durch Bakterien. *S. Immunitaetsforsch.* **55:**311–315.
4. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 221–232. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.
5. **Marraro, R. V., J. L. Mitchell, and C. R. Payet.** 1977. A chromogenic characteristic of an aerobic pseudomonad species in 2% tryptone (indole) broth. *Am. Med. Technol.* **39:**13–19.
6. **Vracko, R., and J. C. Sherris.** 1963. Indole-spot test in bacteriology. *Am. J. Clin. Pathol.* **39:**429–432.

APPENDIX 3.17.23–1

Include QC information on reagent container and in QC records.

Reagent Preparation

Caution: *HCl is toxic and burns. Make indole reagents in a fume hood. Add acid to water; do not add water to acid.*

A. Ehrlich's reagent

p-dimethylaminobenzaldehyde ……1 g
ethyl alcohol, 95% ……95 ml
hydrochloric acid, concentrated ……20 ml

1. Dissolve the aldehyde in the alcohol (this may require gentle heating).
2. Working under a fume hood, slowly add the acid (never add alcohol to acid). Mix constantly.
3. The reagent should be pale yellow and stable for 1 year.

B. Kovács' reagent

p-dimethylaminobenzaldehyde ……10 g
isobutyl or isoamyl alcohol (absolute) …. 150 ml
hydrochloric acid, concentrated ……50 ml

1. Dissolve the aldehyde in the alcohol (this may require gentle heating).
2. Working under a fume hood, slowly add the acid (never add alcohol to acid). Mix constantly.
3. The reagent should be pale yellow and stable for 1 year.

APPENDIX 3.17.23–1 *(continued)*

C. 5% *p*-Dimethylaminobenzaldehyde

p-dimethylaminobenzaldehyde ..... 5 g
10% (vol/vol) hydrochloric acid ..... 100 ml

1. Add 10 ml of concentrated HCl to 90 ml of water.
2. Dissolve the aldehyde in the acid (use a fume hood).
3. Shelf life is 4 months.

D. 1% *p*-Dimethylaminocinnamaldehyde

p-dimethylaminocinnamaldehyde ..... 1 g
10% (vol/vol) hydrochloric acid ..... 100 ml

1. Add 10 ml of HCl to 90 ml of water.
2. Dissolve the aldehyde in the acid (use a fume hood).
3. Shelf life is 2 months (1).

Reference

1. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., P. 221–232. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.

3.17.24 Indoxyl Acetate Disk Test

I. PRINCIPLE

Indoxyl is a tryptophan product of putrefactive decomposition in the intestines of humans by a bacterial esterase (3). The presence of this enzyme can be confirmed in vitro by the bacterial hydrolysis of indoxyl acetate to release indoxyl. Indoxyl then combines with oxygen to spontaneously form indigo. In 1987, Mills and Gherna (2) found this test to be useful to distinguish *Campylobacter jejuni* and *Campylobacter coli* (positive) from *Campylobacter lari* and *Campylobacter fetus* (negative).

II. MICROORGANISMS TESTED

A. Oxidase-positive, motile, curved, gram-negative rods suggestive of *Campylobacter* spp. and related organisms *(Helicobacter* and *Arcobacter)*

B. Gram-negative, oxidase-positive diplococci growing on BAP as white colonies that remain together when lifted with a loop or wire (4)

III. REAGENT AND SUPPLIES

A. Disks

1. Purchase (Hardy Diagnostics, Remel, Inc.) *or*

2. Prepare in-house.

a. 10% Indoxyl acetate (Sigma Chemical Co., St. Louis, MO) in acetone

b. Add 50 µl (25 µl at a time) to a 6-mm blank paper disk (BD Diagnostic Systems, Hardy Diagnostics).

c. Dry the disks at room temperature.

3. Store at 4°C in a brown bottle to protect from light; add desiccant to keep dry.

B. Supplies

1. Sterile wooden sticks

2. Petri dish

3. Sterile distilled water or saline

IV. QUALITY CONTROL

A. Do not use if disks are discolored and no longer white.

B. Perform QC on each lot or shipment of disks using a positive and negative control prior to putting it into use.

C. Organisms

1. *C. jejuni* ATCC 33560 or *Moraxella catarrhalis* ATCC 25240—positive (blue color)

2. *Campylobacter hyointestinalis* ATCC 35217 or *C. fetus* ATCC 27374—negative (no color change)

V. PROCEDURE

A. Place indoxyl acetate disk in a plastic petri dish and transfer a large loopful of 24- to 48-h growth from plate onto the disk.
B. Add a drop of sterile distilled water to the disk for *Campylobacter* (3). For *Moraxella*, premoisten the disk with saline prior to inoculation.
C. Incubate at room temperature for up to 30 min for *Campylobacter* (3) and up to 3 min for *Moraxella* (4).

VI. INTERPRETATION

A. A positive test shows dark blue color development on disk in 3 min for *Moraxella* and 5 to 10 min for *Campylobacter.*
B. A negative test shows no color change.
C. Weakly positive reactions are pale blue in 10 to 30 min.

VII. REPORTING RESULTS

A. *C. jejuni, C. coli, Campylobacter upsaliensis, Arcobacter cryaerophilus,* and *Helicobacter fennelliae* are indoxyl acetate positive (1, 3).
B. *C. lari, Helicobacter pylori, Helicobacter cinaedi,* and *Helicobacter pullorum* are negative for indoxyl acetate (1, 3).
C. Report as *Moraxella catarrhalis* if oxidase-positive, gram-negative diplococci grow on BAP as colonies that remain together when sampled and are strongly indoxyl acetate positive in 3 min (4).

VIII. LIMITATIONS

A. The test is reliable regardless of the media used for growth (2).
B. The disk test is more rapid and reliable than the tube method (3).

REFERENCES

1. **Hodge, D. S., A. Borczyk, and L. L. Wat.** 1990. Evaluation of the indoxyl acetate hydrolysis test for the differentiation of campylobacters. *J. Clin. Microbiol.* **28:**1482–1483.
2. **Mills, C. K., and R. L. Gherna.** 1987. Hydrolysis of indoxyl acetate by *Campylobacter*. *J. Clin. Microbiol.* **25:**1560–1561.
3. **Popovic-Uroic, T., C. M. Patton, M. A. Nicholson, and J. A. Kiehlbauch.** 1990. Evaluation of the indoxyl acetate hydrolysis test for rapid differentiation of *Campylobacter, Helicobacter,* and *Wolinella* species. *J. Clin. Microbiol.* **28:**2335–2339.
4. **Speeleveld, E., J. M. Fosséprè, B. Gordts, and H. W. Van Landuyt.** 1994. Comparison of three rapid methods, tributyrine, 4-methylumbelliferyl butyrate, and indoxyl acetate, for rapid identification of *Moraxella catarrhalis. J. Clin. Microbiol.* **32:**1362–1363.

3.17.25

Kligler's Iron Agar Test and Triple Sugar Iron Agar Test

I. PRINCIPLE

Kligler's iron agar (KIA) contains casein and meat peptones, phenol red as the pH indicator, 0.1% glucose, and 1% lactose for fermentation (4, 5). Ferric or ferrous ions and sodium thiosulfate are present to detect hydrogen sulfide production. Triple sugar iron agar (TSI) is similar in formulation; however, TSI also contains 1% sucrose, which is not included in KIA (3, 7).

Organisms that are non-lactose fermenting initially produce a yellow slant due to the production of acid from the glucose. The small amount of glucose is rapidly depleted. Oxidative metabolism continues in the slant after the low concentration of glucose has been depleted, producing an alkaline pH from the aerobic breakdown of peptone; the slant turns red. There is no oxygen penetration into the butt and no oxidative metabolism; the butt remains acid and yellow. Thus, a non-lactose fermenting organism yields an alkaline (K) slant over an acid (A) butt (K/A; red slant; yellow butt). Lactose-fermenting (and/or sucrose-fermenting in the case of TSI) bacteria continue to produce a large amount of acid in the slant and in the butt so the reaction in both remains acid (A/A; yellow slant; yellow butt). If the slant and the butt remain neutral, the organism is not capable of fermenting glucose or other sugars (K/K; red slant; red butt). Gas production from sugar fermentation is indicated by bubbles, fracturing of the medium, or displacement of the medium.

Hydrogen sulfide is produced by the action of the bacteria with sodium thiosulfate. This is detected by the reduction of ferric ions to produce a black precipitate. While these media are used primarily as screening tests for fecal pathogens, they are useful biochemical tests for H_2S production by *Erysipelothrix* and other organisms, such as *Campylobacter*. In addition, these media are very useful for the determination of the ability of fastidious organisms to ferment glucose, since many organisms will not react in OF medium and appear to be non-glucose fermenting (8).

II. MICROORGANISMS TESTED

A. Organisms
- **1.** Gram-negative rods, especially fastidious organisms, to determine their ability to ferment glucose and lactose (or sucrose, for TSI) and to produce hydrogen sulfide
- **2.** Oxidase-negative, catalase-positive, nonmotile, gram-negative coccobacilli that grow on MAC, to identify *Acinetobacter* spp.
- **3.** Catalase-negative, gram-positive rods to detect H_2S production, to separate lactobacilli from *Erysipelothrix*
- **4.** *Campylobacter*, to detect H_2S production

B. Use as part of the battery to screen for enteric pathogens (procedure 3.8.1).

C. Use to determine whether a gram-negative rod is glucose fermenting, in deciding whether to use an oxidative or fermentative system for the identification of the organism.

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media
- **1.** Characteristics
  - **a.** Agar slants with deep butts
  - **b.** Purchase commercially or prepare from dehydrated powder (6).
    - (1) Rehydrate commercial TSI or KIA according to manufacturer's instructions.
    - (2) Dispense 5- to 7-ml aliquots into 16- by 125-mm screw-cap tubes.

III. MEDIA, REAGENTS, AND SUPPLIES *(continued)*

(3) Autoclave at 121°C for 15 min. Do not overheat.
(4) Cool as slants with deep butts.
(5) Store at 2 to 4°C. Some prefer to store at 25°C so that cracks will not form when medium is warmed prior to inoculation.

2. Options
a. KIA
b. TSI

◩ **NOTE:** For best results with campylobacters, agar must be freshly prepared and used in 1 week. One manufacturer recommends that KIA be prepared fresh or melted and resolidified just before use (6).

B. Supplies
1. Wire or disposable inoculating needles
2. 35°C heat block or incubator without increased CO_2
3. Optional: lead acetate paper

IV. QUALITY CONTROL

A. Perform QC on each new lot and shipment of media prior to putting it into use.
◩ **NOTE:** The Clinical and Laboratory Standards Institute (formerly NCCLS) has proposed elimination of user QC for TSI purchased from commercial sources. Consult with current regulatory agencies prior to discontinuation of user QC (2).

B. Inspect agar for freezing, contamination, cracks, dehydration, and bubbles prior to storage and before use. Discard any tubes with bubbles in the agar. Discard tubes that are not red or that lack a deep butt. The slant and butt should be equal in length.

C. Organisms

Organism	ATCC no.	TSI	KIA	H_2S
Escherichia coli	25922	A/A, gas	A/A, gas	−
Salmonella enterica serovar Typhimurium	14028	K/A, ±[a] gas	K/A, ±[a] gas	+
Pseudomonas aeruginosa	27853	K/K, no gas	K/K, no gas	−
Optional for special species: *Campylobacter hyointestinalis*	35217	K/K, no gas	K/K, no gas	+

[a] ±, with or without.

V. PROCEDURE

A. Warm medium to room temperature and examine for cracks. Do not use if cracks appear.
B. Using a sterile inoculating needle, touch the center of a well-isolated colony.
C. Stab to within 3 to 5 mm from the bottom of the tube.
D. Withdraw the needle, and streak the entire surface of the agar slant.
E. Optional for fastidious organisms: add a strip of lead acetate paper to top of tube and hold in place with the cap of the tube.
F. *Place cap loosely on tube. Do* not *tighten the cap.*
G. Incubate aerobically at 35 to 37°C for 18 to 24 h.
H. Examine the reaction in the slant and the butt. Observe for gas and hydrogen sulfide production.
I. Do not attempt to interpret sugar fermentation reactions after 24 h. Refrigerate tubes if readings will be delayed.
J. If desired, extend incubation only to detect H_2S production. Campylobacters may take 3 days for production of H_2S.

VI. INTERPRETATION

A. Observations
1. Acid reaction: yellow
2. Alkaline reaction: red

VI. INTERPRETATION
(continued)

3. H_2S production: black color throughout the medium, a black ring at the junction of the butt and slant, or a black precipitate in the butt. Low levels of H_2S production present as no blackening of the agar but blackening of the lead acetate paper.

4. Gas production: bubbles, cracks, or displacement of media

B. Interpretation of carbohydrate observations

1. A/A: glucose and lactose (and/or sucrose for TSI) fermented

2. K/A: only glucose fermented (non-lactose fermenter or glucose fermenter).

3. K/K: no carbohydrates fermented (non-glucose fermenter).

VII. REPORTING RESULTS

A. If a catalase-negative, gram-positive rod is vancomycin resistant and H_2S positive, report as "probable *Erysipelothrix rhusiopathiae.*" See Table 3.18.1–5.

B. If a campylobacter-like organism is H_2S positive, report as "probable *Campylobacter hyointestinalis.*" See Table 3.8.2–4 for other tests to confirm identification.

C. For evaluation of the identification of *Salmonella,* see the screening flowchart in Fig. 3.8.1–2.

D. For other organisms, refer to procedure 3.18.2.

VIII. LIMITATIONS

A. Do not read the test before 18 h, since false readings of acid in the slant may result.

B. Copious amounts of H_2S may mask the glucose reaction. If this exists, glucose has been fermented and should be recorded as such. Check for gas production.

C. When using KIA for screening fecal cultures, it is not able to separate some uncommon non-lactose fermenters (e.g., *Providencia*) that ferment sucrose from fecal pathogens which are lactose and sucrose negative.

D. If TSI is used to screen fecal cultures, the sugar reactions of acid/acid may be obtained with *Yersinia enterocolitica* and *Edwardsiella tarda,* since both can ferment sucrose but not lactose (6).

E. Neither KIA nor TSI, without the use of pyrrolidonyl-β-naphthylamide (PYR), will separate lactose-positive *Salmonella* (lysogenized) from *Citrobacter.*

F. Gas production is better in TSI than in KIA. However, H_2S production may be inhibited on TSI for organisms that utilize sucrose and suppress the enzyme mechanism that results in production of H_2S (1).

G. Sulfide-indole-motility (SIM) agar is more sensitive in the detection of H_2S than either TSI or KIA.

REFERENCES

1. **Bulmash, J. M., and M. Fulton.** 1964. Discrepant tests for hydrogen sulfide. *J. Bacteriol.* **88:**1813.
2. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
3. **Hajna, A. A.** 1945. Triple-sugar iron agar medium for identification of the intestinal group of bacteria. *J. Bacteriol.* **49:**516–517.
4. **Kligler, I. J.** 1917. A simple medium for the differentiation of members of the typhoid-paratyphoid group. *Am. J. Public Health* **7:**1042–1044.
5. **Kligler, I. J.** 1918. Modifications of culture media used in the isolation and differentiation of typhoid, dysentery and allied bacilli. *J. Exp. Med.* **28:**319–322.
6. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria,* 3rd ed., p. 239–253. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.
7. **Sulkin, S. E., and J. C. Willett.** 1940. A triple sugar-ferrous sulfate medium for use in identification of enteric organisms. *J. Lab. Clin. Med.* **25:**649–653.
8. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria,* 2nd ed., p. 2–3, 22. Williams & Wilkins, Baltimore, MD.

3.17.26 LAP (Leucine Aminopeptidase) Test

I. PRINCIPLE

Detection of the enzyme leucine aminopeptidase (LAP) is one of the tests in the definitive identification of catalase-negative, gram-positive cocci. Specifically, it differentiates *Aerococcus* and *Leuconostoc* from *Streptococcus*, *Enterococcus*, *Lactococcus*, and *Pediococcus* (1, 2). The former are LAP negative, and the latter are positive. Hydrolysis of the leucine-*p*-naphthylamide substrate on a paper disk inoculated with the bacteria releases leucine and free β-naphthylamide. The β-naphthylamide combines with cinnamaldehyde reagent to form a pink to cherry red pigment.

II. MICROORGANISMS TESTED

Fresh growth less than 24 h old of catalase-negative, gram-positive cocci. If growth is poor, a culture that has been incubated for up to 72 h may be used.

III. MEDIA, REAGENTS, AND SUPPLIES

A. Reagents

1. Disks (Key Scientific Products Company; Hardy Diagnostics; Remel, Inc.; Oxoid, Inc.)

◩ **NOTE:** BactiCard Strep (Remel, Inc.) incorporates both LAP and pyrrolidonyl-β-naphthylamide (PYR) on one card. StrepQuick (Hardy Diagnostics) incorporates LAP, PYR, and esculin on one card.

2. 0.01% *p*-Dimethylaminocinnamaldehyde (the same reagent as used in PYR test)

B. Supplies

1. Sterile loop or stick for harvesting

2. Distilled water (neutral pH)

3. Petri dish

IV. QUALITY CONTROL

A. Perform QC on each new lot or shipment of disks and color reagent prior to putting it into use. Since the test is performed infrequently and most gram-positive cocci are LAP positive, verify potency by testing the positive control each time a negative test is obtained.

B. Organisms

1. *Enterococcus faecalis* ATCC 29212—LAP positive

2. *Aerococcus viridans* ATCC 11563—LAP negative

V. PROCEDURE

A. Place disk onto slide or petri dish. Moisten (do not saturate the disk) with a loopful of distilled water.

B. Using a sterile stick or loop, smear with the suspected isolate.

C. Incubate at room temperature for 5 min.

D. Add 1 drop of cinnamaldehyde developer reagent and wait 2 min to observe color.

VI. INTERPRETATION

A. A positive test after adding reagent shows a deep red to reddish purple color.
B. Weak reactions are pink.
C. A negative test is colorless or yellow.

VII. REPORTING RESULTS

A. *Leuconostoc* is LAP negative, whereas other organisms in the group *(Streptococcus, Enterococcus, Lactococcus,* and *Pediococcus)* are almost always positive. *Aerococcus* is LAP variable.
B. See Table 3.18.1–4 for further testing (2).

VIII. LIMITATIONS

A. This test is only one of a battery of useful tests for identifying catalase-negative, gram-positive cocci and is most helpful when kit tests yield uncommon identifications or Gram stain results do not show chaining cocci.
B. False negatives may result from insufficient inoculum.

REFERENCES

1. **Facklam, R., and J. A. Elliott.** 1995. Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clin. Microbiol. Rev.* **8:**479–495.
2. **LaClaire, L. L., and R. R. Facklam.** 2000. Comparison of three commercial rapid identification systems for the unusual gram-positive cocci *Dolosigranulum pigrum, Ignavigranum ruoffiae,* and *Facklamia* species. *J. Clin. Microbiol.* **38:**2037–2042.

3.17.27 Lecithinase and Lipase Detection

I. PRINCIPLE

Lecithinases or phospholipases are enzymes released by the bacteria that destroy animal tissues. Lecithin is a normal component of egg yolks. In egg yolk agar (EYA) the lipoprotein component, lecithovitellin, can be split by lecithinase into phosphorylcholine and an insoluble diglyceride, which forms a precipitate in the medium (2). This precipitate appears as a white opaque halo surrounding the colony that produces the lecithinase. CCFA agar (procedure 3.8.3), used to identify *Clostridium difficile*, also contains egg yolks.

Bacterial lipases hydrolyze the breakdown of triglycerides into glycerol and free fatty acids. Fatty acids are mostly insoluble and cause opacity on EYA, producing an iridescent sheen on the colonies and the surface of EYA. Unlike lecithinase, lipase is not diffusible, and the reaction occurs only on the surface of the agar in the immediate vicinity of the colony.

II. MICROORGANISMS TESTED

A. Spore-forming gram-positive rods: lecithinase will separate *Bacillus cereus* group (positive) and *Bacillus anthracis* (positive) from other *Bacillus* species (negative) and *Clostridium perfringens* (positive) from some other *Clostridium* spp.

B. Catalase-negative, hemolytic, gram-positive rods: lecithinase separates *Arcanobacterium haemolyticum* from *Actinomyces pyogenes.*

C. Spore-forming, catalase-negative, gram-positive rods: *Clostridium sporogenes* is lipase positive. *C. difficile* is both lipase and lecithinase negative.

D. Non-glucose-fermenting, gram-negative rods of the fluorescent group: lecithinase will separate *Pseudomonas putida* (negative) from *P. fluorescens* (positive).

E. Catalase-negative, gram-variable rods identified as presumptive *Gardnerella vaginalis*, which is often lipase positive

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media: EYA

1. Media available from most medium vendors, or see Appendix 3.17.27–1 for preparation.

2. Store in moist container at 2 to 8°C until expiration date (usually 2 to 4 weeks).

B. Supplies

1. Sterile sticks or inoculating loops

2. Incubators at 42, 35, and 30°C

IV. QUALITY CONTROL

A. Inspect agar for freezing, contamination, cracks, and dehydration prior to storage and before use. Discard plates that are opaque.
B. Perform QC on each new lot or shipment of media prior to putting it into use.
C. Organisms
  1. *B. cereus* ATCC 14579—opaque halo around colony (lecithinase positive)
  2. *Staphylococcus aureus* ATCC 12600—iridescent sheen on and around colony (lipase positive)
  3. *Escherichia coli* ATCC 25922—no change in medium (negative)

V. PROCEDURE

A. After touching several colonies from an 18- to 72-h culture, inoculate a segment of the agar surface with a very *visible circular* amount of organism about the size of a dime. Then, streak an area to obtain isolated colonies.
B. For accurate results, incubate as follows without added CO_2 for 24 to 48 h or anaerobically for up to 72 h for anaerobes.
  1. 35°C for *Bacillus*, other gram-positive rods, and anaerobes
  2. 25°C for fluorescent non-glucose-fermenting, gram-negative rods, except *Pseudomonas aeruginosa*
  3. 30°C for other non-glucose-fermenting, gram-negative rods
  Examine for halo and iridescence.

VI. INTERPRETATION

A. Lecithin
Use transmitted light to observe halo.
  1. Positive test: development of a milky white opaque halo around the colony
  2. Negative test: no halo in the medium or around the colony

B. Lipase
Hold the plate on an angle with good lighting.
  1. Positive test: development of an iridescent sheen (mother-of-pearl) on the surface of the colony and the surrounding agar
  2. Negative test: no change in the medium

VII. REPORTING RESULTS

A. Catalase-positive, spore-forming, gram-positive rods that are lecithinase positive, with large zones of opacity, belong to the *B. cereus* group. Lack of motility separates *B. anthracis* from the other members of the group.
B. *G. vaginalis* organisms are catalase-negative, gram-variable coccobacilli that are hemolytic on human blood and are lipase positive. A negative test does not rule out the identification, since the biotypes that cause bacterial vaginosis are often lipase negative (1).
C. A gram-positive rod that is catalase negative, hemolytic, and lecithinase positive is *A. haemolyticum.*
D. Lecithinase and lipase are useful as part of identification of *Clostridium* to the species level.
  1. *C. perfringens* is lipase negative and lecithinase positive, which can be neutralized by adding anti-α toxin prior to inoculation of the agar (the Nagler reaction).
  2. *C. sporogenes* is lipase positive.
  3. *C. difficile* is both lipase and lecithinase negative.
  4. See section 4 for further tests for identification of *Clostridium* spp.
E. Among the fluorescent group of non-glucose-fermenting, gram-negative rods, *P. putida* is lecithinase negative and most *P. fluorescens* isolates are lecithinase positive. This test can substitute for gelatin hydrolysis.
F. *Burkholderia* organisms are often lecithinase positive.

VIII. LIMITATIONS

A. Ignore a slight clearing around the inoculum.
B. Zones of opacity are small with non-glucose-fermenting rods.
C. A negative lecithinase test should be compared to an uninoculated control plate, as lecithinase can diffuse throughout the entire agar plate and make interpretation difficult.
D. Some microorganisms may require up to 1 week to produce a positive lipase reaction.
E. Not all *P. fluorescens* isolates are lecithinase positive.

REFERENCES

1. **Aroutcheva, A. A., J. A. Simoes, K. Behbakht, and S. Faro.** 2001. *Gardnerella vaginalis* isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems. *Clin. Infect. Dis.* **33:**1022–1027.
2. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria,* 3rd ed., p. 273–281, 286–293. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.

APPENDIX 3.17.27–1

Preparation of Egg Yolk Agar Medium (1)

McClung and Toabe agar, modified for lecithinase and lipase tests

Proteose no. 2 peptone or Polypeptone (BBL)	40.0 g
disodium phosphate	5.0 g
monopotassium phosphate	1.0 g
sodium chloride	2.0 g
magnesium sulfate	0.1 g
glucose	2.0 g
hemin solution, 5 mg/ml	1.0 ml
agar	20.0 g
water	1.0 liter

A. Suspend ingredients, and adjust the pH to 7.6.
B. Mix, and boil to dissolve.
C. Dispense 20 ml per tube, and autoclave at 118°C for 15 min.
D. Cool to 50°C, and to each tube, add 2 ml of commercial egg yolk emulsion or 1 ml of egg yolk emulsion prepared as follows.
   1. Scrub, and then soak, an antimicrobial agent-free hen egg in 95% ethanol for 1 h.
   2. Aseptically aspirate or separate the egg yolk.
   3. Add equal volumes of egg yolk to sterile saline and stir to make smooth suspension.
E. Mix, and pour into plates.

Reference

1. **Phillips, E., and P. Nash.** 1985. Culture media, p. 1064–1065. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), *Manual of Clinical Microbiology,* 4th ed. American Society for Microbiology, Washington, DC.

3.17.28 Lipophilism Test for *Corynebacterium*

I. PRINCIPLE

Some strains of *Corynebacterium* grow better in the presence of added lipid. Such strains are called lipophilic or rabbit serum stimulated. They may also require lipid for growth (3). By adding a drop of serum to a plain Mueller-Hinton agar (MH) plate on which the isolate has been inoculated, the requirement or enhancement of growth with added lipid can be determined. In the tube test, growth is evaluated compared to growth in a broth culture that has not been enriched with rabbit serum. 1.0% Tween 80 can be used as a substitute for serum.

II. MICROORGANISMS TESTED

Catalase-positive, gram-positive rods as part of the identification of *Corynebacterium* to the species level

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media and reagents

1. MH
2. Andrade's glucose broth or BHI
3. Filter-sterilized rabbit serum (no. 16120-099; Invitrogen Life Technologies, Carlsbad, CA)
  a. Store at −20°C in small aliquots.
  b. Heat for 1 h at 60°C prior to use.
4. Sterile 0.85% NaCl

B. Other supplies

1. Sterile sticks and swabs
2. Incubator at 35°C with 5% CO_2
3. 0.5 McFarland standard

IV. QUALITY CONTROL

A. Test each lot of rabbit serum for sterility by inoculating 1 drop into BHI or onto MH. No increase in turbidity should be seen, and Gram stain of broth should show no organisms. No colonies should be present on MH.

B. Test each lot of rabbit plasma to verify that it will enhance the growth of *Corynebacterium.*

C. Organism

Corynebacterium jeikeium ATCC 43734—growth in broth or on MH with rabbit serum but not without it

V. PROCEDURE

A. Plate method

1. Emulsify the test organism in sterile saline to match a no. 0.5 McFarland standard.
2. Dip swab into saline and inoculate MH for confluent growth as for disk susceptibility testing (procedure 5.1). Allow to dry.
3. Place 1 drop of rabbit serum on plate. Allow to dry.
4. Incubate for 24 h at 35°C in 5% CO_2.
5. Observe for growth.

V. PROCEDURE *(continued)*

B. Broth method

1. Warm two tubes of either Andrade's broth with glucose or BHI broth to room temperature.
2. Add 1 drop of rabbit serum to one tube; label as "rabbit serum added."
3. Inoculate both tubes from several colonies of a 24- to 48-h culture.
4. Incubate for 24 h at 35°C in 5% CO_2.
5. Observe for growth.

VI. INTERPRETATION

A. Positive test

1. Plate test: enhanced growth on top of and immediately around the drop size area where the serum was inoculated compared to the areas where no serum was present
2. Tube test: enhanced growth (and red color in Andrade's tube if glucose positive) in tube with serum but no growth or poor growth and no color change in tube without serum

B. Negative test

1. Plate test: no enhancement of growth around the serum drop. Growth will be uniform on MH.
2. Tube test: growth (and red color in Andrade's tube if glucose positive) in both tubes.

VII. REPORTING RESULTS

The following species of *Corynebacterium* are lipophilic (3): *C. afermentans* subsp. *lipophilum, C. accolens, C. bovis, C. diphtheriae/intermedius, C. jeikeium,* CDC group G, *C. lipophiloflavum, C. macginleyi,* CDC group F-1, and *C. urealyticum.*

VIII. LIMITATIONS

A. This test is an excellent adjunct to kit identification of *Corynebacterium;* results with the kit should be consistent with the results of this test (1, 2, 4).

B. This test cannot be performed using CHOC, since most species of *Corynebacterium* will grow on this medium.

C. Tween 80 (polysorbate 80) can be used as an alternative to rabbit serum.

REFERENCES

1. **Funke, G., K. Peters, and M. Aravena-Roman.** 1998. Evaluation of the RapID CB Plus system for identification of coryneform bacteria and *Listeria* spp. *J. Clin. Microbiol.* **36:**2439–2442.
2. **Funke, G., F. N. R. Renaud, J. Freney, and P. Riegel.** 1997. Multicenter evaluation of the updated and extended API (RAPID) Coryne database 2.0. *J. Clin. Microbiol.* **35:**3122–3126.
3. **Funke, G., A. von Graevenitz, J. E. Clarridge III, and K. A. Bernard.** 1997. Clinical microbiology of coryneform bacteria. *Clin. Microbiol. Rev.* **10:**125–159.
4. **Hudspeth, M. K., S. Hunt Gerardo, D. M. Citron, and E. J. C. Goldstein.** 1998. Evaluation of the RapID CB Plus system for identification of *Corynebacterium* species and other gram-positive rods. *J. Clin. Microbiol.* **36:**543–547.

3.17.29 Malonate Test

I. PRINCIPLE

The medium for the malonate test contains sodium malonate (1). Malonate is an enzyme inhibitor and inhibits utilization of succinic acid by bacteria, shutting down the Krebs and glyoxylic cycles. Growth is indicative of malonate utilization as a carbon source; a small amount of glucose is also present to stimulate growth of some organisms. The medium also contains inorganic ammonium salts as the sole source of nitrogen. When the bacterium ferments sodium malonate, sodium hydroxide and sodium bicarbonate are formed, which increases alkalinity of the medium. The shift in pH turns the bromthymol blue indicator in the medium from green to blue (2). Malonate-negative organisms that ferment glucose cause the indicator to turn yellow or acid. This medium is recommended as part of differentiating among the *Enterobacteriaceae*, especially species of *Klebsiella* and *Salmonella.*

II. MICROORGANISMS TESTED

Enterobacteriaceae as part of the identification to the species level

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media

1. Ingredients per liter of deionized water

yeast extract	1.0 g
ammonium sulfate	2.0 g
dipotassium phosphate	0.6 g
monopotassium phosphate	0.4 g
sodium chloride	2.0 g
sodium malonate	3.0 g
glucose	0.25 g
bromthymol blue	0.025 g

a. Final pH, 6.7

b. Store at 2 to 8°C.

2. Malonate tablets are also available (Key Scientific). Refer to http://www.keyscientific.com for procedure.

B. Supplies

1. Sterile inoculating loops or sticks

2. Incubator at 35 to 37°C

IV. QUALITY CONTROL

A. Perform QC on each new lot or shipment of media prior to putting it into use.

B. Inspect broth for contamination prior to storage and before use. Discard any tubes that are blue.

C. Organisms

1. *Klebsiella pneumoniae* ATCC 13883—malonate positive (blue color)

2. *Escherichia coli* ATCC 25922—malonate negative (green color)

V. PROCEDURE

A. Using a loop or stick, inoculate tube with a light inoculum picked from the center of a well-isolated colony (turbidity should be less than a no. 0.5 McFarland standard, i.e., no visible turbidity).

B. Incubate aerobically at 35 to 37°C for up to 48 h.

C. Observe a color change from green to blue along the slant.

VI. INTERPRETATION

A. A positive test is growth and change to light blue or deep Prussian blue color throughout the medium.

B. A negative test is no color change or change from green to yellow due to fermentation of glucose.

VII. REPORTING RESULTS

A. *Klebsiella ozaenae* is malonate negative; *K. pneumoniae* is malonate positive.

B. *Citrobacter amalonaticus* is malonate negative; *Citrobacter koseri* is malonate positive.

C. Use as part of the identification of other *Enterobacteriaceae.*

VIII. LIMITATIONS

A. Some reactions are slight. Compare to an uninoculated tube when reading.

B. Do not read reactions before 48 h.

C. Yeast extract and glucose are needed to stimulate growth of some *Salmonella* organisms but are not generally necessary for other species.

REFERENCES

1. **Leifson, E.** 1933. Fermentation of sodium malonate as a means of differentiating *Aerobacter* and *Escherichia. J. Bacteriol.* **26:**329–330.
2. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria,* 3rd ed., p. 310–315. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.

3.17.30 MGP (Methyl Glucopyranoside) Test

I. PRINCIPLE

MGP broth is used to differentiate enterococci based on the ability to acidify the carbohydrate methyl-α-D-glucopyranoside (MGP). Vancomycin-resistant *Enterococcus faecium* (VREF) is a serious nosocomial problem. *Enterococcus gallinarum* and *Enterococcus casseliflavus,* while not important for purposes of control of nosocomial infections, show intrinsic resistance to vancomycin due to the *vanC* gene (6). *E. gallinarum* and *E. casseliflavus* are difficult to differentiate from VREF by conventional biochemical tests (5), since they are phenotypically closely related.

Typically, *E. gallinarum* and *E. casseliflavus* can be differentiated from *E. faecium* based on motility: *E. gallinarum* and *E. casseliflavus* are motile, while *E. faecium* is not. However, nonmotile strains of *E. gallinarum* have been detected using standard motility agar (2, 8), although this has not been reported when the rapid (2-h) motility test is performed (9). Correct identification of these strains is critical due to their difference in pathogenicity. *E. gallinarum* and *E. casseliflavus* acidify MGP, but *Enterococcus faecalis* and *E. faecium* do not (1, 2, 4, 7, 8). This makes the MGP test useful in preventing the misidentification of vancomycin-resistant *E. gallinarum* as VREF for laboratories that cannot perform the rapid tube motility test.

II. MICROORGANISMS TESTED

A. As an alternative to the motility test
- **1.** Test any gram-positive coccus that is catalase negative and pyrrolidonyl-β-naphthylamide (PYR) positive and grows on plates with 6 μg of vancomycin per ml or demonstrates resistance to vancomycin by antimicrobial susceptibility testing but shows susceptibility to ampicillin.
- **2.** Test any isolate that is identified as *E. faecium* by a commercial kit system but is ampicillin susceptible.

B. Test any nonmotile enterococcus that demonstrates resistance to vancomycin with an MIC less than 32 μg/ml but is ampicillin susceptible.

C. Test can be performed from any BAP or any plate medium with enterococcal growth, including media with bile-esculin or azide.

III. MEDIA AND SUPPLIES

A. Media (Hardy Diagnostics)

1. Standard medium per liter of deionized water

pancreatic digest of casein	10.0 g
sodium chloride	5.0 g
MGP	10.0 g
phenol red	18.0 mg

2. Rapid medium per liter of deionized water

basal medium	15.0 g
indicator	50.0 mg
MGP	50.0 g

3. Store at 2 to 8°C, away from direct light, for up to 6 months.

B. Supplies
- **1.** Incubator
- **2.** Sterile sticks, loops, or inoculating needle

IV. QUALITY CONTROL

A. Examine media for red-orange color (standard tube) or blue color (rapid tube) and lack of turbidity.

B. Test each lot of medium with a positive and negative control prior to putting it into use.

C. Organisms

1. *E. gallinarum* ATCC 49573—medium turns yellow (positive).

2. *E. faecalis* ATCC 29212—medium remains red or orange (negative).

V. PROCEDURE

A. Standard medium

1. Inoculate the MGP broth by lightly touching a single, isolated colony. Do not use a heavy inoculum.

2. Incubate aerobically at 35°C for 24 h.

3. Observe for yellow color change in the media.

B. Rapid medium

1. Using a "sweep" of colonies from an 18 to 24-h pure culture of the organism to be tested, stab the rapid MGP medium with an inoculating needle. There should be a cell paste visible on the needle as the medium is being inoculated.

2. Incubate aerobically at 35°C for 5 h.

3. Observe for the development of a yellow color along the stab line; this is indicative of a positive test.

4. Replace weak or inconclusive reactions in the incubator for a total of 24 h for development of a yellow color.

5. Optional: run controls with each test run to easily distinguish between a negative and positive reaction.

VI. INTERPRETATION

A. A yellow color change is a positive test for acidification of MGP.

B. Red or orange color is a negative test in standard media. Blue color is a negative test in the rapid medium.

VII. REPORTING RESULTS

A. Report as either *Enterococcus gallinarum* or *Enterococcus casseliflavus* if the reaction is positive and the vancomycin susceptibility test shows an intermediate or resistant result for vancomyin. The yellow pigment of *E. casseliflavus* separates these two species. Report the vancomycin MIC result for treatment purposes but do not label or comment that either of these two species, which carry the *vanC* genotype, are vancomycin-resistant enterococci (VRE).

◩ **NOTE:** *E. faecium* and *E. faecalis* carry the *vanA* and *vanB* genotypes, which confer high-level vancomycin resistance and may be transferred within the hospital environment. Patients colonized or infected with true VRE are placed on contact isolation. Patients colonized or infected with either *E.casseliflavus* or *E. gallinarum*, which carry the *vanC* genotype, which confers low-level intrinsic resistance to vancomycin, do not require the same infection control measures to prevent nosocomial spread (3).

B. Report as *Enterococcus faecium* if result is negative and the organism keys out as *E. faecium* by a kit identification method. Also alter infection control to VRE if isolate is vancomycin resistant.

C. Other enterococci are positive for MGP but are not resistant or intermediate in susceptibility to vancomycin. See Table 3.18.1–3.

VIII. LIMITATIONS

A. A heavy inoculum may give false-positive results in the standard test.

B. Extended incubation times may result in false-positive reactions.

C. Do not test organisms identified as *E. faecalis*, since false-positive results have been reported (1).

D. Rapid MGP has a sensitivity of 100% and a specificity of 96.5%. The standard test has reported sensitivities of 100% (2) and 98% (6) and a specificity of 95% (2), but two of the three misidentified *E. faecium* isolates were not VRE.

E. *Vagococcus* gives a positive result (1).

F. Use of an inoculating needle is recommended for the rapid test, as inocula from a loop can overwhelm the media.

G. Negative rapid MGP tests may change from their original color, becoming fainter shades of blue. This color change is easily distinguished if positive and negative control tubes are used in parallel with each test being performed.

REFERENCES

1. **Carvalho, M. G., L. M. Teixeira, and R. R. Facklam.** 1998. Use of tests for acidification of methyl-α-D-glucopyranoside and susceptibility to efrotomycin for differentiation of strains of *Enterococcus* and some related genera. *J. Clin. Microbiol.* **36:**1584–1587.
2. **Chen, D. K., L. Pearce, A. McGeer, D. E. Low, and B. M. Willey.** 2000. Evaluation of D-xylose and 1% methyl-α-D-glucopyranoside fermentation tests for distinguishing *Enterococcus gallinarum* from *Enterococcus faecium. J. Clin. Microbiol.* **38:**3652–3655.
3. **Clinical and Laboratory Standards Institute.** 2008. *Performance Standards for Antimicrobial Susceptibility Testing.* Eighteenth informational supplement. Approved standard M100-S18. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Devriese, L. A., B. Pot, K. Kersters, S. Lauwers, and F. Haesebrouck.** 1996. Acidification of methyl-α-D-glucopyranoside: a useful test to differentiate *Enterococcus casseliflavus* and *Enterococcus gallinarum* from *Enterococcus faecium* species group and from *Enterococcus faecalis. J. Clin. Microbiol.* **34:**2607–2608.
5. **Facklam, R. R., and M. D. Collins.** 1989. Identification of *Enterococcus* species isolated from human infections by a conventional test scheme. *J. Clin. Microbiol.* **27:**731–734.
6. **Gin, A. S., and G. G. Zhanel.** 1996. Vancomycin-resistant enterococci. *Ann. Pharmacother.* **30:**615–624.
7. **Hanson, K. L., and C. P. Cartwright.** 1999. Comparison of simple and rapid methods for identifying enterococci intrinsically resistant to vancomycin. *J. Clin. Microbiol.* **37:**815–817.
8. **Turenne, C. Y., D. J. Hoban, J. A. Karlowsky, G. G. Zhanel, and A. M. Kabani.** 1998. Screening of stool samples for identification of vancomycin-resistant *Enterococcus* isolates should include the methyl-α-D-glucopyranoside test to differentiate nonmotile *Enterococcus gallinarum* from *E. faecium. J. Clin. Microbiol.* **36:**2333–2335.
9. **Van Horn, K. G., and K. M. Rodney.** 1998. Colonization and microbiology of the motile enterococci in a patient population. *Diagn. Microbiol. Infect. Dis.* **31:**525–530.

3.17.31 Motility Tests

I. PRINCIPLE

The motility test is used to detect the presence of flagella by bacteria, allowing them to travel in and out of the microscopic field or beyond their initial inoculation in agar (3). For the wet preparation, a drop of organism in broth is suspended on a clean glass slide, a coverslip is added, and the culture is observed microscopically for motility. Occasionally the organism is incubated in the broth prior to examination.

In the tube test, semisolid motility medium is inoculated in a straight line down through the center of a tube (4). Motile organisms will migrate out from the line of inoculation, causing visible turbidity throughout the tube. Nonmotile organisms will grow only along the line of inoculation. Other substrates which allow simultaneous testing of other biochemical reactions that aid in the identification of microorganisms may be added to the medium.

II. MICROORGANISMS TESTED

Campylobacter; Legionella; enterococci; *Enterobacteriaceae; Listeria; Bacillus;* other gram-positive rods; non-glucose-fermenting, gram-negative rods; and any other organism where motility is useful for identification

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media and reagents

1. Broth medium for wet preparation
- **a.** TSB, nitrate, or BHI broth
- **b.** Voges-Proskauer (VP) cupule of the API strip
- **c.** Any agar slants with TSB or BHI broth added (*see* item VIII)
- **d.** Saline or distilled water

◩ **NOTE:** Cleary (1) reported that some motile organisms can become immobile in distilled water.

2. Motility tube media
- **a.** Combination of sulfide for H_2S detection, indole, and motility test medium (SIM)
- **b.** Motility test medium with or without TTC (triphenyltetrazolium chloride, a colorless vital dye incorporated into the medium, which turns red as it is reduced when incorporated into bacteria; the dye allows visualization of the bacteria)
- **c.** Motility nitrate medium
- **d.** Combination of motility test medium, indole, and lysine decarboxylase
- **e.** Combination of motility test medium, indole, and ornithine decarboxylase (MIO)

◩ **NOTE:** Motility medium contains 5 g of agar per liter and can be prepared in-house in agar plates that are incubated agar side down. Motile organisms move from the point of inoculum to the edges of the plate.

B. Supplies

1. 22- by 22-mm coverslips and microscope slides

2. Phase-contrast or bright-field microscope

3. Sterile inoculating needle or sticks

IV. QUALITY CONTROL

A. Test each new lot or shipment of tube media prior to use with a positively and negatively reacting organism and for sterility.

B. Organisms

Organism	ATCC no.	Media	Motility	Indole	H_2S	Lysine	Ornithine
Escherichia coli	25922	All	+	+		−	+
Klebsiella pneumoniae	13883, 27736	All	−	−	−	+	−
Proteus vulgaris	33420	SIM	+	+	+		

C. Competence of technologists in the hanging-drop test is validated by testing known motile enterococci or *Listeria* in the broth assay.

V. PROCEDURE

A. Wet mount preparation

1. Inoculum: use *fresh* growth from an agar plate and suspend isolated colonies in broth. Use a light inoculum (not visibly turbid).

a. It is acceptable to suspend the organism in a small amount of medium for an initial wet mount, but follow with incubation of a larger amount of broth media if the result is negative.

b. Choosing the medium

(1) Use any broth which does not contain carbohydrate and will support the growth of the organisms (BHI, nitrate broth).

(2) Always use a broth for *Bacillus* spp. (1).

(3) Use 0.5 ml of BHI or TSB for enterococci.

(4) Saline can be used for gram-negative rods.

(5) Use warm sterile tap water for *Legionella.*

(6) A drop from the VP microtube of the incubated API 20E strip may be used before the reagents are added. However, a negative test must be repeated in a non-carbohydrate-containing broth.

(7) For problem organisms, streak an agar slant and then carefully add a few drops of BHI or nutrient broth to the base of the slant, covering just a bit of the streaked slant. After overnight incubation, use a drop of the BHI broth for the wet mount (this works very well for non-fermenting gram-negative rods).

2. Examination

a. While wearing gloves, place a small drop of fresh liquid on the center of a microscope slide; add coverslip. Allow organisms to "settle" for a minute.

b. Observe under high power (40×).

(1) For a light microscope, decrease the light by closing the diaphragm.

(2) Preferably, use a phase-contrast microscope.

3. For all organisms negative for motility by initial wet mount, repeat the wet mount after incubation in broth, or test by tube method below.

a. Incubate at 30°C for nonfermenting, gram-negative rods (24 h).

b. Incubate enterococci and *Listeria* at 30°C for 2 h (5, 8).

c. Other organisms may be incubated at temperatures optimal for their growth, usually 35°C.

B. Tube media for *Enterobacteriaceae;* nonfermenting, gram-negative rods; and *Listeria*

1. With a sterile inoculating wire, pick an isolated colony and stab the medium straight down through the center to a depth of 1/2 in. for small tubes and 1 in. for larger tubes.

V. PROCEDURE *(continued)*

2. Incubate as follows.
   a. At 35°C for *Enterobacteriaceae* for 24 h
   b. At 30°C for nonfermenting, gram-negative rods and enterococci for 24 h
   c. If there is a question regarding a negative result, incubate at 25°C.
   d. For *Listeria* and *Yersinia*, incubate two tubes, one at 35°C and one at 25°C.

VI. INTERPRETATION

A. Wet mount preparation

1. Directional purposeful motility is a positive test. Motile organisms change position with respect to one another. Brownian movement (random jiggling or shaking due to molecular bombardment), where the organisms remain in the same relative position with respect to each other, should not be mistaken for true motility (9).
2. *Campylobacter* organisms display a very active motility which appears as tiny dots darting in and out of the field.

B. Tube media

1. Diffuse growth outward away from stab line or turbidity of the media is a positive test.
2. A clear tube (the same as the uninoculated media) with growth only along the line of inoculation indicates that the organism is nonmotile.
3. In media with TTC, the red color forms in the area of bacterial growth. Motile organisms produce a pink color that diffuses from the stab line. Organisms that are nonmotile produce a pinkish red pigment that is confined to the stab line.

VII. REPORTING RESULTS

A. *Bacillus* spp. should be motile. Lack of motility could indicate *Bacillus anthracis.*

B. *Enterococcus casseliflavus* and *Enterococcus gallinarum* are motile.

C. *Listeria* organisms are motile at 25°C but not at 35°C, with a characteristic umbrella-shaped growth at the top of the tube. On wet mount they exhibit tumbling motility.

D. *Yersinia enterocolitica* is motile at 25°C but not at 35°C.

E. *Acinetobacter* organisms are nonmotile.

F. Nonfermenting, gram-negative rods and *Enterobacteriaceae* vary in their motility.

VIII. LIMITATIONS

A. Studies by Edmondson and Sanford (2) showed that nonmotile, mucoid *Klebsiella* strains may give a false-positive motile reaction in tube media; this is due to mucoid strains spilling between medium and the tube, giving a cloudy appearance which is often confused with motility. False-positive results can be avoided by use of media with adequate tube depth and careful reading with attention to the density of growth in the central stab.

B. *Bacillus* species are best tested directly from a fresh plate. If a fresh plate is not available, inoculate a plate and incubate for 4 h. Then perform wet mount.

C. A large number of *E. casseliflavus* and *E. gallinarum* organisms have been reported as nonmotile using some tube motility agar (6, 7). If a vancomycin MIC is between 4 to 16 µg/ml and the isolate is ampicillin susceptible, but the enterococcus is nonmotile, confirm results with the 2-h broth method or perform the MGP test (procedure 3.17.30).

VIII. LIMITATIONS *(continued)*

D. Motility results for enterococcis with MIO have been reported to have poor sensitivity (7).

E. Excessive heat on a microscope slide can affect the results.

F. False-negative reactions may occur if bacterial flagella are damaged due to heating, shaking, or other trauma. Such environmental shock will render the organism nonmotile.

G. Some microorganisms do not produce flagellar proteins at 35 to 37°C but do so at 22°C.

H. TTC may be inhibitory to certain fastidious bacteria.

REFERENCES

1. **Cleary, T.** 2002. Evaluation of wet-prep motility test for presumptive identification of *Bacillus* species. *J. Clin. Microbiol*. **40:**730. (Letter to the editor.)
2. **Edmondson, E. B., and J. P. Sanford.** 1967. The Klebsiella-Enterobacter (Aerobacter), Serratia group. *Medicine* **46:**323–340.
3. **Leifson, E.** 1960. *Bacterial Flagellation.* Academic Press, New York, NY.
4. **Tittsler, R. P., and L. A. Sandholzer.** 1936. The use of semi-solid agar for the detection of bacterial motility. *J. Bacteriol*. **31:**575–580.
5. **Toth, C., and K. Van Horn.** 1999. Evaluation of motility media for detection of motility in enterococci, abstr. C-442, p. 196. *Abstr. 99th Gen. Meet. Am. Soc. Microbiol.* American Society for Microbiology, Washington, DC.
6. **Turenne, C. Y., D. J. Hoban, J. A. Karlowsky, G. G. Zhanel, and A. M. Kabani.** 1998. Screening of stool samples for identification of vancomycin-resistant *Enterococcus* isolates should include the methyl-α-D-glucopyranoside test to differentiate nonmotile *Enterococcus gallinarum* from *E. faecium. J. Clin. Microbiol.* **36:**2333–2335.
7. **Van Horn, K., C. Tóth, R. Kariyama, R. Mitsuhata, and H. Kumon.** 2002. Evaluation of 15 motility media and a direct microscopic method for detection of motility in enterococci. *J. Clin. Microbiol.* **40:**2476–2479.
8. **Van Horn, K. G., and K. M. Rodney.** 1998. Colonization and microbiology of the motile enterococci in a patient population. *Diagn. Microbiol. Infect. Dis.* **31:**525–530.
9. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria,* 2nd ed., p. 13–14. Williams & Wilkins, Baltimore, MD.

3.17.32 MRS Broth

I. PRINCIPLE

Gas production by gram-positive rods is tested in glucose-containing medium called lactobacillus MRS broth, named for the authors of the publication (1). MRS broth is helpful to differentiate *Leuconostoc* spp. and *Weissella confusa* (previously called *Lactobacillus confusus*) from *Pediococcus*, which is gas negative (2, 4, 5). The gas is detected by a rise in a petroleum jelly plug or displacement of broth with air in the Durham tube. Sufficient gas is not produced in ordinary sugar fermentation tubes to detect these organisms.

II. MICROORGANISMS TESTED

Vancomycin-resistant, catalase-negative, gram-positive coccobacilli that are pyrrolidonyl-β-naphthylamide (PYR) negative and grow aerobically

III. MEDIUM, REAGENTS, AND SUPPLIES

A. MRS broth

Store at 4 to 8°C.

1. Purchase from most vendors.

2. Prepare from dehydrated powder (Difco, BBL, BD Diagnostic Systems, Hardy Diagnostics), dispense into tubes with Durham tube (optional), and autoclave.

B. Gas detection options

1. Vaspar, liquid paraffin, or petroleum jelly, maintained at 56°C in liquid form

2. Durham tube

C. Other supplies

1. Sterile sticks

2. Incubator at 35°C

IV. QUALITY CONTROL

A. Examine media for lack of turbidity. Invert if there is a Durham tube and it contains a bubble.

B. Test each new lot or shipment of medium with a positive and negative control prior to putting it into use.

C. Organisms

1. *Leuconostoc mesenteroides* ATCC 10830—gas produced (positive)

2. *Enterococcus faecalis* ATCC 29212—no gas (negative)

V. PROCEDURE

A. Inoculate MRS broth lightly with one or two colonies from an 18- to 24-h BAP.

B. If a Durham tube is not used, overlay the inoculated MRS broth with a plug of melted Vaspar or petroleum jelly, being careful to cover broth layer entirely without introducing air.

C. Incubate aerobically at 35°C for up to 7 days, and observe daily for gas trapped in the Durham tube or solid plug.

VI. INTERPRETATION

A. A positive test shows a visible lifting of the Vaspar plug resulting in complete separation from the broth surface or trapped gas in the Durham tube. Positive reactions usually raise wax plugs within 48 h.

B. A negative test shows no lifting of the wax plug or no gas bubble in the Durham tube.

VII. REPORTING RESULTS

A. Streptococci, lactobacilli, and *Pediococcus* do not produce gas from glucose.

B. *Leuconostoc* and *W. confusa* are positive for gas.

1. Prepare a Gram stain from the MRS broth.

2. *Leuconostoc* is the only genus that produces coccoid forms as well as coccobacilli and rods. It is arginine deaminase negative and does not grow at 42°C (3).

3. *W. confusa* is a gas producer but does not have coccoid forms, is generally arginine positive, and grows at 42°C (3).

VIII. LIMITATIONS

A. The Durham tube is a safer alternative to petroleum jelly, since strains of *Leuconostoc* can produce copious amounts of gas. However, the Durham tube method does not work as well.

B. The difficulty in separating *Weissella* and *Leuconostoc* may result in a misidentification, if the Gram stain is equivocal and other biochemical tests are not performed.

REFERENCES

1. **De Mann, J. D., M. Rogosa, and M. E. Sharpe.** 1960. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.* **23:**130–135.
2. **Facklam, R., and J. A. Elliott.** 1995. Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clin. Microbiol. Rev.* **8:**479–495.
3. **Facklam, R., D. Hollis, and M. D. Collins.** 1989. Identification of gram-positive coccal and coccobacillary vancomycin-resistant bacteria. *J. Clin. Microbiol.* **27:**724–730.
4. **Isenberg, H. D., E. M. Vellozzi, J. Shapiro, and L. G. Rubin.** 1988. Clinical laboratory challenges in the recognition of *Leuconostoc* spp. *J. Clin. Microbiol.* **26:**479–483.
5. **Olano, A., J. Chua, S. Schroedar, A. Minari, M. La Salvia, and G. Hall.** 2001. *Weissella confusa* bacteremia: a case report. *J. Clin. Microbiol.* **39:**1604–1607.

3.17.33 MR-VP (Methyl Red–Voges-Proskauer) Tests

I. PRINCIPLE

The methyl red (MR) test is used to determine if an organism is able to produce stable acid end products from glucose fermentation (2). Methyl red indicator (red color below pH 4.4; yellow color at pH 5.8) is used to determine the pH after an enteric gram-negative rod has fermented glucose to completion. All members of the *Enterobacteriaceae* give a positive methyl red reaction when tested up to 24 h due to conversion of glucose to pyruvic acid by the Embden-Meyerhof pathway. After further incubation (2 to 5 days) those organisms that are MR positive continue to metabolize pyruvic acid to lactic, acetic, and formic acids by the mixed acid pathway and are able to maintain the acid pH ($<$4.4). Organisms utilizing the butylene glycol pathway produce acetylmethylcarbinol (acetoin) and butanediol, neutral end products that raise the pH towards neutrality (pH $>$ 6) and result in a high final pH. Most *Enterobacteriaceae* demonstrate one or the other metabolic pathway but rarely both.

The Voges-Proskauer (VP) test (9) is used to determine if an organism produces acetylmethylcarbinol from glucose fermentation. If present, acetylmethylcarbinol is converted to diacetyl in the presence of α-naphthol, strong alkali (40% KOH), and atmospheric oxygen. The α-naphthol was not part of the original procedure but was found to act as a color intensifier by Barritt (1) and must be added first. The diacetyl and quanidine-containing compounds found in the peptones of the broth then condense to form a pinkish red polymer (8).

II. MICROORGANISMS TESTED

A. MR test for enteric gram-negative rods, as part of identification to species level

B. VP test for identification to the species level of the following groups of organisms

1. Enteric gram-negative rods, *Aeromonas*, and *Vibrio*

2. Viridans group streptococci (4)

3. Staphylococci

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media

Store broths at 4 to 8°C.

1. Andrade's 1% glucose broth *(to be used for VP testing only, not for MR testing)*

2. MRVP broth (pH 6.9)

Ingredients per liter of deionized water:

buffered peptone ……7.0 g
glucose ……5.0 g
dipotassium phosphate ……5.0 g

3. Volumes

a. Generally dispense approximately 5 ml per tube.

b. Use enough broth to cover an inverted Durham tube, if it is used.

c. Dispense 2 ml of MRVP broth for rapid VP testing and 0.5 ml for rapid MR testing.

4. MRVP tablets with creatine (Key Scientific) for rapid test. Refer to http://www.keyscientific.com for procedures.

B. Reagents

1. 40% Potassium hydroxide

Caution: *KOH is hygroscopic and becomes caustic when moist. Weigh quickly in tared beaker. Store away from acids. Avoid exposure to skin.*

a. Dissolve 40 g of potassium hydroxide pellets in 100 ml of distilled water in a polyethylene bottle. Keep bottle in cool water bath during preparation.

III. MEDIA, REAGENTS, AND SUPPLIES *(continued)*

Include QC information on reagent container and in QC records.

b. Optional: add 0.3 g of creatine (*N*-methyl-*N*-guanylglycine) for more rapid and sensitive reaction (3). Creatine should *not* be added if reagent is used for the API 20E or the tablets from Key Scientific, since it is in the media.
c. Store at 4 to 8°C.
d. Shelf life: 2 to 3 weeks (8)

2. 5% α-Naphthol (5 g/100 ml) in 95% ethyl alcohol (3)
a. Store at 4 to 8°C in the dark.
b. Shelf life: 2 to 3 weeks
c. Reagent should be almost colorless.

3. Methyl red solution, 0.02%
a. Dissolve 0.1 g of methyl red in 300 ml of ethyl alcohol, 95%.
b. Add sufficient distilled water to make 500 ml.
c. Store at 4 to 8°C in a brown bottle.
d. Solution is stable for 1 year.

4. Reagents with extended shelf life are commercially available from most vendors.

C. Supplies
1. Sterile wooden sticks or inoculating loops
2. Test tubes

IV. QUALITY CONTROL

A. Examine broth for signs of contamination, dehydration, and deterioration.

B. Perform QC on each new lot or shipment of media and reagent prior to use with one organism known to demonstrate a positive reaction and one organism known to give a negative reaction.

C. Organisms
1. *Klebsiella pneumoniae* ATCC 13883—MR negative (yellow), VP positive (red)
2. *Escherichia coli* ATCC 25922—MR positive (red), VP negative (no change)

V. PROCEDURE

A. VP test

1. Inoculate a tube of 1% glucose broth (Andrade's base) or MRVP broth with colony of the organism to be tested and incubate at 35°C for 18 to 24 h. *Do not tighten caps.*

◩ **NOTE:** Some organisms may produce acetylmethylcarbinol at room temperature and not 35°C (e.g., *Hafnia alvei, Yersinia, and Listeria*). In this case, inoculate another broth and incubate at room temperature.

2. If a 5-ml broth culture is used, aliquot 2.0 ml of broth into a nonsterile 13- by 100-mm test tube. Hold the remainder for possible reincubation.

◩ **NOTE:** If the glucose broth contains a Durham tube, be careful not to introduce air bubbles into it when the 2.0 ml of broth is poured into the nonsterile tube for testing.

3. Add 6 drops of 5% α-naphthol, and mix well to aerate.

4. Add 2 drops of 40% potassium hydroxide, and mix well to aerate.

5. Observe for a pink-red color at the surface within 30 min. Shake the tube vigorously during the 30-min period.

◩ **NOTE:** If the result is negative, the glucose or MRVP broth can be incubated for up to 48 h and the test repeated.

B. MR test

1. Inoculate tube as for VP test and incubate at 35°C for at least 48 h.

◩ **NOTE:** If the center of one colony is inoculated to a 0.5-ml volume of MRVP broth, the test can be read at 18 to 24 h (8).

2. Remove approximately 1 ml of the 48-h broth to a nonsterile 13- by 100-mm tube. (The remainder should be reserved for testing at 3 to 5 days if necessary.)

3. Add 3 to 6 drops (or 1 drop to 0.5 ml) of methyl red indicator to aliquot.

4. Observe for red color immediately.

VI. INTERPRETATION

A. VP test

1. A pink-red color at the surface is a positive reaction.

2. A lack of a pink-red color is a negative reaction.

3. A copper color should be considered negative. A rust color is a weak positive reaction.

B. MR test

1. A positive reaction is a distinct red color.

2. A negative reaction is a yellow color.

3. A weak positive is red-orange.

4. If an orange color is seen, incubate the remainder of the broth for up to 4 days and repeat the test after further incubation. In this case it may also be helpful to set up a duplicate broth at 25°C.

VII. REPORTING RESULTS

A. Organisms in the *E. coli* group are MR positive, and those in the *Enterobacter-Klebsiella* group are MR negative (5, 7).

B. Most members of the family *Enterobacteriaceae* give opposite MR and VP reactions; however, certain organisms, like *H. alvei* and *Proteus mirabilis,* may give both a positive MR reaction and a positive VP reaction (often delayed) (5, 7).

C. *Streptococcus mitis* group organisms are VP negative, whereas the other viridans group streptococci are VP positive, except *Streptococcus vestibularis,* which is VP variable (4).

D. Refer to procedure 3.8.1 for VP reactions of *Vibrio, Plesiomonas,* and *Aeromonas.*

E. *Listeria* organisms are beta-hemolytic, gram-positive rods that are VP positive at 25°C, but this test is not a key test in the identification.

VIII. LIMITATIONS

A. Increased exposure of the organism to atmospheric oxygen in the microtechnique decreases the incubation period.

B. Avoid overinoculation. Bacterial growth is inhibited when the inoculum exceeds approximately 10^9 viable cells per ml (8).

C. If not enough incubation time is allowed before the methyl red indicator is added, a false-*positive* result may be obtained.

D. With prolonged incubation (>3 days), some VP-positive organisms can produce an acid condition in the medium, yielding weak positive reactions or false-negative VP reactions.

E. Shaking the tubes enhances the VP reaction.

F. Do not add more than 2 drops of KOH per 2 ml of medium. Excess amounts of KOH can give a weak positive reaction, which may be masked by the formation of a copperlike color because of the reaction of KOH with α-naphthol alone.

G. Do not read the test more than 1 h after adding the VP reagents. A copperlike color may develop, resulting in a potential false-positive interpretation.

H. Reagents must be added in the specified order. A reversal of order may result in a weak positive or false-negative VP result.

I. *When performing the VP test for the API 20E, add 1 drop of KOH followed by 1 drop of α-naphthol. This is opposite to the order for adding reagents in the standard test. Creatine is present in the cupule and is not needed in the KOH reagent used for the test (API 20E package insert).*

VIII. LIMITATIONS *(continued)*

J. The original study by Barritt (1) using α-naphthol indicated that it should be prepared in absolute (100%) ethanol, but the Coblentz method (3) using 95% ethanol and creatine in the KOH is more rapid and sensitive (6, 8, 10). For the Coblentz method, use a very heavy inoculum in 2 ml of MRVP broth and creatine in the KOH, and read the test as soon as 6 h (6, 10).

REFERENCES

1. **Barritt, M. M.** 1936. The intensification of the Voges-Proskauer reaction by the addition of α-naphthol. *J. Pathol. Bacteriol.* **42:**441–454.
2. **Clark, W. M., and H. A. Lubs.** 1915. The differentiation of bacteria of the colon-aerogenes family by the use of indicators. *J. Infect. Dis.* **17:**161–173.
3. **Coblentz, L. M.** 1943. Rapid detection of the production of acetyl-methyl-carbinol. *Am. J. Public Health* **33:**815–817.
4. **Coykendall, A.** 1989. Classification and identification of the viridans streptococci. *Clin. Microbiol. Rev.* **2:**315–328.
5. **Ewing, W. H.** 1986. *Edwards and Ewing's Identification of Enterobacteriaceae*, 4th ed. Elsevier Scientific Publishing Co., New York, NY.
6. **Facklam, R., and J. A. Elliott.** 1995. Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clin. Microbiol. Rev.* **8:**479–495.
7. **Farmer, J. J., III, B. R. Davis, F. W. Hickman-Brenner, A. McWhorter, G. P. Huntley-Carter, M. A. Asbury, C. Riddle, H. G. Wathen-Grady, C. Elias, G. R. Fanning, A. G. Steigerwalt, C. M. O'Hara, G. K. Morris, P. B. Smith, and D. J. Brenner.** 1985. Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. *J. Clin. Microbiol.* **21:**46–76.
8. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 321–326, 439–450. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.
9. **Voges, O., and B. Proskauer.** 1898. Beitrag zur Ernärugsphysiologie und zur Differentialdiagnose der Bakterien der hämorrhagischen Septicemia. *Z. Hyg. Infektkr.* **28:**20–37.
10. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed., p. 22–23. Williams & Wilkins, Baltimore, MD.

3.17.34 MUG (4-Methylumbelliferyl-β-D-Glucuronide) Test

I. PRINCIPLE

Escherichia coli and rare other *Enterobacteriaceae (Salmonella, Yersinia,* and *Shigella)* produce the enzyme β-glucuronidase. This enzyme hydrolyzes the substrate 4-methylumbelliferyl-β-D-glucuronide (MUG), releasing 4-methylumbelliferone, which fluoresces blue under long-wave UV light (4). Since 97% of *Escherichia coli* strains possess the β-glucuronidase enzyme, the MUG test can be used for rapid identification of *E. coli*, the most common gram-negative rod seen in clinical specimens (1, 2, 4–6, 8, 10). Since verotoxin-producing *E. coli* strains are among the few *E. coli* strains that do not produce MUG, this test can also be used to detect the absence of the enzyme in a fecal isolate of *E. coli* to alert the microbiologist to the possible presence of a verotoxin-producing strain (7, 9).

II. MICROORGANISMS TESTED

A. Fresh colonies on BAP of possible *E. coli* organisms that are indole-positive, oxidase-negative, gram-negative rods, whether they are lactose positive or negative.

◩ **NOTE:** For the tube test, colonies from EMB, but not MAC, can be used.

B. Do not use this test as part of an algorithm to rapidly identify *E. coli* in abdominal sources, since occasional isolates of both *Salmonella* and *Shigella* can be MUG positive. However, the test can be used to separate potential verotoxin-producing *E. coli* (MUG negative) from other *E. coli* strains (usually MUG positive) in gastrointestinal specimens, once the isolate has been identified as *E. coli.*

III. MEDIA, REAGENTS, AND SUPPLIES

Include QC information on reagent container and in QC records.

A. Reagents

1. Purchase MUG as disks from commercial vendors.
- **a.** MUG disks: Hardy Diagnostics, Inc.; Key Scientific; Oxoid, Inc.; Remel, Inc.
- **b.** BactiCard *E. coli*: MUG and indole on a filter card (Remel, Inc.)

2. Prepare MUG from powder (Sigma Chemical Co.).
- **a.** M/15 Sorensen's phosphate buffer, pH 7.5
  - (1) M/15 Na_2PO_4 (solution A)
    - (a) 4.730 g of Na_2HPO_4
    - (b) q.s. to 500 ml with sterile distilled water.
  - (2) M/15 KH_2PO_4 (solution B)
    - (a) 4.535 g of KH_2PO_4
    - (b) q.s. to 500 ml with sterile distilled water.
  - (3) Combine 85 ml of solution A and 15 ml of solution B.

    ◩ **NOTE:** Check final pH; adjust to pH 7.5 with either solution if necessary (solution A to make more alkaline; solution B to acidify).
  - (4) Store at room temperature.

III. MEDIA, REAGENTS, AND SUPPLIES *(continued)*

b. MUG liquid reagent or stock for disks
  (1) Dissolve 50 mg of MUG in 100 ml of Sorensen's buffer.
  (2) This is a 500-µg/ml stock solution.
  (3) Label, aliquot, and store at −20°C in convenient amounts.
  (4) Shelf life: 1 year at −20°C or 1 week at 4°C in the dark (cover tubes in refrigerator with foil to keep from light).

c. Disk preparation
  (1) Add 0.2 ml of stock MUG solution to 3.2 ml of M/15 Sorensen's phosphate buffer to prepare a 1:16 dilution.
  (2) Add 1.25 ml to a vial of 50 sterile 6-mm-diameter paper disks (BD Diagnostic Systems; Hardy, Inc.). Allow to thoroughly saturate disks so that no fluid hangs on the sides of vial (0.78 µg/disk).
  (3) Spread the saturated disks onto a metal screen and place in a dry environment until the disks are completely dry. Protect from light.
  (4) Store disks at −20°C, away from the light, for up to 1 year or at 4°C for up to 1 month.

B. Supplies
1. Long-wave UV light (366 nm; e.g., Wood's lamp)
2. Wooden sticks or inoculating loops
3. 35 to 37°C incubator
4. Empty petri dish or Durham tubes

IV. QUALITY CONTROL

A. Perform QC on each new lot or shipment of disks or liquid reagent prior to putting it into use.

B. Organisms
1. *E. coli* ATCC 25922—blue fluorescence (positive)
2. *Klebsiella pneumoniae* ATCC 13883 or ATCC 27736—no fluorescence (negative)

V. PROCEDURE

A. MUG tube test
1. Place 2 drops of MUG reagent in Durham tube.
2. Remove one colony from BAP or EMB with sterile stick and inoculate tube.
◩ **NOTE:** For commercial products, place 0.25 ml of deionized water in a glass (not soda lime glass) tube and add the MUG disk to the tube after inoculation with the colony.
3. Incubate for at least 2 h at 35°C without added CO_2
◩ **NOTE:** MUG reactions occasionally take 4 h to turn positive.
4. Observe fluorescence using long-wave UV light in a darkroom.

B. MUG disk method
1. Place disk in sterile empty petri dish and wet with 1 drop of water.
◩ **NOTE:** If excess water is used, test may be falsely negative.
2. Using a wooden stick or bacteriological loop, roll colony from a BAP onto the disk.
◩ **NOTE:** Alternatively, place the disk directly over the colony and incubate for 30 min. Observe for fluorescence and reincubate disk for up to 2 h.
3. Incubate at 35°C for a minimum of 2 h (test can be read up to 24 h, but 2 h is usually sufficient).
4. Observe the disk using long-wave UV light in a darkened room.

VI. INTERPRETATION

A. A positive test is bright blue-white fluorescence.

B. A negative test is lack of blue fluorescence.

◩ **NOTE:** It may be helpful, when interpreting the tube test, to observe an uninoculated tube when evaluating the fluorescence. When interpreting weak reactions with the disk test, after incubation, place the disk in 2 ml of sterile saline. Observe as for tube test after 10 min.

VII. REPORTING RESULTS

A. *E. coli* is definitively identified if a gram-negative rod is indole positive, oxidase negative, and MUG positive (11).

B. *E. coli* O157:H7 is indole positive and MUG negative.

VIII. LIMITATIONS

A. Not all *E. coli* organisms are MUG positive. A negative test does not mean that the organism is not *E. coli.*

B. Do not use media that contain dyes (e.g., EMB, MAC) for the disk test, although the dyes do not interfere with the tube test.

C. Of *Shigella* species that are indole positive, approximately 8% are also MUG positive. Rare isolates of *Salmonella* and *Yersinia* are also MUG positive. However, they are rarely indole positive. Thus, to avoid a misidentification, lactose-negative organisms from abdominal sources or from blood should not be tested using this method.

D. Some fluorescing organisms, such as *Pseudomonas aeruginosa,* may resemble a positive MUG result. Therefore, the test should not be performed on oxidase-positive organisms.

E. Some organisms fluoresce orange, which is not considered a positive reaction.

F. Rare MUG-positive *E. coli* O157 strains have been reported (3).

REFERENCES

1. **Edberg, S. C., and C. M. Kontnick.** 1986. Comparison of β-glucuronidase-based substrate systems for identification of *Escherichia coli. J. Clin. Microbiol.* **24:**368–371.
2. **Feng, P. C. S., and P. A. Hartman.** 1982. Fluorogenic assays for immediate confirmation of *Escherichia coli. Appl. Environ. Microbiol.* **43:**1320–1329.
3. **Hayes, P. S., K. Blom, P. Feng, J. Lewis, N. A. Strockbine, and B. Swaminathan.** 1995. Isolation and characterization of a beta-D-glucuronidase-producing strain of *Escherichia coli* serotype O157:H7 in the United States. *J. Clin. Microbiol.* **33:**3347–3348.
4. **Iritani, B., and T. J. Inzana.** 1988. Evaluation of a rapid tube assay for presumptive identification of *Escherichia coli* from veterinary specimens. *J. Clin. Microbiol.* **26:**564–566.
5. **Kilian, M., and P. Bülow.** 1976. Rapid diagnosis of Enterobacteriaceae. I. Detection of bacterial glucosidases. *Acta Pathol. Microbiol. Scand. Sect. B* **84:**245–251.
6. **Perez, J. L., C. I. Berrocal, and L. Berrocal.** 1986. Evaluation of a commercial β-glucuronidase test for rapid and economical identification of *Escherichia coli. J. Appl. Bacteriol.* **61:**541–545.
7. **Ratnam, S., S. B. March, R. Ahmed, G. S. Bezanson, and S. Kasatiya.** 1988. Characterization of *Escherichia coli* serotype O157:H7. *J. Clin. Microbiol.* **26:**2006–2012.
8. **Thaller, M. C., F. Berlutti, B. Dainelli, and R. Pezzi.** 1988. New plate medium for screening and presumptive identification of gram-negative urinary tract pathogens. *J. Clin. Microbiol.* **26:**791–793.
9. **Thompson, J. S., D. S. Hodge, and A. A. Borczyk.** 1990. Rapid biochemical test to identify verocytotoxin-positive strains of *Escherichia coli* serotype O157. *J. Clin. Microbiol.* **28:**2165–2168.
10. **Trepeta, R. W., and S. C. Edberg.** 1984. Methylumbelliferyl-β-D-glucuronide-based medium for rapid isolation and identification of *Escherichia coli. J. Clin. Microbiol.* **19:**172–174.
11. **York, M. K., E. J. Baron, M. Weinstein, R. Thomson, and J. E. Clarridge.** 2000. A multilaboratory validation of rapid spot tests for identification of *Escherichia coli. J. Clin. Microbiol.* **38:**3394–3398.

3.17.35 Nitrate/Nitrite Reduction Test

I. PRINCIPLE

Nitrate broth and nitrite broth are used to determine if an organism can reduce nitrate (NO_3^-) to nitrites (NO_2^-) and reduce nitrites further. The reduction of nitrate is associated with anaerobic respiration in which the organism derives its oxygen from nitrate. The end products of nitrate reduction include nitrite, ammonia, molecular nitrogen (N_2), hydroxylamine, and other related compounds.

The presence of nitrites resulting from the reduction of nitrate is detected by the formation of a red diazonium compound when sulfanilic acid, α-naphthylamine, and nitrite react. The nitrite combines with acetic acid to form nitrous acid which will diazotize sulfanilic acid to a diazonium salt. The dimethyl-naphthylamine couples with the diazo compound to form a red dye. Nitrogen gas is detected as gas trapped in a Durham vial inverted in the nitrate or nitrite broth (3).

The reduced nitrogen compounds are not detected directly but are inferred if there is no gas or nitrite but nitrate has been reduced. This is tested by adding zinc dust to the reaction tube to detect unreduced nitrate after the sulfanilic acid and α-naphthylamine have been added but did not produce the red color. The zinc dust will reduce the remaining nitrate to nitrite and the red color develops. If nitrite is not present, because it has been previously reduced to nitrogen compounds, no red color develops after addition of zinc and the test is positive for both nitrate and nitrite reduction.

The test is useful in the evaluation of non-glucose-fermenting and fastidious gram-negative rods and in differentiating *Moraxella catarrhalis* from *Neisseria* (2). The test also confirms membership in the family *Enterobacteriaceae.* The disk test is used for anaerobic nitrate testing (*see* section 4).

II. MICROORGANISMS TESTED

A. Gram-negative rods and gram-negative cocci (*Neisseria* spp.), as part of their identification (2, 4)

B. Gram-positive, non-spore-forming bacilli that grow aerobically

C. Anaerobic organisms

III. MEDIA, REAGENTS, AND SUPPLIES

Include QC information on reagent container and in QC records.

A. Media

1. Nitrate broth: store at 2 to 8°C.

peptone ..... 20 g *or*
heart infusion broth for fastidious organisms .... 25 g
potassium nitrate .....2 g
distilled water ..... 1,000 ml

Dispense 4-ml aliquots in 16- by 125-mm screw-cap tubes with Durham tube. Autoclave at 121°C for 15 min.

2. Nitrite broth: store at 2 to 8°C.

heart infusion broth ..... 25 g
potassium nitrite .....0.1 to 1 g
distilled water ..... 1,000 ml

Dispense 4-ml aliquots in 16- by 125-mm screw-cap tubes with Durham tube. Autoclave at 121°C for 15 min.

3. Glucose cupule of the API 20E strip

4. Nitrate disk (available commercially) for anaerobic organisms

5. Nitrate tablets (Key Scientific) for both media and reagents

B. Reagents

1. 0.8% Sulfanilic acid (reagent A)

sulfanilic acid .....0.8 g
distilled water ..... 70 ml
glacial acetic acid ..... 30 ml

a. Mix sulfanilic acid with water; heat to dissolve.

b. Cool, and then add acetic acid.

III. MEDIA, REAGENTS, AND SUPPLIES *(continued)*

c. Store at 2 to 8°C.
d. Shelf life is 3 months.

2. 0.5% *N,N*-Dimethyl-α-naphthylamine (reagent B)

glacial acetic acid …… 30 ml
distilled water …… 70 ml
N,N-dimethyl-α-naphthylamine ……0.5 g

a. Combine acetic acid and water. Add α-naphthylamine.
b. Store at 2 to 8°C.
c. Shelf life is 3 months.

◩ **NOTE:** The purchase of this reagent is preferred to preparation in-house, since special precautions must be taken in handling concentrated *N,N*-dimethyl-α-naphthylamine, a potential carcinogen.

3. Zinc metal dust

IV. QUALITY CONTROL

A. Examine tubes for contamination. Invert to remove bubbles in the Durham tube prior to storage of product.

B. Test each new lot or shipment of media and reagents with a positively and negatively reacting organism prior to putting it into use.

◩ **NOTE:** New lots of reagent can be checked without inoculation either with nitrate broth by adding zinc after the reagents are added or with nitrite broth directly. If the reagents give a red color, they are acceptable and have detected nitrite.

C. Organisms
1. *Escherichia coli* ATCC 25922—nitrate positive, gas negative
2. *Pseudomonas aeruginosa* ATCC 27853—nitrate positive, gas positive
3. *Acinetobacter baumannii* ATCC 19606—nitrate negative
4. Alternative positive control: *Campylobacter jejuni* ATCC 33560—nitrate positive

V. PROCEDURE

A. Tube method

1. Before inoculating the broth, check the Durham tube for trapped air bubbles. If necessary, invert the tube until the air bubbles are gone.
2. Inoculate the tube of nitrate broth from an isolated colony; a pure subculture or, preferably, 1 or 2 drops of an overnight broth culture of the organism. Subsequently, if nitrate has *not* been reduced, determine reduction of nitrite by inoculation of nitrite broth in the same manner.
3. Incubation
a. Nonfermenting, gram-negative rods, 25 to 30°C
b. Other organisms, 35°C
c. Two to five days of incubation may be necessary for some organisms.
d. Incubate *Campylobacter* at 35°C in a microaerobic atmosphere for 72 h.
4. Look for and record any gas in the Durham vial and growth in the tube. *Do not add reagents if there is no visible growth in the tube.*
5. *If gas is present* and the organism does not ferment glucose in Kligler's iron agar or triple sugar iron agar, do not add reagents because the test is considered positive for nitrate reduction, nitrite reduction, and gas.
6. If no gas is present *or* the organism is a glucose-fermenting, gram-negative rod (i.e., *Enterobacteriaceae*), remove approximately 0.5 ml of broth into a nonsterile 13- by 100-mm tube.
a. Add 2 or 3 drops of reagent A. Mix well by tapping or shaking tube.
b. Then, add 2 or 3 drops of reagent B. Mix again.
c. Look for a red color within 1 to 2 min.

V. PROCEDURE *(continued)*

7. If no red color is observed, add a small amount of zinc dust to the nitrate tube. *Do not add zinc dust to nitrite.*
   a. The amount of zinc dust should not exceed that which adheres to the end of an applicator stick.
   b. Examine for red color within 10 min.
8. If nitrate or nitrite has not been reduced or there is no gas production, reincubate the remaining broth and retest at 48 h. Test nitrite broth additionally at 5 days.

B. Disk method (used only for anaerobes)

1. Make a fresh subculture of the organism to be tested onto a supportive agar and place a nitrate disk in the heavy inoculum area. Incubate anaerobically for 24 to 48 h.
2. Remove the disk from the surface of the plate and place it in a clean petri dish or on a slide.
3. Add 1 drop each of reagents A and B. If no color develops in a few minutes, drop a small amount of zinc dust onto the surface of the disk and observe for up to 5 min.

C. Rapid method

1. Prepare 0.5 ml of broth or use tablet (Key Scientific). Refer to http://www.keyscientific.com for instructions on tablet preparation and testing.
2. Inoculate *heavily* with organism.
3. Incubate for 2 h at 35°C.
4. Add reagents A and B and observe for red color.
5. Interpret as for standard test.

VI. INTERPRETATION

◩ **NOTE:** If nitrate has been reduced, inoculation of nitrite broth is unnecessary.

A. Gas bubbles (even a single gas bubble) in the Durham vial, except for glucose-fermenting, gram-negative rods (no reagent added)

1. In nitrate tube: nitrate reduction positive, nitrite reduction positive, gas positive
2. In nitrite tube: nitrite reduction positive, gas positive

B. No gas bubbles: gas negative; add reagents for evaluation of reduction.

C. Organisms that ferment glucose and non-glucose-fermenting, gram-negative rods that are gas negative

1. In nitrate tube
   a. Red color after the addition of reagents: nitrate reduction positive, nitrite reduction negative
   b. No red color after the addition of reagents *plus* no red color after the addition of zinc to nitrate broth: nitrate reduction positive, nitrite reduction positive
2. In nitrite tube—no color development after addition of reagents: nitrite reduction positive

D. Negative results (final reading at ≥48 h)

1. In nitrate broth—no color development after adding reagents and red color development after adding zinc (zinc catalyzes the change from nitrate to nitrite): nitrate reduction negative
2. In nitrite broth—a red color after addition of reagents: nitrite reduction negative

VII. REPORTING RESULTS

A. Report as nitrate reduction positive or negative and nitrite reduction positive or negative.
B. Report the production of gas for non-glucose-fermenting rods.
C. Use the results in the determination of the species, using charts in procedures 3.18.1 and 3.18.2.
D. EF-4 is among the few unique microorganisms that reduce nitrate and nitrite, but no gas is produced (4).
E. Among the gram-negative diplococci that infect humans, only *Neisseria mucosa* and *M. catarrhalis* are able to reduce nitrate (2).

VIII. LIMITATIONS

A. Gas production in *Enterobacteriaceae* is not due to nitrate reduction but is hydrogen gas.
B. Failure to recognize that the organism did not grow in the medium will result in possible false-negative test(s).
C. Interpretation of color reactions should be made immediately, as color reactions with a positive test may fade rapidly.
D. Failure to remove air bubbles prior to inoculation may result in reading the result as a false-positive reaction for gas reduction.
E. A faint pink color may be produced following addition of the nitrate reagents. This is not a positive result.
F. A negative zinc reduction (no color change) test, in combination with a negative nitrite reaction, is presumptive indication that the nitrate was reduced beyond the nitrite stage. Although a very common end product of nitrite reduction is nitrogen gas, other end products may be formed.
G. Excess zinc dust has been reported to cause false-positive nitrite reduction reactions due to complete reduction of previously unreduced nitrate to ammonia.
H. Most positive reactions are detected in 12 to 24 h; however, rare isolates may take up to 5 days (1).

REFERENCES

1. **Cowan, S. T.** 1974. *Cowan & Steels Manual for the Identification of Medical Bacteria,* 2nd ed., p. 38–39, 167. Cambridge University Press, Cambridge, United Kingdom.
2. **Janda, W. M., and J. S. Knapp.** 2003. *Neisseria* and *Moraxella catarrhalis,* p. 585–608. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology,* 8th ed. ASM Press, Washington, DC.
3. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria,* 3rd ed., p. 348–358. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.
4. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria,* 2nd ed., p. 14–15. Williams & Wilkins, Baltimore, MD.

3.17.36

O/129 Disk Susceptibility Testing for *Vibrio* and *Aeromonas* spp.

I. PRINCIPLE

Disks containing 10 and 150 µg of O/129, the vibriostatic agent 2,4-diamino-6,7-diisopropylpteridine phosphate, are used in a method resembling disk susceptibility testing to differentiate *Vibrio* spp. from *Aeromonas* spp. (3–5). *Aeromonas* species are resistant, with no zone of inhibition at 24 h, with both disks. *Vibrio* and *Plesiomonas* species will show susceptibility, with a distinct zone of inhibition with the 150-µg disk; results for the 10-µg disk will vary among the *Vibrio* and *Plesiomonas* species.

Some *Vibrio* spp. require salt for growth. Therefore, the test is run in duplicate on Mueller-Hinton media (MH) with low salt (approximately 0.5%) and with added NaCl (4%) to ensure growth on at least one of the plates.

II. MICROORGANISMS TESTED

A. Gram-negative rods which are oxidase positive, indole positive, and nonpigmented and grow on MAC

B. Microorganisms identified by kits as either *Vibrio* or *Aeromonas* species

C. *Corynebacterium*, to separate *Corynebacterium amycolatum* from *Corynebacterium xerosis*

III. MEDIA, REAGENTS, AND SUPPLIES

Include QC information on reagent container and in QC records.

A. O/129 disks

1. Purchase (Oxoid, Inc.; Hardy Diagnostics) *or*

2. Prepare in-house.

a. Using sterile technique, dissolve 30 mg of O/129 (2,4-diamino-6,7-diisopropylpteridine phosphate, Sigma Chemical Company, St. Louis, MO) in a small amount of sterile deionized water.

b. Add sufficient water to make a total of 4.0 ml at 7.5 mg/ml. This is solution A.

c. Dilute this 1:15 (add 0.5 ml of stock solution to 7.0 ml of H_2O) to make solution B of 0.5 mg/ml.

d. Lay out blank 1/4-in. paper disks (6 mm) (BD Diagnostic Systems, Hardy Diagnostics) in an empty petri dish.

e. Pipette 20 µl of solution A onto each disks to produce 150-µg disks.

f. Pipette 20 µl of solution B onto each disk to produce 10-µg disks.

g. Place petri dishes with lids slightly ajar at 35°C until disks are dry, 10 to 30 min.

h. Return disks to blank vials and close tops tightly.

i. Label each vial with type of content (10-µg O/129 disks or 150-µg O/129 disks).

j. Expiration date is set at 1 year from date of preparation but can be extended if the zone sizes of controls remain within a 5-mm range over time.

k. Place each vial in a separate container half-filled with desiccant.

l. Label this container similarly.

m. Store at −20°C.

B. Media

1. MH

2. MH with 4% NaCl (sold by most vendors for oxacillin screening)

C. Supplies

1. Swabs

2. Broth for inoculum

IV. QUALITY CONTROL

A. Perform QC on each new lot of disks and with each use with both an organism that is susceptible and one that is resistant to O/129.

B. Organisms

Test organism	MH		MH with 4% salt	
	10 µg	150 µg	10 µg	150 µg
Aeromonas hydrophila ATCC 7966	6 mm	6 mm	NA	NA
Vibrio fluvialis ATCC 33809	NA[a]	NA	7–15 mm	≥20 mm

[a] NA, not applicable because the organism will not grow on the medium.

V. PROCEDURE

A. Remove disks from freezer and equilibrate to room temperature.

B. Allow test media (MH and MH with 4% NaCl) to equilibrate to room temperature. If the plates have excess moisture, dry them for 10 to 30 min at 35°C with lids slightly ajar.

C. Suspend an overnight growth of the organism and each of the controls in TSB to a turbidity of a no. 0.5 McFarland standard.

D. Divide each plate in half with a marker.

E. Using a swab dipped into the broth, inoculate the surface of one-half of each plate with organism suspension in three planes as for antimicrobial susceptibility disk testing.

F. In the same manner, inoculate the other half of the MH plate with 4% salt with the *Vibrio* control and the MH plate without salt with the *Aeromonas* control.

G. Allow plates to dry for 3 to 5 min but no longer than 15 min.

H. Place a 10-µg O/129 disk and a 150-µg O/129 disk on each half of the two plates in well-separated locations. Add antimicrobial susceptibility disks, if desired.

I. Using sterile forceps, press each disk gently onto the agar surface to ensure good contact.

J. Incubate for 18 to 24 h at 35°C in a non-CO_2 incubator.

K. Read zone of inhibition on plate with best growth and most clear-cut zone(s).

VI. INTERPRETATION

A. The organism is O/129 resistant if there is no zone of inhibition around the respective disk.

B. The organism is O/129 susceptible if there is any zone around the respective disk.

C. The organism is generally salt requiring if it does not grow on MH.

D. The organism is salt tolerant or enhanced if it grows on MH with 4% salt.

E. The organism is inhibited by salt if it does not grow on MH with 4% salt.

VII. REPORTING RESULTS

A. Gram-negative rods that are oxidase positive, grow on MAC, and ferment glucose (most are indole positive) are reported as follows.

1. *Aeromonas*, if resistant to 150 µg of O/129, grows without salt, and is arginine positive. Strains are also usually DNase positive.

a. *Aeromonas veronii* bv. Veronii is arginine negative.

b. *Vibrio cholerae* is arginine negative, and some strains are resistant to O/129 and may not grow on media with 4% salt (1). Perform further testing on strains with these characteristics, or submit to reference laboratory to separate *V. cholerae* from *A. veronii* bv. Veronii.

c. *Vibrio fluvialis* is arginine positive and may be resistant to 150 µg of O/129, but it usually grows only with 4% salt.

VII. REPORTING RESULTS
(continued)

2. *Plesiomonas shigelloides,* if susceptible to 150 μg of O/129 and positive for indole, arginine dihydrolase, and ornithine and lysine decarboxylases. *P. shigelloides* is also inhibited on 4% salt agar and is DNase negative.

3. *Vibrio* spp., if susceptible to 150 μg of O/129 even if they do *not* grow on MH with 4% salt but are either negative for arginine dihydrolase or negative for ornithine and lysine decarboxylases

◩ **NOTE:** Some vibrios may be resistant to 150 μg of O/129, but they will be arginine negative.

B. Identify *Vibrio* to the species level with commercial kit using saline diluent.

1. If isolate is resistant to 10 μg of O/129, it is likely to be *V. fluvialis, Vibrio alginolyticus,* or *Vibrio parahaemolyticus,* the most common isolates.

2. If isolate is susceptible to 10 μg of O/129, it is likely to be *Vibrio vulnificus.*

C. Unless the organism identification from the kit agrees with the results of the O/129 and salt tolerance tests, submit to a reference laboratory for further identification. See Fig. 3.8.1–1 in procedure 3.8.1 for flowchart.

D. Submit *V. cholerae* and possibly *V. vulnificus* to the local health department, in accordance with local health department policies, or to a reference laboratory for confirmation of toxicity and identification.

E. *C. amycolatum* is usually resistant to 150 μg of O/129, with no zone of inhibition, while *C. xerosis, Corynebacterium minutissimum,* and *Corynebacterium striatum* are susceptible.

VIII. LIMITATIONS

A. Kits can misidentify *V. vulnificus* as *V. parahaemolyticus, Aeromonas* as *V. fluvialis,* and *Vibrio damselae* as *Vibrio cholerae* (2). Use both O/129 disks and growth with salt testing to prevent initial misidentifications and potential public health consequences.

B. *V. cholerae* strains that are resistant to O/129 have been reported, which is probably related to antimicrobial therapy (1).

C. The 10-μg disk is not as important in the identification as the 150-μg disk and may be omitted.

D. Some vibrios do not grow on MH with 4% salt, and some grow without added salt. All *Aeromonas* organisms grow without salt but may grow on medium with salt.

REFERENCES

1. **Abbott, S. L., W. K. Cheung, B. A. Portoni, and J. M. Janda.** 1992. Isolation of vibriostatic agent O/129-resistant *Vibrio cholerae* non-O1 from a patient with gastroenteritis. *J. Clin. Microbiol.* **30:**1598–1599.
2. **Abbott, S. L., L. S. Seli, M. Catino, Jr., M. A. Hartley, and J. M. Janda.** 1998. Misidentification of unusual *Aeromonas* species as members of the genus *Vibrio:* a continuing problem. *J. Clin. Microbiol.* **36:**1103–1104.
3. **Furniss, A. L., J. V. Lee, and T. J. Donovan.** 1978. *The Vibrios.* Public Health Laboratory Service monograph ser. no. 11. Her Majesty's Stationery Office, London, United Kingdom.
4. **Janda, J. M., C. Powers, R. G. Bryant, and S. L. Abbott.** 1988. Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. *Clin. Microbiol. Rev.* **1:**245–267.
5. **Janda, J. M.** 1991. Recent advances in the study of the taxonomy, pathogenicity, and infectious syndromes associated with the genus *Aeromonas. Clin. Microbiol. Rev.* **4:**397–410.

3.17.37 ONPG (*o*-Nitrophenyl-β-D-Galactopyranoside) Test

I. PRINCIPLE

o-Nitrophenyl-β-D-galactopyranoside (ONPG) is used in differentiating members of the *Enterobacteriaceae* and *Neisseria* based on β-D-galactosidase activity. The ability of a bacterium to ferment lactose depends on two enzymes, permease and β-galactosidase. Permease allows lactose to enter the bacterial cell wall, where it is then broken down into glucose and galactose by β-galactosidase. The glucose and galactose can then be metabolized by the bacteria. The enzymes are inducible and are only present when lactose, rather than glucose, is available to the organism for metabolism. Some organisms lack permease and appear as late lactose fermenters or non-lactose fermenters. The ONPG test will detect true lactose fermenters that have the β-galactosidase enzyme, even if they lack the permease enzyme. A lactose fermentation test will not detect organisms lacking the permease.

ONPG is a colorless substrate, similar in structure to lactose, used in this test as the substrate for β-galactosidase. If the organism possesses β-galactosidase, the enzyme will split the β-galactoside bond, releasing galactose and *o*-nitrophenol, which is a yellow compound. The activity of the galactosidase enzyme is increased in the presence of sodium ions (1). Other substrates can be used to detect β-galactosidase and are found in the commercial identification kits, especially those used to identify *Neisseria* species.

II. MICROORGANISMS TESTED

A. Gram-negative rods growing aerobically
B. Gram-negative diplococci growing aerobically

III. MATERIALS, REAGENTS, AND SUPPLIES

A. Reagents
1. Because of the instability of ONPG, the reagent should not be prepared but purchased.
2. Purchase ONPG as broth, disks, or tablets.
   - **a.** Store as directed by the manufacturer. Usually store broth at less than −10°C and disks and tablets at 4°C.
   - **b.** Store away from direct light; ONPG is *light sensitive.*

B. Supplies/equipment
1. Plastic or glass tubes for disk test
2. Wooden sticks or inoculating loops
3. Sterile saline
4. Incubator at 35 to 37°C

IV. QUALITY CONTROL

A. Do not use if broth media or disk, after reconstitution, is not light amber and clear, with no precipitates.
B. Perform QC on each new lot or shipment prior to putting it into use.
C. Organisms
1. *Escherichia coli* ATCC 25922—ONPG positive
2. *Proteus mirabilis* ATCC 12453—ONPG negative

or

3. *Neisseria lactamica* ATCC 23971—ONPG positive
4. *Neisseria gonorrhoeae* ATCC 43069—ONPG negative

V. PROCEDURE

A. Tube medium

1. Bring test medium to room temperature.
2. Using a heavy inoculum (no. 2 McFarland) from a pure 18- to 24-h culture, inoculate the test medium.
3. Incubate aerobically at 35°C.
4. Examine for yellow color development at 1 h.
5. If the tube has not changed color after 1 h of incubation or if the colony was not taken from lactose-containing medium, continue incubation up to 24 h.

B. Disk test

1. Inoculate colonies into 0.5 ml of saline to produce a heavy suspension (no. 2 McFarland).
2. Add disk to tube.
3. Incubate aerobically at 35°C for up to 6 h.
4. Observe for yellow color change.

VI. INTERPRETATION

A. A yellow color change is a positive test for ONPG.
B. A lack of color change is a negative test.

VII. REPORTING RESULTS

A. An organism that produces a yellow color is considered ONPG positive and generally a lactose fermenter.
B. An organism that does not produce a yellow color is considered ONPG negative and non-lactose fermenting.
C. Among the *Neisseria* spp., *N. lactamica* is identified by its ONPG-positive reaction (2).

VIII. LIMITATIONS

A. For the rapid test, colonies should be from a lactose-containing medium (e.g., triple sugar iron agar or MAC).
B. Cultures that naturally produce a yellow pigment cannot be tested with this medium.
C. If medium is not properly buffered, results may be inaccurate.
D. Do not use if medium is yellow.
E. Since glucose inhibits lactose fermentation by bacteria, organisms growing on glucose-containing medium show less activity than they would in the presence of lactose.

REFERENCES

1. **Negut, M., and G. Hermann.** 1975. A comparison of two methods for detecting β-D-galactosidase. *Public Health Lab.* **33:**190–193.
2. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed., p. 16. Williams & Wilkins, Baltimore, MD.

3.17.38 Optochin Susceptibility Test

I. PRINCIPLE

Streptococcus pneumoniae is found commonly in the human respiratory tract, as are other streptococci, and has a hemolytic pattern indistinguishable from that of other alpha-hemolytic streptococci and lactobacilli. Optochin susceptibility is used to differentiate *S. pneumoniae* from other alpha-hemolytic streptococci, either as isolated colonies or as a test to recognize *S. pneumoniae* colonies directly on cultures of respiratory specimens.

Sensitivity to optochin (ethylhydrocupreine hydrochloride) has been well-established for *S. pneumoniae* since the early 20th century (5). In 1955 Bowen and Jeffries impregnated disks with the reagent to demonstrate the susceptibility of the pneumococcus for identification purposes (2). A positive identification in an organism with Gram stain and colony morphology consistent with *S. pneumoniae* is made when a well-defined zone of inhibition forms around the impregnated disk. Other alpha-hemolytic streptococci do not display this clear zone of inhibition in the presence of optochin.

II. MICROORGANISMS TESTED

A. Test any fresh culture of alpha-hemolytic, catalase-negative, gram-positive cocci in pairs, growing on BAP or Columbia colistin-nalidixic acid agar (CNA) and having the characteristic central depression (flattened center) or mucoid colony morphology suggestive of *S. pneumoniae*.

B. As a rapid detection and identification method, place the disks on plates inoculated with either respiratory specimens or subcultures from blood cultures. However, results for disks placed on plates directly inoculated with respiratory specimens can be very misleading unless a Gram-stained smear indicates an almost singular presence of lanceolate gram-positive diplococci.

C. As a test of purity of culture, add the disk for any known *S. pneumoniae* isolate that is being tested for antimicrobial resistance.

III. REAGENTS AND SUPPLIES

A. Reagents

1. Optochin disks—purchase from most vendors.

a. Each disk is impregnated with 5 µg of optochin.

b. Store stock at 2 to 8°C. Protect from light, excessive heat, and moisture.

c. The expiration date is at least 9 months (2) but may be considerably longer.

2. BAP—use only 5% sheep blood agar for identification test.

B. Supplies/equipment

1. Standard microbiological loops

2. CO_2 incubator at 35 to 37°C

3. Sterile forceps

IV. QUALITY CONTROL

A. Perform QC with known positive and negative controls prior to use of new lots or shipments and, optionally, at weekly intervals.

B. Organisms and zone sizes

1. *S. pneumoniae* ATCC 49619, ≥14 mm

2. *Enterococcus faecalis* ATCC 29212, no zone

◩ **NOTE:** For a 10-mm disk, use ≥16 mm as the breakpoint for susceptibility. Compare weekly zone size results and if the size is decreasing, open a new vial, as the current supply in use may be deteriorating even before the expiration date.

V. PROCEDURE

A. Using an inoculating loop, select a well-isolated colony of the alpha-hemolytic organism to be tested. Alternatively, a broth culture with a turbidity corresponding to a 0.5 McFarland standard can be used (3).

B. Using a loop from a colony, or swab from a broth culture, streak the isolate onto BAP in at least two directions so as to obtain confluent growth. Several isolates may be placed on one plate by dividing the plate into quadrants and streaking one isolate per quadrant.

◩ **NOTE:** Use of media other than 5% sheep blood agar is not recommended, as smaller zone sizes can result in lack of definitive identification (3). However, when performing a susceptibility test for purposes of detection of contamination of a culture of known *S. pneumoniae,* the Mueller-Hinton blood agar used for the susceptibility testing can be used without strict measurement of the zone size.

C. Using sterile forceps, place an optochin disk onto the inoculated surface of the agar.

D. Press disk gently with the sterile forceps or loop so that the disk adheres firmly to the agar surface.

E. Incubate the plate at 35 to 37°C for 18 to 24 h in 5 to 10% CO_2.

F. If zone of inhibition is present, measure the diameter with a millimeter ruler or caliper.

VI. INTERPRETATION

A. A zone of inhibition of ≥14 mm (15 to 30 mm) around the disk is considered a susceptible result.

B. Organisms with zone sizes of <14 mm around the disk should be considered intermediate in susceptibility to optochin.

C. If there is no zone around the disk, the organism is considered resistant to optochin.

D. Colonies *within* the zone may or may not be *S. pneumoniae,* since this phenomenon can occur with *S. pneumoniae* (7).

VII. REPORTING RESULTS

A. Report an identification of *S. pneumoniae* if the alpha-hemolytic colony from gram-positive cocci in pairs is catalase negative and susceptible to optochin.

B. For any alpha-hemolytic colonies from gram-positive cocci in pairs that are catalase negative but produce zone sizes with *intermediate* results, perform spot bile solubility test for confirmation of identification. Report as *Streptococcus pneumoniae* if positive.

C. If the organism is optochin resistant and is a catalase-negative, alpha-hemolytic colony from gram-positive cocci in pairs, report as a viridans group streptococcus.

D. For purposes of determination of contamination of a susceptibility test, any zone of inhibition of ≥10 mm indicates lack of contamination.

VIII. LIMITATIONS

A. *S. pneumoniae* isolates should be incubated in a CO_2-enriched environment, as some isolates will grow poorly or not at all without increased CO_2 (3, 8).

B. If the organism is optochin resistant, it is likely to be a nonpneumococcal alpha-hemolytic streptococcus; however, rare exceptions have been reported (1, 7).

C. Optochin susceptibility is an excellent test to identify *S. pneumoniae,* with a 99% sensitivity for *encapsulated* strains and 98 to 99% specificity (4, 6). However, Mundy et al. (6) reported that only 10 of 33 nonencapsulated strains positive by DNA probe were detected as susceptible by optochin; the remainder had intermediate zone diameters with optochin.

D. If the tube bile test is performed to confirm strains with zone of inhibition between 7 and 14 mm, the sensitivity in detection of nonencapsulated strains is increased; however, bile-resistant strains with intermediate zone sizes may still be *S. pneumoniae.* Further testing with a DNA probe (GenProbe, Inc.) is needed for definitive identification (6). Since most (85%) will *not* be *S. pneumoniae* and most nonencapsulated strains are from noninvasive sites, this approach may not be cost-effective (3).

E. If there are colonies present within the zone of inhibition, these colonies may or may not be pneumococci. Pikis et al. (7) reported that resistant strains may exhibit this phenomenon; however, it is rare. True optochin-resistant strains have only been reported for three cases (7). The resistance was shown to be a point mutation in strains from patients that had been treated with antimicrobial agents (7). Subculture such colonies and confirm with Gram stain, catalase test, and bile solubility to determine if they are contaminants or pneumococci prior to reporting susceptibility results.

REFERENCES

1. **Borek, A. P., D. C. Dressel, J. Hussong, and L. R. Peterson.** 1997. Evolving clinical problems with *Streptococcus pneumoniae:* increasing resistance to antimicrobial agents, and failure of traditional optochin identification in Chicago, Illinois, between 1993 and 1996. *Diagn. Microbiol. Infect. Dis.* **29:**209–214.
2. **Bowen, E. F., and L. R. Jeffries.** 1955. Optochin in the identification of *Str. pneumoniae. J. Clin. Pathol.* **8:**58–60.
3. **Gardam, M. A., and M. A. Miller.** 1998. Optochin revisited: defining the optimal type of blood agar for presumptive identification of *Streptococcus pneumoniae. J. Clin. Microbiol.* **36:**833–834.
4. **Kellogg, J. A., D. A. Bankert, C. J. Elder, J. L. Gibbs, and M. C. Smith.** 2001. Identification of *Streptococcus pneumoniae* revisited. *J. Clin. Microbiol.* **39:**3373–3375.
5. **Moore, H. F.** 1915. The action of ethylhydrocupreine (optochin) on type strains of pneumococci *in vitro* and *in vivo,* and on some other microorganisms *in vitro. J. Exp. Med.* **22:**269–285.
6. **Mundy, L. S., E. N. Janoff, K. E. Schwebke, C. J. Shanholtzer, and K. E. Willard.** 1998. Ambiguity in the identification of *Streptococcus pneumoniae.* Optochin, bile solubility, quellung, and the AccuProbe DNA probe tests. *Am. J. Clin. Pathol.* **109:**55–61.
7. **Pikis, A., J. M. Campos, W. J. Rodriguez, and J. M. Keith.** 2001. Optochin resistance in *Streptococcus pneumoniae:* mechanism, significance, and clinical implications. *J. Infect. Dis.* **184:**582–590.
8. **Ragsdale, A. R., and J. P. Sanford.** 1971. Interfering effect of incubation in carbon dioxide on the identification of pneumococci by optochin discs. *Appl. Microbiol.* **22:**854–855.

3.17.39 Oxidase Test

I. PRINCIPLE

In the presence of atmospheric oxygen, a bacterium's intracellular cytochrome oxidase enzymes oxidize the phenylenediamine reagent (an electron acceptor) to form a deep purple compound, indol phenol (4). The test is useful in the initial characterization of gram-negative bacteria (3).

II. MICROORGANISMS TESTED

A. Aerobic, facultatively anaerobic, or microaerobic gram-negative rods and cocci from BAP, Mueller-Hinton agar, brucella agar, BHI, or CHOC

B. Restrictions

1. Do not use media with dyes, such as EMB or MAC.

2. Do not test strictly anaerobic organisms.

3. Do not test organisms growing on media that contain glucose.

III. REAGENTS AND SUPPLIES

A. Reagents

1. Kovács' reagent

◩ **NOTE:** Other formulas exist, but Kovács' is the most sensitive reagent.

a. For 0.5 to 1% solution, dissolve 0.1 g (size of a pea) *N*,*N*,*N*,*N*-tetramethyl-*p*-phenylenediamine dihydrochloride in 10 ml of sterile distilled water.

b. Mix well and allow to sit for 15 min.

c. Make fresh daily (preferred method), or store aliquots of the reagent in foil-wrapped test tubes at −20°C. Remove from the freezer and thaw before use.

d. Discard unused portion daily.

2. Dried filter paper disks or strips impregnated with reagent

3. Disposable glass ampoules (available from most vendors)

B. Supplies

1. Filter paper or swab

2. Disposable petri dish

3. Sterile wooden sticks or plastic or platinum loops or wires

4. Deionized water

IV. QUALITY CONTROL

A. Do not use once the pale purple color of the reagent or the filter paper begins to darken.

B. Perform QC on each new lot of powdered reagent prior to putting it into use.

C. Organisms

1. *Pseudomonas aeruginosa* ATCC 27853—oxidase positive

2. *Escherichia coli* ATCC 25922—oxidase negative

V. PROCEDURE

A. Filter paper method

1. Optional preparations

a. Place a small square of Whatman no. 1 filter paper in a petri dish and moisten with 1 or 2 drops of prepared Kovács' oxidase reagent *or*

b. Place impregnated dry disk or strip in petri dish and moisten with deionized water.

◩ **NOTE:** Some manufacturers may not require that the strip be moistened.

2. Optional methods of colony testing

a. Pick an isolated colony with a stick and smear onto Kovács' reagent-dampened filter paper. Observe paper for purple color.

b. For fastidious bacteria, swipe colony onto white cotton swab and rub onto the dampened filter paper. Observe *swab* for purple color.

c. Touch moistened filter paper to colony and observe paper for purple color.

B. Plate method

1. Drop a few drops of reagent directly on top of a few suspected colonies. Do not flood entire plate because bacteria covered by the reagent generally are not viable for subculture.

2. Expose the colonies to air by tilting the culture after flooding with oxidase reagent to allow oxygen to reach the colonies.

3. Observe colony for purple color. Ignore any discoloration of surrounding medium.

◩ **NOTE:** Colonies tested by this method are quickly nonviable. Subculture immediately.

VI. INTERPRETATION

A. Positive test

1. Development of a deep blue to purple color in 10 to 30 s is a positive reaction.

2. Development of the color in 30 to 60 s is a weak positive reaction, characteristic of many *Pasteurella* spp.

3. Do not read after 60 s.

B. Negative test is no color change in 60 s.

VII. REPORTING RESULTS

A. To avoid misidentifications, perform oxidase test on *all* gram-negative rods, except those that swarm.

◩ **NOTE:** This test is especially important in separating *Aeromonas* from *Enterobacteriaceae.* Most commercial kits do not include an oxidase test as part of their biochemical reactions but require the user to record the result as part of their identification scheme. If the test is omitted, errors in identification can be made. In addition, oxidase testing can aid in rapid identifications, avoiding the need for costlier kit identifications (1).

B. Gram-negative diplococci should give a positive reaction, since all members of the genus *Neisseria* are oxidase positive. *Moraxella* spp. which are either gram-negative diplococci or coccobacilli are also oxidase positive.

VII. REPORTING RESULTS *(continued)*

C. Gram-negative rods that are oxidase positive do not belong to the *Enterobacteriaceae*, with the exception of *Plesiomonas shigelloides*, which is both oxidase and indole positive (*see* Table 3.18.2–8).

D. *Campylobacter* spp. are oxidase positive.

E. Use this test as a major characteristic for identification of gram-negative rods that are not in the *Enterobacteriaceae* family.

VIII. LIMITATIONS

A. To avoid false-positive results

1. Do not use a Nichrome wire to pick colony.

2. Do not test organisms growing on media that contain glucose or dyes (e.g., MAC or EMB).

3. Do not use if reagent or filter paper is purple.

B. Mixed cultures of *Neisseria* and pseudomonads can give false-negative results, since the pseudomonads can elaborate an inhibitory substance that interferes with the production of oxidase by *Neisseria.*

C. Timing is critical to accurate testing.

D. Dry filter paper may be rewet with reagent for further use until it is purple.

E. Some organisms may require subculture to produce a positive reaction.

F. The modified oxidase test (Microdase disks; Remel, Inc.) to detect cytochrome *c* is one of several tests that can be used to separate *Micrococcus* (positive) from staphylococci (negative). The test is performed in the same manner as the oxidase test, except that the reagent is 6% tetramethylphenylenediamine hydrochloride in dimethyl sulfoxide (2).

REFERENCES

1. **Clinical and Laboratory Standards Institute.** 2008. *Abbreviated Identification of Bacteria and Yeasts*, 2nd ed. Approved guideline M35-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
2. **Faller, A., and K. H. Schleifer.** 1981. Modified oxidase and benzidine tests for separation of staphylococci from micrococci. *J. Clin. Microbiol.* **13:**1031–1035.
3. **Kovács, N.** 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. *Nature* **178:**703.
4. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria*, 3rd ed., p. 368–378. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.

3.17.40 Phenylalanine Deaminase Test

I. PRINCIPLE

The phenylalanine deaminase (PDA) test is used to differentiate among the urea-positive gram-negative bacilli based on the ability of the microorganisms to produce phenylpyruvic acid by oxidative deamination. Phenylalanine is an amino acid that, upon deamination by oxidase enzymes, results in the formation of phenylpyruvic acid. The deamination of phenylalanine to phenylpyruvic acid is detected by the addition of a ferric chloride solution that acts as a chelating agent with the α-keto acid by-product to produce a light to deep green cyclic compound.

Hendriksen, in 1950, demonstrated that *Proteus* spp. were able to convert phenylalanine to phenylpyruvic acid (3). This observation was incorporated into a medium by Ewing et al. (2) and into a disk along with urea by Ederer et al. (1). Of the *Enterobacteriaceae* that are urea positive, only members of the *Proteus, Providencia,* and *Morganella* group are capable of deaminating phenylalanine (2). The test can also be used to identify other *Enterobacteriaceae, Buttiauxella, Rahnella,* and *Tatumella,* which are PDA positive but are urea negative.

Tryptophan can be substituted for phenylalanine; tryptophan deamination releases indole-pyruvic acid, which results in a purple to black color with the addition of ferric chloride. Either tryptophan or phenylalanine can be used to differentiate among the *Proteus* group of gram-negative rods.

II. MICROORGANISMS TESTED

A. Gram-negative rods that grow well on MAC, are oxidase negative, and are usually urea positive

B. This test can be used as part of screening for fecal pathogens to rule out *Proteus, Providencia,* and *Morganella.*

C. Oxidase-positive, gram-negative coccobacilli and rods that are urea positive, to separate *Oligella* spp. and *Psychrobacter phenylpyruvicus* and others from PDA-negative strains

III. MEDIA, REAGENT, AND SUPPLIES

Include QC information on reagent container and in QC records.

A. Media

1. Phenylalanine agar slants (available from most medium vendors)
- **a.** Contain L-phenylalanine and yeast extract in a buffered agar
- **b.** Store at 15 to 30°C. Shelf life, 1 year

2. Rapid tests
- **a.** Urea-PDA disks (Hardy Diagnostics; Remel, Inc.; or prepare in-house [*see* reference 1])
- **b.** Phenylalanine tablets (Key Scientific)
- **c.** Indole-IPA tablets containing tryptophan (Key Scientific)
- **d.** ONGP–PDA broth (Hardy Diagnostics)—store frozen at less than −10°C.

◩ **NOTE:** Commercial multitest systems often include a substrate to detect either PDA or tryptophan deaminase (TDA).

B. Reagent

10% Ferric chloride, acidified

1. Dissolve 12 g of ferric chloride in 97.5 ml of water.

2. Slowly add 2.5 ml of concentrated HCl in a fume hood.

3. Store in brown bottle at 4°C.

III. MEDIA, REAGENT, AND SUPPLIES *(continued)*

4. For in-house-prepared reagent, test monthly and discard when reaction is weak.

◩ **NOTE:** Acidified ferric chloride is recommended, but 10% aqueous ferric chloride (10 g in 100 ml of deionized water) can be used (4).

C. Supplies/equipment

1. Sterile wooden sticks or inoculating loops
2. Saline or water in small plastic tube for disk test
3. Incubator at 35 to 37°C

IV. QUALITY CONTROL

A. Examine phenylalanine agar to be sure that it is slightly opalescent and light amber. Check for signs of prior freezing, contamination, cracks, dehydration, and deterioration.

B. Perform QC on each new lot or shipment of medium, disks, and ferric chloride prior to use with one organism known to demonstrate a positive reaction and one organism known to give a negative reaction.

C. Organisms

1. *Proteus mirabilis* ATCC 12453—turns green after the addition of 4 or 5 drops of ferric chloride with agitation; may take 1 to 5 min (positive)
2. *Escherichia coli* ATCC 25922—remains yellow after addition of ferric chloride (negative)

V. PROCEDURE

A. Agar test

1. Prior to inoculation, allow the medium to equilibrate to room temperature.
2. Using a heavy inoculum from an 18- to 24-h pure culture, streak the slant surface using a fishtail motion.
3. Incubate the inoculated slant aerobically at 35°C for 18 to 24 h. If a heavy inoculum is used, incubate for 4 to 6 h.
4. Following incubation, apply 4 or 5 drops of ferric chloride directly to the slant.
5. Gently roll the reagent over the slant to dislodge surface colonies, and observe for the development of a green color within 1 to 5 min.

B. Rapid tests

Check the package insert for differences from procedure below.

1. Prepare a small test tube with 0.25 ml (5 drops) of saline or water.
   ◩ **NOTE:** Preferably use plastic tubes for disk tests.
2. Make a heavy suspension of the actively growing organism.
3. Add disk (tablets are already in tube).
4. Incubate aerobically at 37°C for 1 h or up to 2 h for the urea-PDA test.
5. If urea is present in disk, observe for pink color indicative of a positive urea reaction. Add 2 drops of 1 N HCl to acidify the alkaline urea reaction (optional).
6. Add 2 drops of 10% ferric chloride, shake, and observe for green color.

VI. INTERPRETATION

A. A positive phenylalanine deamination reaction is indicated by the development of a light to dark green color (PDA) or purple to black color (TDA) within 1 to 5 min after applying ferric chloride reagent.

B. A negative test is indicated by the absence of a green color reaction. Negative results will take on a yellow color due to the color of the ferric chloride.

VII. REPORTING RESULTS

A. A positive or negative PDA test is only one test in the identification of urea-positive *Proteus, Providencia,* and *Morganella.*

B. If the PDA test is used as a screening test, PDA-positive *Enterobacteriaceae* can be eliminated as fecal pathogens.

C. A positive PDA test can eliminate the identification of *Brucella* from oxidase-positive, urea-positive, gram-negative coccobacilli.

VIII. LIMITATIONS

A. The green color reaction of a positive test fades rapidly. Test results must be interpreted within 5 min following the application of ferric chloride or false-negative results may occur.

B. Slight agitation of the tube containing ferric chloride will produce a faster, more pronounced color reaction.

C. TDA can be detected instead of PDA with the same interpretation of results.

REFERENCES

1. **Ederer, G. M., J. H. Chu, and D. J. Blazevic.** 1971. Rapid test for urease and phenylalanine deaminase production. *Appl. Microbiol.* **21:**545.
2. **Ewing, E. H., B. R. Davis, and R. W. Reaves.** 1957. Phenylalanine and malonate media and their use in enteric bacteriology. *Public Health Lab.* **15:**153–160.
3. **Hendriksen, S. D.** 1950. A comparison of the phenylalanine acid reaction and urease test in the differentiation of *Proteus* from other enteric organisms. *J. Bacteriol.* **60:**225–231.
4. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria,* 3rd ed., p. 388–393. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.

3.17.41 PYR (L-Pyrrolidonyl-β-Naphthylamide) Test

I. PRINCIPLE

L-Pyrrolidonyl-β-naphthylamide (PYR) serves as a substrate for the detection of pyrrolidonyl peptidase. Following hydrolysis of the substrate by the peptidase, the resulting β-naphthylamide produces a red color upon the addition of 0.01% cinnamaldehyde reagent. When a visible inoculum of microorganism is rubbed onto a small area of a disk impregnated with the substrate, the hydrolysis occurs within 2 min, at which time the cinnamaldehyde reagent is added to detect the reaction by a color change to purple.

Godsey et al. (5) first reported that the hydrolysis of this substrate serves as a useful tool in the identification of *Streptococcus pyogenes* (beta-hemolytic group A) and enterococci. Others soon tested the method and demonstrated its accuracy (1, 3, 4). York et al. (7) and Chagla et al. (2) also found a high degree of accuracy using PYR for identification of *Escherichia coli*, separating it from other indole-positive, lactose-positive, gram-negative rods. Hébert et al. (6) demonstrated the utility of using PYR to differentiate among the coagulase-negative staphylococci.

II. MICROORGANISMS TESTED

A. Catalase-negative, beta-hemolytic, gram-positive cocci with typical group A streptococcal morphology

B. Catalase-negative, gamma- or alpha-hemolytic, gram-positive cocci with typical enterococcal morphology

C. Oxidase-negative, indole-positive, gram-negative rods that are lactose positive on MAC, to identify *E. coli*

D. Coagulase-negative staphylococci, to screen for *Staphylococcus lugdunensis* and identify other staphylococci to the species level (6)

III. MATERIALS, REAGENTS, AND SUPPLIES

A. Reagents

1. Disks impregnated with PYR

a. Store at 2 to 8°C in the dark.

b. Vendors: BD Diagnostic Systems; EY Laboratories; Hardy Diagnostics; Key Scientific; Oxoid, Inc.; PML; Remel, Inc.
Caution: *PYR powder or liquid is a potential carcinogen; making the reagent in the clinical laboratory is discouraged.*

c. A number of vendors combine several spot tests for identification of streptococci into a card with PYR. PYR in some kits is a liquid reagent, which is more suitable for staphylococci (6).

2. 0.01% *p*-Dimethylaminocinnamaldehyde

B. Other supplies

1. Sterile water

2. Sterile sticks

3. Petri dish and forceps

IV. QUALITY CONTROL

A. Perform QC on each new lot or shipment of disks and color reagent prior to putting it into use.

B. Organisms

1. *Enterococcus faecalis* ATCC 29212—PYR positive

2. *E. coli* ATCC 25923—PYR negative

V. PROCEDURE

A. Using forceps, place PYR disk in petri dish.
B. Moisten, but do not saturate, disk with sterile water.
C. Using a sterile stick, remove one or two loopfuls of culture from a blood agar plate that is 24 to 48 h old. Use several loopfuls for organisms that take 48 h or more to grow.
D. Rub onto PYR disk.
E. Allow to react for 2 min (extend time to 10 min for poorly growing organisms).
F. After incubation period, add 1 drop of cinnamaldehyde reagent and observe for red color.

VI. INTERPRETATION

A. A positive test is indicated by the appearance of a bright pink or cherry red color usually within 1 min.
B. A negative test is indicated by no color change or a blue color due to a positive indole reaction.
C. A pale pink reaction (weak) is considered negative.

VII. REPORTING RESULTS

A. An identification of *S. pyogenes* may be made for PYR-positive, catalase-negative, beta-hemolytic, gram-positive cocci with typical group A streptococcal morphology from throat cultures.
  1. Confirm negative PYR tests on beta-hemolytic streptococci with further testing or a fresh subculture if inoculum is low or results are questionable.
  2. Confirm positive tests from nonrespiratory sites with either negative bile-esculin, negative esculin, or serologic typing for the group A antigen.
B. An identification of *Enterococcus* may be made on PYR-positive, catalase-negative, nonhemolytic, gram-positive cocci with typical enterococcal morphology, short chains, and diplococci, *not* tetrads or clusters.
C. Gram-negative rods are identified as *E. coli* if they are oxidase-negative, indole-positive, lactose-positive colonies that are PYR negative.
D. Slide coagulase-positive staphylococci that are PYR positive could be *Staphylococcus lugdunensis.* Use a positive ornithine or other tests to confirm (*see* Table 3.18.1–1).
E. *Citrobacter* is PYR positive and can be differentiated from H_2S-positive *Salmonella,* which is PYR negative.

VIII. LIMITATIONS

A. Odd gram-positive cocci will be positive in this test but are not enterococci. Gram stain is most helpful with this issue. They are generally in tetrads or clusters in the smear, are tiny colonies, or are not significant pathogens.
B. A false-negative test can result if the disk is too moist.
C. Weak, pale results occur with the disk test for *Staphylococcus aureus;* positive results may need to be confirmed with other tests or with the tube PYR test, which is available in rapid identification kits, such as API Rapid Strep (6).
D. False-negative tests result if selective media or tube biochemical agars are used to provide inocula.

REFERENCES

1. **Bosley, G. S., R. R. Facklam, and D. Grossman.** 1983. Rapid identification of enterococci. *J. Clin. Microbiol.* **18:**1275–1277.
2. **Chagla, A. H., A. A. Borczyk, J. E. Aldom, S. Dalla Rosa, and D. D. Cole.** 1993. Evaluation of the L-pyrrolidonyl β-naphthylamide hydrolysis test for the differentiation of members of the families *Enterobacteriaceae* and *Vibrionaceae*. *J. Clin. Microbiol.* **31:**1946–1948.
3. **Ellner, P. D., D. A. Williams, M. E. Hosmer, and M. A. Cohenford.** 1985. Preliminary evaluation of a rapid colorimetric method for the presumptive identification of group A streptococci and enterococci. *J. Clin. Microbiol.* **22:**880–881.
4. **Facklam, R. R., L. G. Thacker, B. Fox, and L. Eriquez.** 1982. Presumptive identification of streptococci with a new test system. *J. Clin. Microbiol.* **15:**987–990.
5. **Godsey, J., R. Schulman, and L. A. Eriquez.** 1981. The hydrolysis of L-pyrrolidonyl-β-naphthylamide as an aid in the rapid identification of *Streptococcus pyogenes*, *S. avium*, and group D enterococci, abstr. C84, p. 276. *Abstr. 81st Annu. Meet. Am. Soc. Microbiol. 1981*. American Society for Microbiology, Washington, DC.
6. **Hébert, G. A., C. G. Crowder, G. A. Hancock, W. R. Jarvis, and C. Thornsberry.** 1988. Characteristics of coagulase-negative staphylococci that help differentiate these species and other members of the family *Micrococcaceae*. *J. Clin. Microbiol.* **26:**1939–1949.
7. **York, M. K., E. J. Baron, M. Weinstein, R. Thomson, and J. E. Clarridge.** 2000. A multilaboratory validation of rapid spot tests for identification of *Escherichia coli*. *J. Clin. Microbiol.* **38:**3394–3398.

3.17.42 Quellung Reaction for Pneumococci

I. PRINCIPLE

As a rapid method to definitively identify *Streptococcus pneumoniae* directly in sputum, pleural fluid, CSF, blood, or other body fluids, capsular swelling of the pneumococcus in the presence of specific capsular antibody is observed (3, 5). This procedure can also be used to identify difficult isolates of presumptive pneumococci that are bile insoluble or optochin negative or to type these organisms after they have already been isolated and identified, using specific capsular typing sera, for evaluation of vaccine efficacy. Antigen-antibody reaction between antiserum and the capsule causes the capsule to appear to swell, although the mechanism is probably due to a change in the refractive index of the capsule that enhances its visibility (Neufeld reaction [1]).

II. SPECIMEN OR MICROORGANISM TESTED

A. Positive blood cultures containing lancet-shaped, gram-positive cocci in pairs suggestive of pneumococci

B. Respiratory specimens or CSF with direct Gram stains positive for lancet-shaped, gram-positive cocci in pairs

C. Colonies with characteristic pneumococcal morphology that are bile resistant

III. REAGENTS AND SUPPLIES

A. Reagents

1. Polyvalent *S. pneumoniae* antisera from Omniserum (Erna Lund, Pneumococcus Department, Statens Seruminstitut, Copenhagen, Denmark; distributed by DAKO, Carpinteria, CA).
- **a.** Contents: antisera to 84 pneumococcal types in a small amount of methylene blue
- **b.** Store at 4°C.

2. Methylene blue solution (store at room temperature)
- **a.** 0.3 g of methylene blue
- **b.** 100 ml of H_2O

3. Rabbit serum (store at −20°C)

B. Supplies

1. Loops

2. Slides and coverslips

3. Pasteur pipettes

IV. QUALITY CONTROL

A. Prior to use and every 6 months thereafter, test a fresh isolate of a bile-soluble colony (preferably from a blood culture) and a bile-insoluble colony to obtain the expected positive Quellung reaction (bile-soluble colony) and negative Quellung reaction (bile-insoluble colony).

B. QC strains are not suitable for controls, as they easily lose their capsule.

V. PROCEDURE

A. Divide a slide into two sections with a wax pencil. Label one section as the test and the other as the control. (Two slides may be used instead.)

B. Specimen

1. Dilute growth from blood culture bottles containing pneumococci 1:10 in broth or saline before the slide is made, depending on the number of organisms seen in smear. Four organisms per field is ideal.

2. Prepare a small saline or broth suspension of a colony; it should be barely visibly turbid.

3. Dilute other direct specimens if needed in saline, especially if they are viscous.

C. Using a sterile 10-µl loop (1:100 ml), place a drop of specimen suspension onto each of two sections of a slide; spread out and allow to air dry.

D. Using a sterile 10-µl loop (1:100 ml), place a small drop of antiserum on the first section of the slide and spread out over the specimen.

◩ **NOTE:** Antiserum is very expensive, and a small amount will give the desired reaction.

E. Using a 10-µl loop, place a similar-size drop of rabbit serum on the second section of the slide.

F. Put a small drop of methylene blue solution on each of two coverslips. Invert the coverslips onto each section of the slide.

◩ **NOTE:** Adding additional methylene blue solution to the pneumococcal omniserum will give a more readable preparation, as there is only a small amount of methylene blue in the reagent.

G. After 10 min, read the test and control slides.

1. Adjust the microscope to obtain oblique light by adjusting the iris diaphragm so that only one-third of the light passes through the condenser at low power (p. 163 in reference 2).

2. Switch to oil immersion lens to view capsules.

H. If the test is negative

1. Repeat if inoculum is too heavy. Approximately four organisms per field is ideal.

2. Hold for 30 min in a wet chamber before discarding as negative. (A petri dish with damp gauze upon which to rest slide[s] works well.)

VI. INTERPRETATION

A. A positive test shows marked swelling of the capsules in comparison to the control, giving a sharply demarcated halo. Capsules may be visible in the control but do not produce a clear demarcation and glassy appearance.

B. A negative test is marked by no difference between the test and control cells.

VII. REPORTING RESULTS

A. Report a positive test as "*Streptococcus pneumoniae* by Quellung test."

B. Report a negative test as "Negative for *Streptococcus pneumoniae* by Quellung test."

VIII. LIMITATIONS

A. A negative test does not indicate that the organism is not *S. pneumoniae,* since the organism could have lost the ability to express a capsule (6).

B. The test can be falsely negative if there are too many organisms on the slide (antigen excess); if this occurs, the test should be repeated with fewer organisms.

VIII. LIMITATIONS *(continued)*

C. The Phadebact pneumococcus test (Boule Diagnostics, Huddinge, Sweden) is a coagglutination test using antibodies directed against the capsular antigens of *S. pneumoniae.* In the package insert, the manufacturer reports an 8% false-positive rate with other viridans group streptococci, but it had a 100% sensitivity and 98% specificity in one published study (4). The Directogen and Pneumoslide (BD Diagnostic Systems) had an 85 to 87% specificity in the same study (4).

REFERENCES

1. **Austrian, R.** 1976. The quellung reaction, a neglected microbiologic technique. *Mt. Sinai J. Med.* **43:**699–709.
2. **Facklam, R. R., and R. B. Carey.** 1985. Streptococci and aerococci, p. 154–175. *In* E. H. Lennette, A. Ballows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), *Manual of Clinical Microbiology,* 4th ed. American Society for Microbiology, Washington, DC.
3. **Heineman, H. S.** 1973. Quellung test for pneumonia. *N. Engl. J. Med.* **288:**1027.
4. **Kellogg, J. A., D. A. Bankert, C. J. Elder, J. L. Gibbs, and M. C. Smith.** 2001. Identification of *Streptococcus pneumoniae* revisited. *J. Clin. Microbiol.* **39:**3373–3375.
5. **Merrill, C. W., J. M. Gwaltney, Jr., J. W. Hendley, and M. A. Sande.** 1973. Rapid identification of pneumococci. Gram stain vs. the quellung reaction. *N. Engl. J. Med.* **288:**510–512.
6. **Mundy, L. S., E. N. Janoff, K. E. Schwebke, C. J. Shanholtzer, and K. E. Willard.** 1998. Ambiguity in the identification of *Streptococcus pneumoniae.* Optochin, bile solubility, quellung, and the AccuProbe DNA probe tests. *Am. J. Clin. Pathol.* **109:**55–61.

3.17.43 6.5% Salt and Temperature Tolerance Test

I. PRINCIPLE

As part of the differentiation of catalase-negative, gram-positive cocci, growth in 6.5% salt and growth at low (10°C) and high (42 to 45°C) temperatures are used (1). Salt tolerance broth, containing heart infusion (HI), glucose, and bromcresol purple indicator with 6.5% salt, was initially designed by Qadri et al. (4) to aid in the differentiation of *Enterococcus* spp. from streptococci. Enterococci can grow in the presence of 6.5% NaCl, but streptococci do not. The salt concentration, acting as a selective agent, interferes with membrane permeability and osmotic equilibrium for most bacteria. Salt-tolerant organisms produce heavy growth in the medium within 48 h. Organisms that are capable of growing in the high salt concentration will also ferment glucose. Glucose fermentation produces an acid reaction which results in the bromcresol purple indicator turning yellow. Appearance of a yellow color change in broth with indicator is indicative of a positive salt tolerance test, although turbidity of the media is also considered a positive reaction. The broth media, HI with dextrose and indicator (without additional salt), can be used as a growth control medium for the organism, for comparison of its growth in the same formula with the added salt. The control medium can also be used to demonstrate growth of the microorganism at various temperatures.

The pyrrolidonyl-β-naphthylamide (PYR) test has replaced the salt agar test to differentiate enterococci, but salt agar continues to be useful in the separation of the more recently described genera of catalase-negative cocci (1–3).

II. MICROORGANISMS TESTED

A. Catalase-negative, aerobic, gram-positive cocci, to aid in the identification of organisms that do not key out by multitest kits as streptococci and enterococci

B. Catalase-negative, aerobic, gram-positive cocci in tetrads and clusters, as part of the identification, including *Aerococcus* and *Alloiococcus*

C. For some gram-negative rods, growth at various temperatures is used in their identification (reference 5 and procedure 3.8.2).

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media

Store at 4 to 8°C.

1. HI (Difco, BD Diagnostic Systems; Remel, Inc.)
Approximate formula per liter of purified water

beef HI	500.0 g
tryptose	10.0 g
sodium chloride	5.0 g

2. Add 0.1 to 1% glucose and bromcresol purple indicator (1 ml of 1.6-g/100 ml 95% ethanol to a liter of medium) for temperature studies.

3. Add 0.5 to 1% glucose and bromcresol purple indicator plus 6% NaCl (HI contains 0.5% NaCl).

◩ **NOTE:** Agar may be added to solidify the medium. Slant tube while cooling. Color reactions are easier to observe when agar, rather than broth, medium is used since reaction (yellow color change) on the slant is visible before entire broth tube changes pH to produce yellow color.

4. Commercial HI broth with 6.5% salt is available from Remel, Inc. BHI with 6.5% salt is available from BD Diagnostic Systems and Hardy Diagnostics. SF broth (BD Diagnostic Systems) is similar but contains 5% salt and sodium azide to inhibit organisms other than *Enterococcus* spp.

B. Other supplies/equipment

1. Sterile sticks or inoculation loop

2. Beakers of water

3. Incubator at 35°C

4. Refrigerator at 10°C

5. Incubator or heat block at 42 to 45°C

IV. QUALITY CONTROL

A. Examine medium for purple color and lack of turbidity.
B. Test each new lot or shipment of medium with a positive and negative control prior to putting it into use.
C. Organism
  1. *Enterococcus faecalis* ATCC 29212—growth at all temperatures and in salt (positive)
  2. *Streptococcus bovis* ATCC 33317—no growth at 10°C or in salt broth (negative)

V. PROCEDURE

A. Add 1 or 2 drops from an overnight broth culture or one or two colonies from a fresh plate to the broth.
B. Tighten tube caps.
C. Incubate aerobically for up to 7 days and observe for growth (turbidity) and color change.
  1. To hold the temperatures, place the tube in a beaker of water at 10°C and at 42 to 45°C if an incubator, rather than a heat block, is used.
  2. Incubation temperatures
    a. HI at 35°C
    b. HI with 6.5% salt at 35°C
    c. HI at 10°C
    d. HI at 42 to 45°C

VI. INTERPRETATION

A. A positive test shows visible turbidity and/or change in color from purple to yellow under the above-listed conditions; this occurs usually within 24 h, but tests can be incubated for up to 14 days.
B. A negative test shows no turbidity or yellow color.

VII. REPORTING RESULTS

A. Viridans group streptococci (including *S. bovis*), *Granulicatella*, *Dolosicoccus*, and *Gemella* do not grow in 6.5% salt.
B. *Streptococcus agalactiae* may grow in salt broth.
C. *Enterococcus*, *Lactococcus*, *Globicatella*, and *Vagococcus* will grow at 10°C, but other catalase-negative, gram-positive cocci generally will not (3).
D. *S. bovis* and *Enterococcus* will grow at 45°C (1).
E. *Pediococcus* and *Leuconostoc* have variable reactions but are easily separated by their vancomycin resistance and PYR-negative reactions.
F. *Weissella confusa* usually grows at 42°C (2).

VIII. LIMITATIONS

A. Adding agar to 6.5% salt broth and slanting the tube will provide a medium that will easily allow visualization of weak reactions that start on the top of the slant.
B. Use of BHI, instead of HI, has not been tested with the unusual gram-positive cocci that are confused with streptococci and enterococci.

REFERENCES

1. **Facklam, R., and J. A. Elliott.** 1995. Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clin. Microbiol. Rev.* **8:**479–495.
2. **Facklam, R., D. Hollis, and M. D. Collins.** 1989. Identification of gram-positive coccal and coccobacillary vancomycin-resistant bacteria. *J. Clin. Microbiol.* **27:**724–730.
3. **LaClaire, L., and R. Facklam.** 2000. Comparison of three commercial rapid identification systems for the unusual gram-positive cocci *Dolosigranulum pigrum*, *Ignavigranum ruoffiae*, and *Facklamia* species. *J. Clin. Microbiol.* **38:**2037–2042.
4. **Qadri, S. M., C. W. Nichols, and S. G. Qadri.** 1978. Rapid sodium chloride tolerance test for presumptive identification of enterococci. *J. Clin. Microbiol.* **7:**238.
5. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed. Williams & Wilkins, Baltimore, MD.

3.17.44 Satellite Test

I. PRINCIPLE

Haemophilus influenzae requires two factors, hemin and NAD, for growth. NAD is also referred to as V factor. BAP supplies hemin but not NAD. Although *H. influenzae* requires both hemin and NAD, other *Haemophilus* species require only NAD for growth. Species that require NAD will not grow on BAP unless they are hemolytic and can release the NAD from the RBCs. However, they will grow well on BAP near colonies of staphylococci, which are capable of providing NAD. The NAD diffuses into the surrounding medium and stimulates growth of *Haemophilus* in the vicinity of the staphylococcus colony. This is known as satelliting. For *Haemophilus* spp., the satellite test substitutes for the V factor test and does not require the purchase or QC of any reagents or strips (3, 4).

Similarly, nutritionally variant streptococci (NVS; include *Abiotrophia defectiva* and *Granulicatella adiacens, Granulicatella elegans,* and *Granulicatella balaenopterae*, previously in the genus *Abiotrophia*) require either L-cysteine or pyridoxal to grow in vitro (2). These organisms do not grow on BAP, since it lacks the nutritional requirements, but will grow in the medium surrounding staphylococci, which supplies the needed nutrients.

II. MICROORGANISM TESTED

A. Any organism growing only on CHOC and not on BAP that meets the following criteria
  1. Is suggestive of *Haemophilus* by Gram stain (gram-negative coccobacilli or short rods)
  2. Is a possible NVS by Gram stain (e.g., cocci, filaments, and bulbous forms in smear)

B. Perform test only in conjunction with Gram stain. If bizarre forms are seen on Gram stain, repeat the smear after subculture to fresh CHOC (1).

C. Use for detection of *Haemophilus* from lower respiratory specimens by dotting directly on the primary BAP.

III. ORGANISMS, MEDIUM, AND SUPPLIES

A. Microorganisms
  1. *Staphylococcus aureus* ATCC 25923 *or*
  2. *Staphylococcus epidermidis* strain that is nonhemolytic and penicillinase negative

  ◩ **NOTE:** Most any staphylococcus will work for the test. However, if using on primary BAP, it might be useful to choose coagulase-negative, beta-lactamase-negative, nonhemolytic staphylococci to be able to easily detect contamination of primary media and see hemolysis of colonies that grow from the specimen.

B. BAP

C. Supplies/equipment
  1. Sterile sticks or inoculating loop
  2. Incubator at 35°C with 5 to 10% CO_2 or a CO_2-generating system

IV. QUALITY CONTROL

A. Demonstrate satelliting on *each lot* of BAP using the staphylococcus and *H. influenzae* ATCC 43065. Record lot numbers of BAP and results in QC log.

B. Verify satellitism with an NVS (e.g., *A. defectiva* ATCC 49176) when choosing a staphylococcus for the test.

C. Periodically verify that the strain has not become contaminated, especially if it is being used on the direct specimen plates for sputum cultures. Alternatively, get a new stock of staphylococci from the freezer monthly.

◩ **NOTE:** It is convenient to do the QC test when new lots of CHOC are received and must be quality controlled. The fresh growth of *Haemophilus* on the CHOC provides a stock for use in QC of the satellite test.

V. PROCEDURE

A. Gram stain suspected colony.

B. Subculture colony to BAP and CHOC.

1. Use a sterile stick to dot the staphylococcus on the inoculated BAP.

2. Incubate plates for 24 h at 35 to 37°C in 5% CO_2.

3. Examine for the presence of colonies that satellite around staphylococcus dots on BAP.

4. Use colonies from CHOC to perform further tests for identification to the species level or for susceptibility testing.

VI. INTERPRETATION

A. Growth of tiny colonies surrounding the staphylococcus dots is a positive test.

B. Growth on BAP without the staphylococcus dots is a negative test. Colonies appear on media with no concentration around staphylococcus dots or there is no growth on BAP.

VII. REPORTING RESULTS

A. A positive result for a tiny gram-negative rod or coccobacillus indicates that the organism is in the genus *Haemophilus.*

B. A positive result for a gram-positive coccus indicates a *Granulicatella* or *Abiotrophia* sp. For practical purposes, report as "Nutritionally variant streptococci."

C. Some microorganisms grow only on CHOC and will not grow on BAP even with a staphylococcus dot. These include *Francisella tularensis, Haemophilus ducreyi,* and some *Methylobacterium* spp.

VIII. LIMITATIONS

A. *Haemophilus ducreyi* does not require V factor (NAD) but due to its fastidious nature does not grow on BAP even with the staphylococcus dot.

B. *Haemophilus haemolyticus* and *Haemophilus parahaemolyticus* may grow without the staphylococcus and thus may not demonstrate the satellite phenomenon even though they require V factor (NAD). Since they are hemolytic, they can release NAD into the medium.

C. *Brucella* can be confused with *Haemophilus;* this test separates these genera, since *Brucella* spp. grow on BAP without staphylococci to supply NAD.

D. A positive satellite test for NVS does not definitely demonstrate that the organism requires pyridoxal to grow. Disks for this purpose are available commercially (Hardy Diagnostics; Remel, Inc.).

REFERENCES

1. **Bottone, E. J., C. A. Thomas, D. Lindquist, and J. M. Janda.** 1995. Difficulties encountered in identification of a nutritionally deficient streptococcus on the basis of its failure to revert to streptococcal morphology. *J. Clin. Microbiol.* **33:**1022–1024.
2. **Collins, M. D., and P. A. Lawson.** 2000. The genus *Abiotrophia* (Kawamura et al.) is not monophyletic: proposal of *Granulicatella* gen. nov., *Granulicatella adiacens* comb. nov., *Granulicatella elegans* comb. nov., and *Granulicatella balaenopterae* comb. nov. *Int. J. Syst. Evol. Microbiol.* **50:**365–369.
3. **Kilian, M.** 1974. A rapid method for the differentiation of *Haemophilus* strains—the porphyrin test. *Acta Pathol. Microbiol. Scand. Sect. B* **82:**835–842.
4. **Kilian, M.** 2003. *Haemophilus*, p. 623–635. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, DC.

3.17.45 SPS (Sodium Polyanethol Sulfonate) Disk Test

I. PRINCIPLE

Gardnerella vaginalis is believed to be a contributing cause of bacterial vaginitis (BV). It has also occasionally been reported to cause bacteremia in postpartum women and in men after transurethral resection of the prostate. Differentiation of *G. vaginalis* from other catalase-negative, gram-variable coccobacilli is achieved by demonstration of the susceptibility of *G. vaginalis* to sodium polyanethol sulfonate (SPS), a common anticoagulant. Using a standardized inoculum, susceptibility to SPS is easily determined by measuring a zone of inhibition around a disk containing SPS (3).

Other microorganisms are susceptible to SPS, including *Haemophilus ducreyi*, which can be demonstrated by this test. Unfortunately, the use of SPS as an anticoagulant in blood cultures may interfere with the ability to isolate SPS-susceptible organisms from bacteremic patients.

II. MICROORGANISMS TESTED

A. Catalase-negative, gram-variable coccobacilli presumed to be *G. vaginalis*

B. Catalase-negative, gram-negative rods, to separate *H. ducreyi* from other *Haemophilus* spp.

C. See section 4 for use of this test to identify anaerobic cocci.

III. MEDIA AND SUPPLIES

A. Medium and reagent

1. Brucella or other agar on which the organism will grow

2. SPS disks—1 mg

B. Other supplies/equipment

1. Sterile sticks, swabs, or inoculation loops

2. Sterile saline

3. Incubator at 35°C with 5 to 10% CO_2 or CO_2-generating system

4. 0.5 McFarland standard

IV. QUALITY CONTROL

A. Test each new lot and/or shipment of disks with a positive and negative control prior to putting it into use and monthly or with use if test is performed less often.

B. Organisms

1. *G. vaginalis* ATCC 14108, ≥12 mm

2. *Streptococcus sanguinis* ATCC 35557, <12 mm

V. PROCEDURE

A. Using a 24- to 48-h culture, prepare a suspension of the test organism in sterile saline to match a no. 0.5 McFarland standard.

B. Dip a sterile swab into the saline suspension and inoculate brucella or other agar in three directions for confluent growth, as for disk susceptibility testing.

◩ **NOTE:** Reimer and Reller (3) recommend use of brucella agar for *G. vaginalis.* Other media may work well and may be preferred for other organisms.

C. Place SPS disk on plate.

D. Incubate for 24 to 48 h at 35°C in 5% CO_2.

E. Observe zone of inhibition.

VI. INTERPRETATION

A. Susceptible is a zone of inhibition of ≥12 mm.

B. Resistant is a zone of inhibition of ≤11 mm.

VII. REPORTING RESULTS

A. The definitive identification of *G. vaginalis* consists of the following (1).

1. Colonies appear pinpoint and transparent, with no greening of the agar on BAP, Columbia colistin-nalidixic acid agar (CNA), or CHOC.

2. Gram-variable to gram-negative small, pleomorphic coccobacilli that do not elongate into filaments or chains

3. Catalase negative

4. SPS sensitive

5. Beta-hemolytic on human blood agar

B. The identification of *G. vaginalis* may include positive hippurate and lipase reactions, but negative results for these tests do not rule out the identification.

C. *H. ducreyi* is susceptible, with a zone of ≥12 mm; no other *Haemophilus* species are susceptible. The addition of 0.002% Tween 80 may aid in dispersion of the cells (4). *Capnocytophaga* is reported to be SPS susceptible, but it is aminolevulinic acid (ALA) positive (4).

VIII. LIMITATIONS

A. A critical factor in performance of the test is the inoculum size, which must be 10^8 CFU/ml (3).

B. It is not necessary to confirm the identification of *G. vaginalis* with tests other than colony morphology, catalase, and typical smear, if the direct Gram stain of a vaginal specimen is consistent with diagnosis of BV (2).

REFERENCES

1. **Aroutcheva, A. A., J. A. Simoes, K. Behbakht, and S. Faro.** 2001. *Gardnerella vaginalis* isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems. *Clin. Infect. Dis.* **33:**1022–1027.
2. **Catlin, B. W.** 1992. *Gardnerella vaginalis:* characteristics, clinical considerations, and controversies. *Clin. Microbiol. Rev.* **5:**213–237.
3. **Reimer, L. G., and L. B. Reller.** 1985. Use of a sodium polyanetholesulfonate disk for the identification of *Gardnerella vaginalis. J. Clin. Microbiol.* **21:**146–149.
4. **Shawar, R., J. Sepulveda, and J. E. Clarridge.** 1990. Use of the RapID-ANA system and sodium polyanetholesulfonate disk susceptibility testing in identifying *Haemophilus ducreyi. J. Clin. Microbiol.* **28:**108–111.

3.17.46 SS (Salmonella-Shigella) Agar Test for Growth

I. PRINCIPLE

Salmonella-shigella (SS) agar is commonly used for the selective isolation and differentiation of *Salmonella* and *Shigella* in both clinical and nonclinical samples. However, SS agar is also useful as part of an overall identification scheme for other, more unusual gram-negative organisms based on their ability to grow on the medium (3). SS agar is inhibitory to most gram-positive and many gram-negative organisms, because it contains bile salts and brilliant green and neutral red dyes. SS agar is similar to MAC and can differentiate among the lactose fermenting and non-lactose-fermenting organisms, but it is more inhibitory. It is a modification of a formula originally described by Leifson (2). Sodium thiosulfate and ferric citrate enable the detection of hydrogen sulfide production as evidenced by colonies with black centers.

II. MICROORGANISMS TESTED

A. Gram-negative rods that are able to grow on MAC, if needed, as part of the identification (3)

B. Gram-negative rods identified as either *Ralstonia paucula* (CDC group IVc-2) or *Bordetella bronchiseptica*

III. MEDIUM AND SUPPLIES

A. SS agar

1. Store at 2 to 8°C.

2. If purchased or prepared in tubes, the shelf life is extended beyond 6 months.

3. Prepare from dehydrated medium (Difco, BBL; BD Diagnostic Systems; Hardy Diagnostics).

a. Weigh desired amount per manufacturer's instructions.

b. Boil for 2 to 3 min to dissolve. Cool to 50 to 60°C.

c. Dispense in tubes, slant, and tighten caps. Do not autoclave medium.

B. Other supplies/equipment

1. Sterile sticks or inoculating loop

2. Incubator at 35°C

IV. QUALITY CONTROL

A. Check plates for signs of contamination, cracks, dehydration, and deterioration.

B. Test each lot or shipment of medium with a positive and negative control prior to putting it into use. End-user QC testing of commercially prepared SS agar is not required, if the manufacturer certifies that each lot has been verified to meet or exceed Clinical and Laboratory Standards Institute (formerly NCCLS) guidelines (1).

C. Organisms

1. *Shigella flexneri* ATCC 12022—growth and colorless colonies (positive)

2. *Escherichia coli* ATCC 25922—partial or complete inhibition of growth (negative); colonies are pink to rose.

V. PROCEDURE

A. Inoculate 1 or 2 drops from an overnight broth culture or one or two colonies from a fresh plate to the agar slant or plate. Streak slant in a zigzag direction across the slant and streak plate in quadrants for isolation of colonies.

B. Incubate aerobically for up to 7 days at 35°C, and observe for visible growth.

VI. INTERPRETATION

A. A positive test shows visible growth, usually within 24 h, but tests can be incubated for up to 7 days (3).

B. A negative test shows no growth on the agar slant.

VII. REPORTING RESULTS

A. For oxidase-positive, urea-positive, gram-negative, non-glucose-oxidizing rods that are nonpigmented and grow on MAC, separation of species is accomplished with SS agar.

1. *R. paucula* (CDC IVc-2) does not grow on SS agar (negative).

2. *B. bronchiseptica* grows on SS agar (positive).

B. Refer to Weyant et al. (3) for other gram-negative rods for which SS agar is useful in identification (e.g., *Alcaligenes faecalis, Achromobacter xylosoxidans, Bordetella avium/hinzii, Pseudomonas mendocina* [Vb-2], and *Ralstonia eutropha*).

VIII. LIMITATIONS

A. *E. coli* may grow, but will grow poorly, on SS agar. *Enterococcus faecalis* will not grow.

B. Organisms that do not grow on MAC will not grow on SS agar.

C. Due to the dyes and bile salts, growth of some *Shigella* strains is inhibited.

REFERENCES

1. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.

2. **Leifson, E.** 1935. New culture media based on sodium desoxycholate for the isolation of intestinal pathogens and for the enumeration of colon bacilli in milk and water. *J. Pathol. Bacteriol.* **40:**581–599.

3. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria,* 2nd ed., p. 20. Williams & Wilkins, Baltimore, MD.

3.17.47 Starch Hydrolysis Test

I. PRINCIPLE

Bacteria can excrete an amylase, endoamylase, which hydrolyzes amylose to maltose and glucose. If there is no enzyme present, and therefore no hydrolysis, the starch agar will turn blue in the presence of iodine, due to the action of iodine and the helical structure of amylose. When hydrolysis occurs and thus no amylose is present, there is no color development in the medium immediately surrounding the colony. To observe this hydrolysis, an organism is grown on medium containing amylose. Since amylose and iodine react together to form a blue color, the plates can be flooded with iodine to visually detect the absence or presence of amylose, one of the components of starch. Mueller-Hinton agar (MH) contains amylose and can be used to test for starch hydrolysis, avoiding the purchase of additional media.

II. MICROORGANISMS TESTED

A. To separate *Streptococcus bovis* (positive) from other viridans group streptococci that are bile-esculin positive, 6.5% NaCl negative, and pyrrolidonyl-β-naphthylamide (PYR) negative

B. To separate *Chryseobacterium meningosepticum* (negative) from *Chryseobacterium indologenes* (positive)

III. MEDIA, REAGENT, AND SUPPLIES

A. Media

1. MH

2. Heart infusion agar with 2% starch

B. Reagent

Gram's iodine (Appendix 3.2.1–1)

C. Supplies/equipment

1. Sterile sticks or inoculating loops

2. Incubator at 35°C

IV. QUALITY CONTROL

A. Inspect agar for freezing, contamination, cracks, and dehydration prior to storage and before use.

B. Perform QC on each new lot or shipment of starch agar prior to putting it into use. MH agar can be tested with use for starch hydrolysis.

C. Organisms

1. *S. bovis* ATCC 33317—clear halo around colony with addition of iodine (starch positive)

2. *Enterococcus faecalis* ATCC 25922—blue color with addition of iodine (starch negative)

V. PROCEDURE

A. After touching several colonies from an 18-h culture, inoculate several segments of one-half of the agar surface with a very *visible circular* amount of organism about the size of a dime on each spot. *Be sure plate has no surface moisture.*

B. Inoculate a positive and negative control on the other half of the plate in the same manner.

V. PROCEDURE *(continued)*

C. Incubate without increased CO_2 for at least 48 h.

D. To detect starch hydrolysis, transfer a piece of agar from one of the growth dots of the test or control organism to an empty sterile petri dish. Flood dropwise with Gram's iodine and observe for halos around colony.

E. If negative for starch hydrolysis, reincubate and retest additional pieces of agar at 72 h or later.

VI. INTERPRETATION

A. A positive test is development of a clear halo without color around the colony and a blue to blue-purple color in the surrounding medium after the addition of Gram's iodine.

B. A negative test shows no clear halo around the colony. The medium around the colony turns blue to blue-purple after the addition of Gram's iodine.

VII. REPORTING RESULTS

A. Catalase-negative, PYR-negative, gram-positive cocci that are bile-esculin positive, do not grow on 6.5% salt, and hydrolyze starch are *S. bovis.* If they are positive for group D antigen, they are definitively identified, without starch or salt testing.

B. Catalase-negative, PYR-negative, gram-positive cocci that are bile-esculin positive, do not grow on 6.5% salt, and do not hydrolyze starch may be *S. bovis* variants, but other tests need to be done to confirm the identification.

C. Among the yellow-pigmented indole-, catalase-, esculin-, and oxidase-positive gram-negative rods, *Chryseobacterium gleum/indologenes* is starch positive.

VIII. LIMITATIONS

A. Avoid using glucose starch medium, since the metabolism of glucose may interfere with the assay (3).

B. Read plates immediately after addition of iodine, as the blue color fades.

C. Once the iodine is added, the organisms are nonviable.

D. A red-violet color is due to partial hydrolysis, and the test should be repeated after further incubation.

E. The identification of *S. bovis* is difficult, and the organism can be confused with *Streptococcus mutans* (starch negative) and *Streptococcus salivarius* (1, 4).

F. *S. bovis* strains from humans are said to be biotype I if they hydrolyze starch. *S. bovis* biotype II strains do not hydrolyze starch (2).

G. *Gardnerella vaginalis* organisms are starch positive but this test is not a reliable method to identify them, since they do not grow on MH and require special starch media to grow.

REFERENCES

1. **Coykendall, A.** 1989. Classification and identification of the viridans streptococci. *Clin. Microbiol. Rev.* **2:**315–328.
2. **Facklam, R. R.** 1972. Recognition of group D streptococcal species of human origin by biochemical and physiological tests. *Appl. Microbiol.* **23:**1131–1139.
3. **MacFaddin, J. F.** 2000. *Biochemical Tests for the Identification of Medical Bacteria,* 3rd ed., p. 412–423. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.
4. **Ruoff, K., S. I. Miller, C. V. Garner, M. J. Ferraro, and S. B. Calderwood.** 1989. Bacteremia with *Streptococcus bovis* and *Streptococcus salivarius:* clinical correlates of more accurate identification of isolates. *J. Clin. Microbiol.* **27:**305–308.

3.17.48 Urea Test

I. PRINCIPLE

Urea medium, whether a broth or agar, contains urea and the pH indicator phenol red. Many organisms, especially those that infect the urinary tract, have a urease enzyme, which is able to split urea in the presence of water to release two molecules of ammonia and carbon dioxide. The ammonia combines with the carbon dioxide and water to form ammonium carbonate, which turns the medium alkaline (6), turning the indicator from its original orange-yellow color to bright pink (2).

This test can be used as part of the identification of several genera and species of *Enterobacteriaceae*, including *Proteus, Klebsiella,* and some *Yersinia* and *Citrobacter* species, as well as some *Corynebacterium* species. It is also useful to identify *Cryptococcus* spp. (*see* section 8), *Brucella, Helicobacter pylori,* and many other bacteria that produce the urease enzyme.

Disks are available that combine urea and phenylalanine deaminase (PDA), allowing a one-disk test to identify *Proteus, Providencia,* and *Morganella* and separate them from *Klebsiella* and *Yersinia enterocolitica* (4). The disk reactions are rapid and sensitive and allow for the rapid detection of agents of serious infections, e.g., *Brucella* and *Cryptococcus.*

II. MICROORGANISMS TESTED

A. The urea test is part of the battery of tests to identify the following.
- **1.** Gram-negative enteric pathogens, including *Yersinia* spp.
- **2.** Fastidious gram-negative rods—*Brucella, H. pylori,* and *Pasteurella*
- **3.** Gram-positive rods—*Corynebacterium* and *Rhodococcus* spp.
- **4.** Yeasts—*Cryptococcus* spp.

B. Directly, this test is performed on gastric biopsy samples to detect the presence of *H. pylori* (*see* procedure 3.8.4 for details).

III. MEDIA, REAGENTS, AND SUPPLIES

A. Media and reagents

Use one of the following.
- **1.** Christensen's urea agar slants containing urea, peptone, dextrose, and agar in buffered phenol red solution
  ◩ **NOTE:** Urea cannot be autoclaved but must be filter sterilized and added to agar. Christensen's urea agar base 10× concentrate (BD Diagnostic Systems) can be added to autoclaved and cooled agar to prepare medium.
- **2.** Christensen's urea broth
  - **a.** Dilute Christensen's urea agar base 10× concentrate 1:10 with sterile distilled water.
  - **b.** Dispense in 0.5-ml amounts into 13- by 100-mm tubes.
- **3.** Rapid urea medium (Remel, Inc.; Hardy Diagnostics) containing agar and urea in buffered phenol red solution. This medium can also be used for gastric biopsy specimens (1, 5).
- **4.** Rapid urea broth (Remel, Inc.)
- **5.** RSU broth containing sucrose and urea for rapid identification of *Corynebacterium* (Hardy Diagnostics)
- **6.** Urea disks or tablets—stored at 2 to 8°C
  - **a.** Supplied by Remel, Inc.; Key Scientific; or Hardy Diagnostics
  - **b.** Supplied as urea-PDA disks (Remel, Inc.; Hardy Diagnostics)

B. Supplies/equipment
- **1.** Sterile wooden sticks or loops
- **2.** Saline or water in small plastic tube for disk test
- **3.** Incubator at 35 and 30°C

IV. QUALITY CONTROL

A. Inspect agar for evidence of prior freezing, contamination, cracks, and dehydration prior to storage and before use.

B. Each time the test is performed for direct gastric specimens, test negative reactions for the ability of the medium to split urea and turn the medium red.

1. Insert a urease tablet (Kimberly Clark, Draper, UT) into the negative patient test, as you would a biopsy sample.

2. After 5 min, inspect for a positive red color change.

C. Test each new lot or shipment of medium or reagent with a positive and negative control prior to putting it into use.

◩ **NOTE:** The Clinical and Laboratory Standards Institute (formerly NCCLS) has proposed elimination of user QC for urease agar purchased from commercial sources. Consult with current regulatory agencies prior to discontinuation of user QC (3).

D. Organisms

1. *Proteus mirabilis* ATCC 12453—positive (red color)

2. *Escherichia coli* ATCC 25922—negative (no color change)

◩ **NOTE:** Alternate controls should be used if test is being done for detection of *H. pylori.* The reaction time of *H. pylori* is much shorter than that of *P. mirabilis.*

H. pylori ATCC 43504—positive (red color)

Campylobacter jejuni ATCC 33560—negative (no color change)

V. PROCEDURE

A. Christensen's urea agar

1. Using a sterile stick or loop, inoculate the agar slant surface from a well-isolated colony. Do not stab the butt.

2. Incubate, with cap loosened, aerobically at 35 to 37°C.

3. For nonfermenters, incubate at 30°C.

4. Examine for the development of a pink color for as long as 7 days.

◩ **NOTE:** If the organism does not grow on the slant, inoculate heavily with growth from the plate to detect preformed urease.

B. Urea agar deeps or rapid urea broth

1. Pick up colonies with either a stick or needle.

2. Stab the agar or rotate in the broth with the inoculated stick or needle.

3. For biopsy samples, refer to procedure 3.8.2.

C. Urea disks or tablets

Check the package insert for differences from procedure below.

1. Prepare a small test tube with 0.25 ml (5 drops) of saline or water.

◩ **NOTE:** Preferably use plastic tubes for disk tests.

2. Make a heavy suspension of the actively growing organism.

3. Add urea disk. (Tablets are already in tube.)

4. Incubate aerobically at 35°C for 1 to 24 h.

D. Gastric specimens

See procedure 3.8.4.

VI. INTERPRETATION

A. A positive test is development of an intense magenta to bright pink color in 15 min to 24 h.

B. A negative test shows no color change.

VII. REPORTING RESULTS

It is imperative that these cultures be handled in a biological safety cabinet.

A. This test will help differentiate among gram-negative rods that grow well on MAC and are likely members of the *Enterobacteriaceae.*

B. Urea-positive, oxidase-positive, gram-negative coccobacilli that do not grow on MAC in 24 h are presumptively identified as *Brucella*, unless they are isolated from urine. *Immediately transfer cultures to a biosafety cabinet.*

C. Urea-positive, oxidase-positive, gram-negative coccobacilli that are isolated from the urinary tract may be *Oligella ureolytica.*

D. Identify further any significant numbers of urea-positive corynebacteria from respiratory or urine specimens. See procedure 3.18.1.

E. Urea-positive, oxidase-positive, curved rods from gastric specimens are identified as *H. pylori* (7).

F. Use urea reaction as part of the identification of other microorganisms, following charts in procedure 3.18.2.

VIII. LIMITATIONS

A. Some organisms rapidly split urea *(Brucella* and *H. pylori),* while others react slowly.

B. When performing overnight tests from medium that contains peptone, the alkaline reaction may be due not to urease but to hydrolysis of peptone.

C. Urea is light sensitive and can undergo autohydrolysis. Store at 2 to 8°C in the dark.

D. The test is less sensitive if the medium is not buffered.

REFERENCES

1. **Abdalla, S., F. Marco, R. M. Perez, J. M. Pique, J. M. Bordas, M. T. Jimenez de Anta, and J. Teres.** 1989. Rapid detection of gastric *Campylobacter pylori* colonization by a simple biochemical test. *J. Clin. Microbiol.* **27:**2604–2605.
2. **Christensen, W. B.** 1946. Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. *J. Bacteriol.* **52:**461–466.
3. **Clinical and Laboratory Standards Institute.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Ederer, G. M., J. H. Chu, and D. J. Blazevic.** 1971. Rapid test for urease and phenylalanine deaminase production. *Appl. Microbiol.* **21:**545.
5. **Goldie, J., S. J. O. Veldhuyzen van Zanten, S. Jalali, J. Hollingsworth, R. H. Riddell, H. Richardson, and R. H. Hunt.** 1989. Optimization of medium for rapid urease test for detection of *Campylobacter pylori* in gastric antral biopsies. *J. Clin. Microbiol.* **27:**2080–2082.
6. **Mobley, H. L., M. D. Island, and R. P. Hausinger.** 1995. Molecular biology of microbial ureases. *Microbiol. Rev.* **59:**451–480.
7. **Owen, R. J., S. R. Martin, and P. Boman.** 1985. Rapid urea hydrolysis by gastric campylobacters. *Lancet* **i:**111.

3.18.1 Identification of Gram-Positive Bacteria

I. PRINCIPLE

Unlike gram-negative rods, it can be very difficult to sort out the identification of gram-positive cocci and rods. Many kits for staphylococcal identification have proved to be less sensitive than desired, and new DNA studies indicate that we have misidentified many streptococci. Gram-positive rods have been difficult to identify because there are hundreds of named species and thousands of genotypes or biochemical variants found in the environment and the normal microbiota of the human body, including skin, mucosal membranes, oropharynx, and genitourinary and gastrointestinal tracts. Thus, it is not within the scope of this handbook to identify all isolates, but to detect and identify the known pathogenic microorganisms in the human biosphere and to limit other identifications to those bacteria that are involved in disease from invasively collected specimens. The figures and tables that follow are designed to rapidly determine the agents of infection and to provide guidance for when to perform a kit identification or pursue other microorganisms. Because of the increasing microbial diversity and emergence of common pathogens having rare or unique phenotypic characteristics and the identification of new pathogens with incompletely defined phenotypes, more laboratories are relying on a combination of phenotypic and genotypic methods to report an accurate identification of many bacteria, so indications where molecular identification using DNA target sequencing may be useful are included (8, 32).

The charts may not include all the options at each step, since the observance of a colony morphology may lead to performing a test but the lack of that morphology may suggest doing a different test. The figures are designed to do the minimum to arrive at an excellent identification, without doing further tests unless the morphology warrants them. For species identification of viridans group streptococci, enterococci, and corynebacteria, commercial kits are easier to perform than standard tube biochemical tests (*see* Tables 3.16–1, 3.16–3, and 3.16–4) but are still limited in their accuracy. No system available can do as good a job as cell wall analysis and DNA studies, but most of these technologies remain beyond the scope of most small laboratories. For identification of other aerobic gram-positive rods in the actinomycete group, refer to section 6 of this handbook; for further information on *Actinomyces* spp., refer to section 4 and reference 7.

II. MICROORGANISMS TESTED

Gram-positive rods or cocci as determined by Gram stain.

◩ **NOTE:** Because of the lack of a unique colony morphology of many gram-positive microorganisms, the Gram stain *must* be performed on all isolates. Colonies of *Streptococcus* and *Aerococcus* may appear similar, but these organisms can be differentiated by the arrangement of the cells in the Gram stain. Group B streptococci and *Listeria* colonies may also have a similar appearance. Gram-variable microorganisms are considered to be gram positive. When in doubt, the Gram reaction enzymatic test (procedure 3.17.20) may be helpful.

III. MEDIA, REAGENTS, AND SUPPLIES

◩ **NOTE:** See individual tests in procedure 3.17 for methods for use of tests. To the extent that tests are available in kits, it is not necessary to stock the separate tests.

A. Media

1. Bile-esculin (procedure 3.17.5)
2. Glucans (optional) (procedure 3.17.19)
3. H_2S production medium (procedure 3.17.22)
4. Human blood agar for *Gardnerella*
5. Lipophilism test (procedure 3.17.28)
6. Ornithine and arginine decarboxylase (procedure 3.17.15)
7. 6.5% NaCl broth (procedure 3.17.43)

III. MEDIA, REAGENTS, AND SUPPLIES *(continued)*

8. Broth for motility (procedure 3.17.31)
9. Lecithinase agar (procedure 3.17.27)

B. Reagents and supplies

1. Catalase (3% H_2O_2) (procedure 3.17.10)
2. Polymyxin B (300 U), novobiocin (5 g), bacitracin (0.4 U), and vancomycin disks (procedure 3.17.4)
3. CAMP test (procedure 3.17.8)
4. Coagulase by rabbit plasma and (optionally) agglutination (procedures 3.17.13 and 3.17.14)
5. Spot bile reagent (procedure 3.17.6)
6. Hippurate (optional) (procedure 3.17.21)
7. Leucine aminopeptidase (LAP) (procedure 3.17.26)
8. Optochin disks (procedure 3.17.38)
9. Streptococcal grouping antisera (section 11)
10. Pyrrolidonyl-β-naphthylamide (PYR) (procedure 3.17.41)
11. Urea test (procedure 3.17.48)
12. Kits for identification of enterococci and viridans group streptococcus gram-positive and gram-positive rod identifications (corynebacterium and anaerobe kits) (Tables 3.16–1, 3.16–3, and 3.16–4)

IV. PROCEDURE

A. Observe colony morphology on BAP and CHOC if growth is lacking on BAP.

B. Perform catalase and Gram stain from BAP or CHOC.

◩ **NOTE:** To avoid misidentifications, do not skip this step.

C. Use Fig. 3.18.1–1 if the organism is a catalase-positive, gram-positive coccus.

D. Use Fig. 3.18.1–2 if organism is a beta-hemolytic, catalase-negative, gram-positive coccus.

E. Use Fig. 3.18.1–3 if organism is a catalase-negative, gram-positive coccus that is not hemolytic (except those with characteristic group B streptococcus morphology) and either is PYR negative or does not grow on BAP, except around staphylococci.

F. Proceed to Fig. 3.18.1–4 if the catalase-negative, gram-positive coccus is PYR positive and not identified from the other figures.

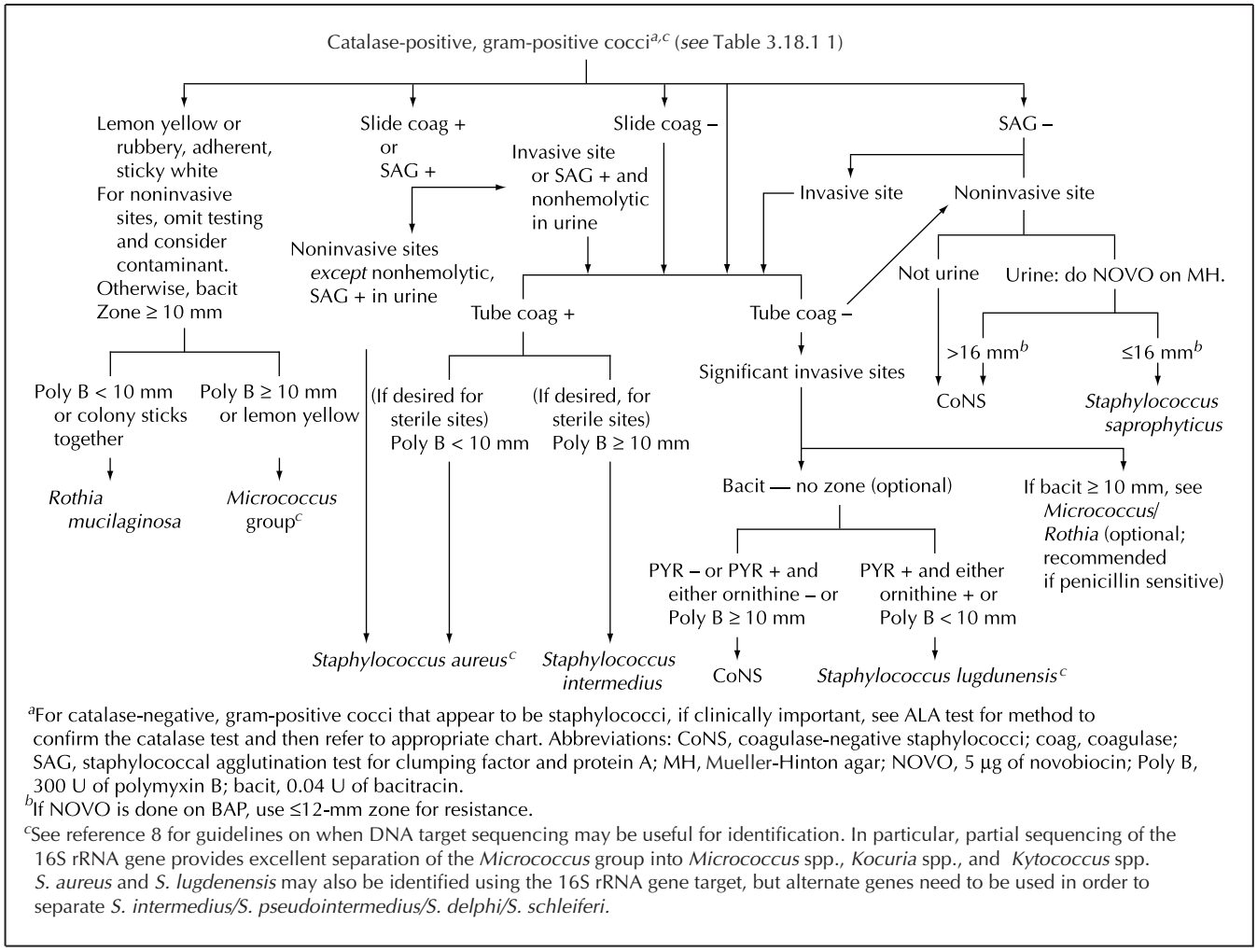
G. For gram-positive rods, proceed to Fig. 3.18.1–5.

1. If isolate is catalase positive
   - **a.** Perform CAMP test (and test for lipophilism, if microorganism demonstrates small colonies or poor growth at 24 h).
   - **b.** Check for motility by wet mount from young growth of either broth or agar cultures.
2. If isolate is catalase negative, follow Fig. 3.18.1–5 based on the anatomical site of isolation.

V. REPORTING AND INTERPRETATION OF RESULTS

A. Follow tables and kit identifications to report genus and species as appropriate without delay.

B. Use commercial kits for identification of *Enterococcus faecalis* and *Enterococcus faecium,* but perform motility testing on vancomycin-intermediate or -resistant *E. faecium.*


Catalase-positive, gram-positive cocci[a,c] (see Table 3.18.1 1)
Lemon yellow or rubbery, adherent, sticky white
For noninvasive sites, omit testing and consider contaminant.
Otherwise, bacit
Zone ≥ 10 mm
Slide coag + or SAG +
Noninvasive sites except nonhemolytic, SAG + in urine
Slide coag –
Invasive site or SAG + and nonhemolytic in urine
SAG –
Invasive site
Noninvasive site
Not urine
Urine: do NOVO on MH.
>16 mm[b]
≤16 mm[b]
CoNS
Staphylococcus saprophyticus
Tube coag +
Tube coag –
Significant invasive sites
Poly B < 10 mm or colony sticks together
Poly B ≥ 10 mm or lemon yellow
Rothia mucilaginosa
Micrococcus group[c]
(If desired for sterile sites) Poly B < 10 mm
(If desired, for sterile sites) Poly B ≥ 10 mm
Staphylococcus aureus[c]
Staphylococcus intermedius
Bacit — no zone (optional)
If bacit ≥ 10 mm, see Micrococcus/Rothia (optional; recommended if penicillin sensitive)
PYR – or PYR + and either ornithine – or Poly B ≥ 10 mm
PYR + and either ornithine + or Poly B < 10 mm
CoNS
Staphylococcus lugdunensis[c]

[a]For catalase-negative, gram-positive cocci that appear to be staphylococci, if clinically important, see ALA test for method to confirm the catalase test and then refer to appropriate chart. Abbreviations: CoNS, coagulase-negative staphylococci; coag, coagulase; SAG, staphylococcal agglutination test for clumping factor and protein A; MH, Mueller-Hinton agar; NOVO, 5 µg of novobiocin; Poly B, 300 U of polymyxin B; bacit, 0.04 U of bacitracin.

[b]If NOVO is done on BAP, use ≤12-mm zone for resistance.

[c]See reference 8 for guidelines on when DNA target sequencing may be useful for identification. In particular, partial sequencing of the 16S rRNA gene provides excellent separation of the *Micrococcus* group into *Micrococcus* spp., *Kocuria* spp., and *Kytococcus* spp. *S. aureus* and *S. lugdenensis* may also be identified using the 16S rRNA gene target, but alternate genes need to be used in order to separate *S. intermedius/S. pseudointermedius/S. delphi/S. schleiferi.*

Figure 3.18.1–1 Flowchart for identification of catalase-positive, gram-positive cocci.

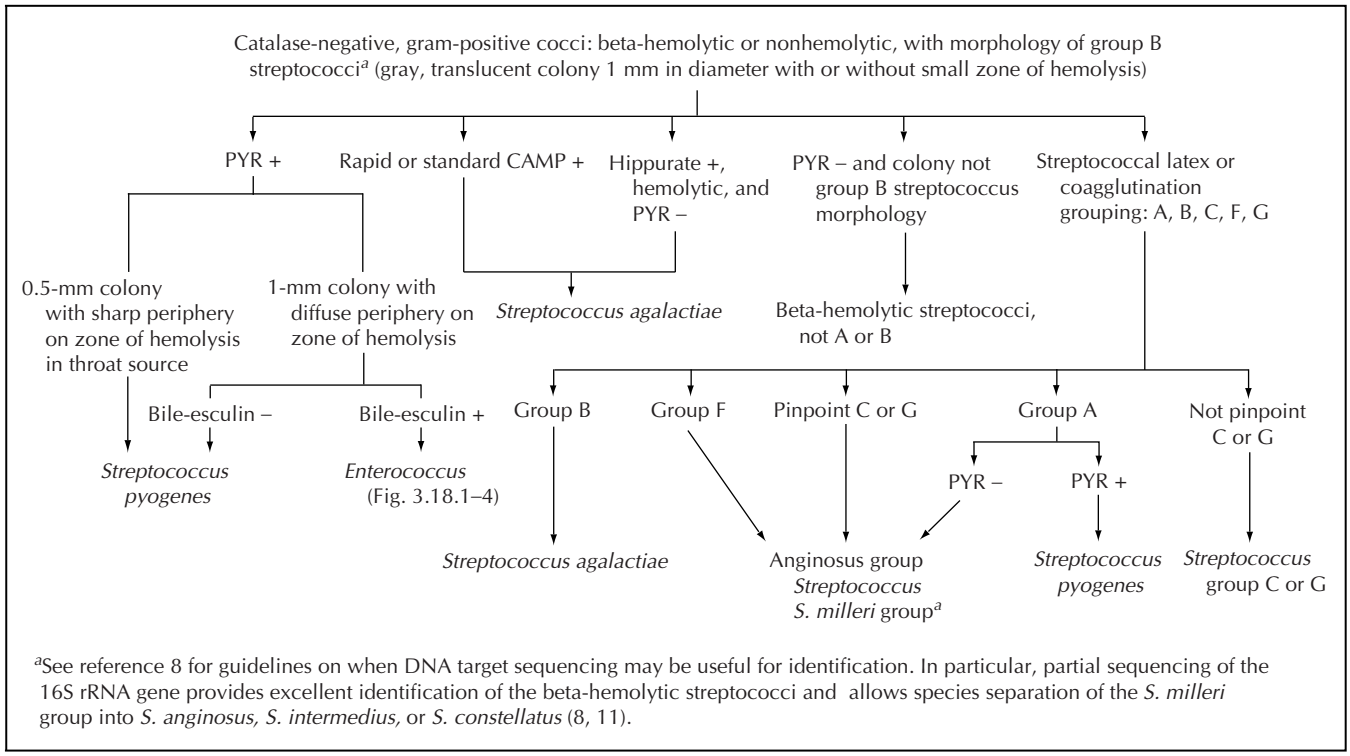
VI. LIMITATIONS

A. A number of gram-positive cocci are either coagulase or agglutination positive but are not *Staphylococcus aureus*, making identification problematic.

B. Some staphylococci are catalase-negative. See the aminolevulinic acid (ALA) test for options.

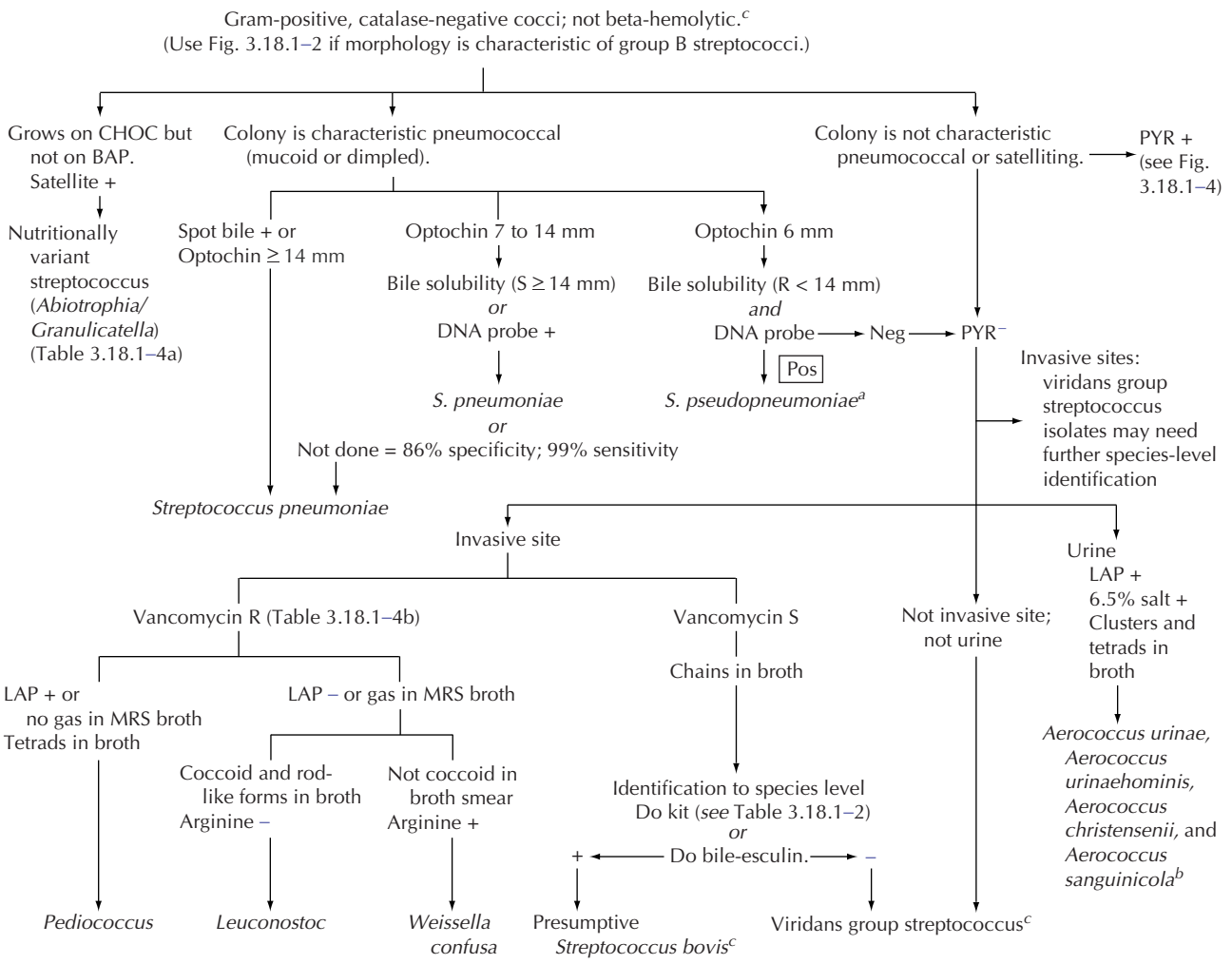
C. Streptococci are increasingly difficult to identify to the species level, even with commercial kits. The LAP test is important to at least confirm the genus of streptococci or enterococci.

D. Gram-positive rods are most difficult to identify. Every laboratorian should be able to recognize *Listeria monocytogenes*, *Erysipelothrix rhusiopathiae*, *Bacillus cereus*, *Arcanobacterium haemolyticum*, and *Gardnerella vaginalis* and be able to presumptively recognize *Bacillus anthracis* and *Corynebacterium diphtheriae*. Some other *Corynebacterium* species are identified using a commercial kit. For other gram-positive rods of significance, a reference laboratory is usually needed.


Catalase-negative, gram-positive cocci: beta-hemolytic or nonhemolytic, with morphology of group B streptococci[a] (gray, translucent colony 1 mm in diameter with or without small zone of hemolysis)
PYR +
Rapid or standard CAMP +
Hippurate +, hemolytic, and PYR –
PYR – and colony not group B streptococcus morphology
Streptococcal latex or coagglutination grouping: A, B, C, F, G
0.5-mm colony with sharp periphery on zone of hemolysis in throat source
1-mm colony with diffuse periphery on zone of hemolysis
Streptococcus agalactiae
Beta-hemolytic streptococci, not A or B
Bile-esculin –
Bile-esculin +
Group B
Group F
Pinpoint C or G
Group A
Not pinpoint C or G
Streptococcus pyogenes
Enterococcus (Fig. 3.18.1–4)
PYR –
PYR +
Streptococcus agalactiae
Anginosus group Streptococcus S. milleri group[a]
Streptococcus pyogenes
Streptococcus group C or G

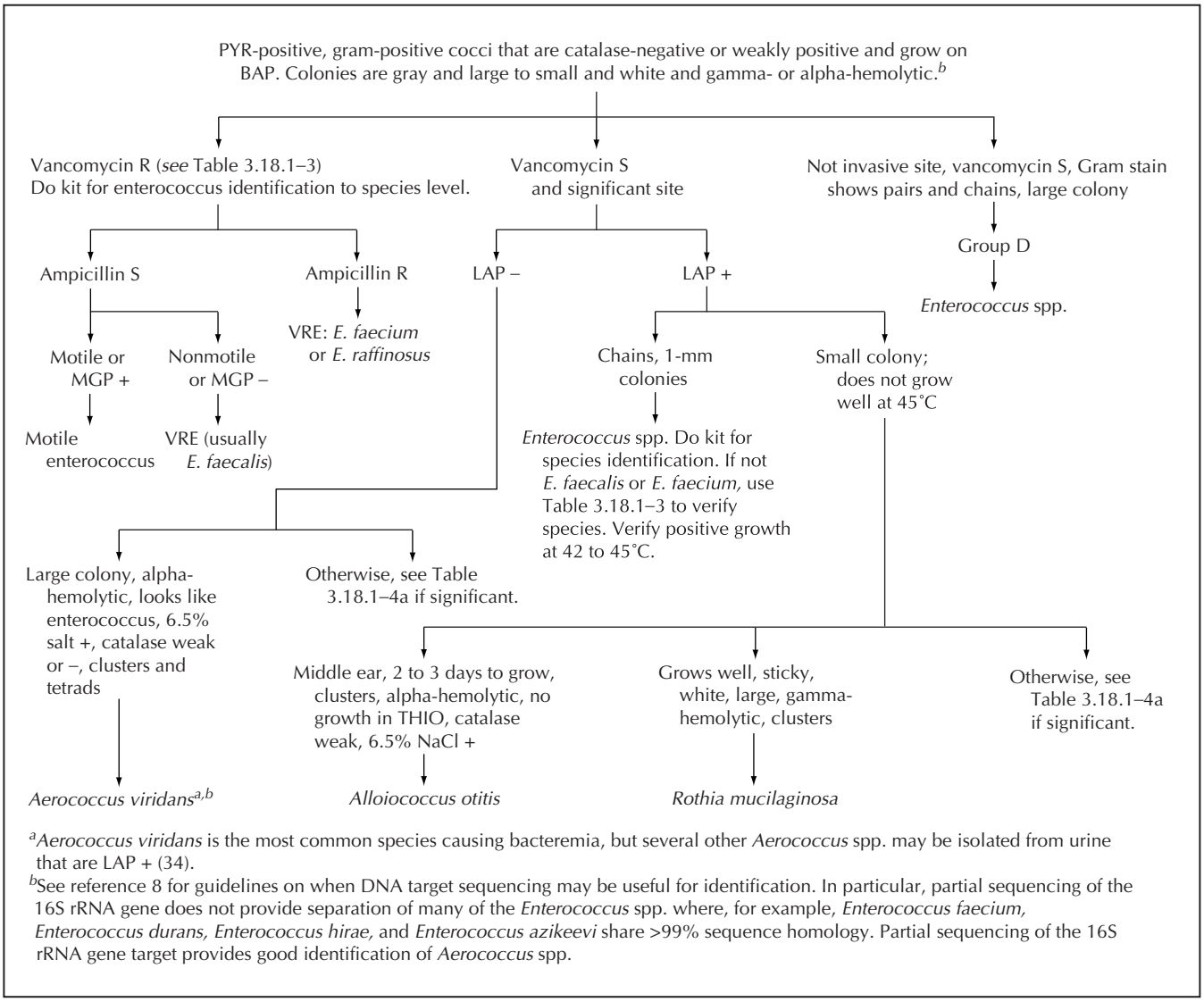
[a]See reference 8 for guidelines on when DNA target sequencing may be useful for identification. In particular, partial sequencing of the 16S rRNA gene provides excellent identification of the beta-hemolytic streptococci and allows species separation of the *S. milleri* group into *S. anginosus, S. intermedius,* or *S. constellatus* (8, 11).

Figure 3.18.1–2 Flowchart for identification of catalase-negative, gram-positive cocci, either beta-hemolytic or nonhemolytic, with morphology of group B streptococci.


Gram-positive, catalase-negative cocci; not beta-hemolytic.[c]
(Use Fig. 3.18.1–2 if morphology is characteristic of group B streptococci.)
Grows on CHOC but not on BAP. Satellite +
Nutritionally variant streptococcus (*Abiotrophia/ Granulicatella*) (Table 3.18.1–4a)
Colony is characteristic pneumococcal (mucoid or dimpled).
Spot bile + or Optochin ≥ 14 mm
Optochin 7 to 14 mm
Bile solubility (S ≥ 14 mm) *or* DNA probe +
S. pneumoniae *or* Not done = 86% specificity; 99% sensitivity
Optochin 6 mm
Bile solubility (R < 14 mm) *and* DNA probe
Neg
Pos
S. pseudopneumoniae[a]
Streptococcus pneumoniae
Colony is not characteristic pneumococcal or satelliting.
PYR +
(see Fig. 3.18.1–4)
PYR–
Invasive sites: viridans group streptococcus isolates may need further species-level identification
Invasive site
Vancomycin R (Table 3.18.1–4b)
LAP + or no gas in MRS broth
Tetrads in broth
Pediococcus
LAP – or gas in MRS broth
Coccoid and rod-like forms in broth
Arginine –
Leuconostoc
Not coccoid in broth smear
Arginine +
Weissella confusa
Vancomycin S
Chains in broth
Identification to species level
Do kit (*see* Table 3.18.1–2) *or*
Do bile-esculin.
+
Presumptive *Streptococcus bovis*[c]
–
Not invasive site; not urine
Viridans group streptococcus[c]
Urine
LAP +
6.5% salt +
Clusters and tetrads in broth
Aerococcus urinae, Aerococcus urinaehominis, Aerococcus christensenii, and *Aerococcus sanguinicola*[b]

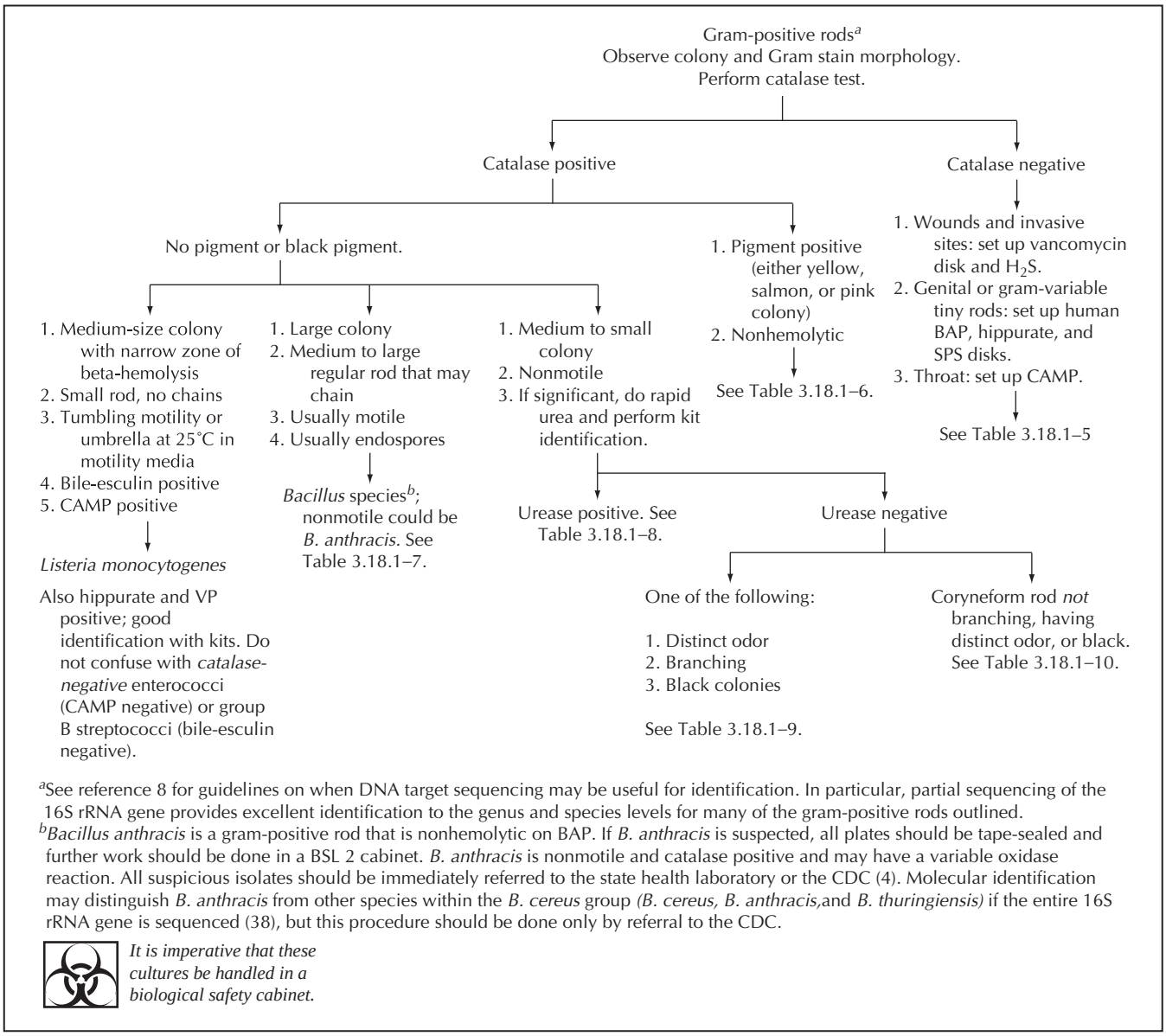
[a]See reference 1.
[b]See reference 34.
[c]See reference 8 for guidelines on when DNA target sequencing may be useful for identification. In particular, partial sequencing of the 16S rRNA gene does not provide separation of many of the species within the viridans streptococcus groups, including the *S. mitis* group, particularly *S. pneumoniae/S. pseudopneumoniae* and *S. sanguinis,* the *S. salivarius* group, and the *S. mutans* group (8). Partial sequencing of the 16S rRNA gene will allow separation of *S. bovis* biotypes I and II (6).

Figure 3.18.1–3 Flowchart for identification of gram-positive, catalase-negative cocci that are not beta-hemolytic. R, resistant; S, susceptible; Pos, positive; Neg, negative.


PYR-positive, gram-positive cocci that are catalase-negative or weakly positive and grow on BAP. Colonies are gray and large to small and white and gamma- or alpha-hemolytic.[b]
Vancomycin R (see Table 3.18.1–3) Do kit for enterococcus identification to species level.
Vancomycin S and significant site
Not invasive site, vancomycin S, Gram stain shows pairs and chains, large colony
Group D
Enterococcus spp.
Ampicillin S
Ampicillin R
LAP –
LAP +
VRE: E. faecium or E. raffinosus
Motile or MGP +
Nonmotile or MGP –
Chains, 1-mm colonies
Small colony; does not grow well at 45°C
Motile enterococcus
VRE (usually E. faecalis)
Enterococcus spp. Do kit for species identification. If not E. faecalis or E. faecium, use Table 3.18.1–3 to verify species. Verify positive growth at 42 to 45°C.
Large colony, alpha-hemolytic, looks like enterococcus, 6.5% salt +, catalase weak or –, clusters and tetrads
Otherwise, see Table 3.18.1–4a if significant.
Middle ear, 2 to 3 days to grow, clusters, alpha-hemolytic, no growth in THIO, catalase weak, 6.5% NaCl +
Grows well, sticky, white, large, gamma-hemolytic, clusters
Otherwise, see Table 3.18.1–4a if significant.
Aerococcus viridans[a,b]
Alloiococcus otitis
Rothia mucilaginosa

[a] *Aerococcus viridans* is the most common species causing bacteremia, but several other *Aerococcus* spp. may be isolated from urine that are LAP + (34).
[b] See reference 8 for guidelines on when DNA target sequencing may be useful for identification. In particular, partial sequencing of the 16S rRNA gene does not provide separation of many of the *Enterococcus* spp. where, for example, *Enterococcus faecium, Enterococcus durans, Enterococcus hirae,* and *Enterococcus azikeevi* share >99% sequence homology. Partial sequencing of the 16S rRNA gene target provides good identification of *Aerococcus* spp.

Figure 3.18.1–4 Flowchart for identification of PYR-positive, catalase-negative, gram-positive cocci. R, resistant; S, susceptible; VRE, vancomycin-resistant enterococci; MGP, methyl-α-D-glucopyranoside.


Gram-positive rods[a]
Observe colony and Gram stain morphology.
Perform catalase test.
Catalase positive
Catalase negative
No pigment or black pigment.
1. Pigment positive (either yellow, salmon, or pink colony)
2. Nonhemolytic
See Table 3.18.1–6.
1. Wounds and invasive sites: set up vancomycin disk and H_2S.
2. Genital or gram-variable tiny rods: set up human BAP, hippurate, and SPS disks.
3. Throat: set up CAMP.
See Table 3.18.1–5
1. Medium-size colony with narrow zone of beta-hemolysis
2. Small rod, no chains
3. Tumbling motility or umbrella at 25°C in motility media
4. Bile-esculin positive
5. CAMP positive
Listeria monocytogenes
Also hippurate and VP positive; good identification with kits. Do not confuse with catalase-negative enterococci (CAMP negative) or group B streptococci (bile-esculin negative).
1. Large colony
2. Medium to large regular rod that may chain
3. Usually motile
4. Usually endospores
Bacillus species[b]; nonmotile could be B. anthracis. See Table 3.18.1–7.
1. Medium to small colony
2. Nonmotile
3. If significant, do rapid urea and perform kit identification.
Urease positive. See Table 3.18.1–8.
Urease negative
One of the following:
1. Distinct odor
2. Branching
3. Black colonies
See Table 3.18.1–9.
Coryneform rod not branching, having distinct odor, or black. See Table 3.18.1–10.

[a]See reference 8 for guidelines on when DNA target sequencing may be useful for identification. In particular, partial sequencing of the 16S rRNA gene provides excellent identification to the genus and species levels for many of the gram-positive rods outlined.
[b]*Bacillus anthracis* is a gram-positive rod that is nonhemolytic on BAP. If *B. anthracis* is suspected, all plates should be tape-sealed and further work should be done in a BSL 2 cabinet. *B. anthracis* is nonmotile and catalase positive and may have a variable oxidase reaction. All suspicious isolates should be immediately referred to the state health laboratory or the CDC (4). Molecular identification may distinguish *B. anthracis* from other species within the *B. cereus* group *(B. cereus, B. anthracis,*and *B. thuringiensis)* if the entire 16S rRNA gene is sequenced (38), but this procedure should be done only by referral to the CDC.

It is imperative that these cultures be handled in a biological safety cabinet.

Figure 3.18.1–5 Guide to distinguish genera and significant species of gram-positive rods. VP, Voges-Proskauer. (Refer to section 6 for aerobic actinomycetes.)

Table 3.18.1–1 Key biochemical reactions of the common and/or significant gram-positive cocci that are catalase positive with large white to yellow colonies[a]

Organism(s)	Selected characteristic(s)	Slide coag	SAG	Tube coag	Bacit (0.04 U)	Poly B (300 U)	PYR[b]
Rothia mucilaginosa	Adherent, sticky				S	R	V
Micrococcus group[c]	Often yellow				S	S	V
S. aureus	VP +	V	+	+	R	R	−
S. intermedius (dogs)	VP −	V	V	+	R	S	+
Staphylococcus delphini (dolphins)	VP −	−	NA	+	R	NA	NA
Staphylococcus hyicus (pigs)	VP −	−	NA	V	R	R	−
S. lugdunensis	Ornithine +	V	V	−	R	R	+
S. schleiferi	Ornithine −	+	V	−+	R	S	+
S. saprophyticus	Urine; novo R; nonhemolytic	−	V	−	R	S	−
Staphylococcus epidermidis	Nonhemolytic	−	−	−	R	R	−
S. haemolyticus	Urease −, VP +, DNase −	−	−	−	R	S	+
Staphylococcus caprae	Urease +, DNase +	−	−	−	R	S	+
Other coagulase-negative staphylococci	Novo V, urease V; nonhemolytic or delayed hemolysis	−	V	−	R	S	V

[a] Symbols and abbreviations: +, greater than 90% of strains positive in 48 h; −, greater than 90% of strains negative; V, results are between 90 and 10% positive; −+, most strains are negative but rare positive strains exist; NA, not applicable or available; R, resistant; S, susceptible; Bacit, bacitracin; Poly B, polymyxin B; coag, coagulase; SAG, staphylococcal protein A or clumping factor agglutination; VP, Voges-Proskauer; Novo, novobiocin. Data are from references 2, 9, 16, 20, 21, 26, 31, and 40. Catalase can be weak for *Rothia.*

[b] PYR data are for broth test. Weak positive results with *S. aureus* ATCC 29213 and ATCC 25923 occur with the disk test, suggesting that this test is unreliable to separate *S. aureus* from *S. intermedius* (M. York, personal communication).

[c] Includes related taxa. The genus *Micrococcus* has been divided into additional genera, including *Kytococcus* and *Kocuria.*

Table 3.18.1–2 Separation of the common groups of viridans group streptococci isolated from human clinical specimens (PYR-negative, LAP-positive, 6.5% NaCl-negative cocci in chains)[a]

Group[b]	Species	VP	ARG	MAN	SOR	Esculin	Glucans	Hemolysis and comments
Mutans	*S. mutans, S. sobrinus, S. ratti, S. criceti*	+	−	+	+	V	+, puddles and droplets	α, β. Sometimes dry and adherent. *S. sobrinus* can be sorbitol negative; *S. ratti* is arginine positive.
Salivarius	*S. salivarius, S. vestibularis, S. thermophilus*	+	−	−	−	+	+, mucoid, firm	α, γ. *S. vestibularis* is α, VP and esculin variable, and glucan negative
Bovis	I	+	−	+	−	+	+, watery, spreads	γ. Bile-esculin positive (5). Taxonomic revisions suggest that human *S. bovis* I isolates be renamed as *S. gallolyticus,* and biotypes II/2 and II/1 have proposed species names *S. pasteurianus* and *S. infantarius,* respectively (5, 6, 14, 37)
	II/1	+	−	−	−	+	+, watery, spreads	
	II/2	+	−	−	−	+	−	
Anginosus ("*S. milleri*")	*S. anginosus, S. constellatus, S. intermedius*	+	+	−+	−	+−	−	α, β, γ. *S. constellatus* is divided into subspecies *constellatus* and *pharyngis.* All species are esculin positive, except that *S. constellatus* subsp. *constellatus* is esculin variable (41).
Mitis	*S. sanguinis, S. parasanguinis, S. gordonii, S. cristatus, S. peroris, S. intermedius*	−	+	−	V	+−	V, hard, adheres to agar	α. *S. cristatus* may be arginine and esculin negative. *S. parasanguinis* may be esculin negative. *S. sanguinis* may be sorbitol positive.
	S. mitis, S. oralis	−	−	−	−	−+	V	α. Can be pencillin resistant. *S. oralis* can be esculin positive.

[a] Abbreviations: ARG, hydrolysis of arginine; MAN, acid production from mannitol; SOR, acid production from sorbitol; +−, most strains positive but rare negative strains exist. See Table 3.18.1–1, footnote *a* for other abbreviations and symbols. Strains do not always produce glucans; test is only useful if positive. Commercial kits for identification of streptococci are helpful to resolve variable reactions. Data are extrapolated from reference 46. Also see references 10, 14, 35, and 41.

[b] Only the most commonly isolated species are included within each group; the other species within each group are listed here. *S. mutans* group species *(S. downei, S. ferus, S. macacae,* and *S. hyvaginalis)* have been found only in animals. The *S. salivarus* group includes *S. thermophilus,* which has been identified only from dairy products. *S. bovis* group species *(S. equnius, S. gallolyticus, S. infantarius,* and *S. alactolyticus)* are all human pathogens and are frequently isolated from blood cultures. *S. mitis* group species *(S. infantis* and *S. peroris)* have rarely been isolated from clinical specimens (22, 41).

Table 3.18.1–3 Common species of enterococci and related PYR-positive cocci in chains[a]

Organism	Motility[b]	Pigment[c]	MGP	Arginine dihydrolase	Arabinose	Mannitol	Lactose	Raffinose	Ribose	Sorbitol	45°C growth	Vancomycin
E. avium	−	−	V	−	+	+	+	−	+	+	+	S
E. raffinosus	−	−	V	−	+	+	+	+	+	+	+	V
Vagococcus fluvialis	+	−	+	−	−	+	−	−	+	+	−	S
E. faecium	−	−	−	+	+	+ −	+	V	+	V	+	V
E. gallinarum	+ −	−	+	+ −	+	+	+	+	+	D	+	R
E. casseliflavus	+ −	+ −	+	+ −	+	+	+	D	V	V	+	R
E. mundtii	−	+	−	+	+	+	+	+	+	V	+	S
E. faecalis[d]	−	−	−	+ −	−	+ −	+ [d]	−	+	+	+	V
Lactococcus garvieae	−	−	−	+	−	+	+	−	+	−	−	S
E. durans	−	−	−	+	−	−	+	−	+	−	+	S
E. hirae	−	−	−	+	−	−	+	+	+	−	+	S
E. dispar	−	−	+	+	−	−	+	+	+	−	−	S

[a] All species grow well on BAP and in 6.5% NaCl and are PYR, bile-esculin, and LAP positive. PYR-negative species *E. cecorum, E. columbae,* and *E. saccharolyticus* are not included and have not been isolated from humans. PYR-positive strains *E. malodoratus, E. pseudoavium, E. asini,* and *E. sulfureus* (H_2S^+) also are not listed since they have not been isolated from humans. *E. gilvus* and *E. pallens* have been described to occur in humans but are extremely rare. Abbreviations: MGP, methyl-α-D-glucopyranoside; D, different reactions in references. See footnote *a* to Tables 3.18.1–1 and 3.18.1–2 for other abbreviations and symbols. Table adapted from references 15, 36, and 44. Also see references 27, 28, and 43.

[b] Motility is done in 0.5 ml of BHI or TSB incubated at 30°C for 2 h.

[c] Pigment (yellow) is observed by swabbing a blood agar plate incubated at 35°C in 5% CO_2 for 24 to 48 h and observing swab for bright yellow color (+).

[d] Lactose-negative asaccharolytic *E. faecalis* exists.

Table 3.18.1–4a Biochemical reactions of PYR-positive, catalase-negative or weakly positive, gram-positive cocci (excluding *Streptococcus pyogenes*)

Genus or species[b]	Phenotypic characteristics[a]								
	Gram stain	CAT	LAP	NaCl	10°C	45°C	Colony on BAP	Hemolysis	Bile-esculin
Enterococccus (some motile)	CH	−	+	+	+	+	Large	α, γ, β	+
Lactococcus	CH	−	+	V	+	−	Large	α, γ	+
Vagococcus (motile)	CH	−	+	+	+	−	Large	α, γ	+
Abiotrophia/Granulicatella	CH	−	+	−	−	−	Satellite	α, γ	−
Globicatella	CH	−	−	+	−	−	Small	α	V
Dolosicoccus	CH	−	−	−	−	−	Small	α	NA
Aerococcus viridans	CL/T	−, W	−	+	−	−	Large	α	V
Helcococcus kunzii	CL/T	−	−	V	−	−	Tiny	γ	−
Gemella	CL/T/CH	−	V	−	−	−	Tiny, 48 h to grow	α, γ	−
Facklamia (hippurate +)	CL/CH	−	+	+	−	−	Small	γ	−
Alloiococcus otitis	CL/T	W, +	+	+	−	−	Tiny, 72 h to grow	α	NA
Ignavigranum (hippurate −)	CL/CH	−	+	+	−	−	Satellite (V) or small	γ	−
Rothia mucilaginosa	CL	−, W, +	+	−	NA	NA	Sticky	γ	V
Dolosigranulum	CL/T	−	+	+	−	−	Small	γ	NA

[a] CAT, catalase production; NaCl, growth in broth containing 6.5% NaCl; 10°C and 45°C, growth at 10 and 45°C, respectively (for the latter, use campylobacter incubator if heat block not available). Abbreviations for cell arrangement in Gram stain: CL, clusters; T, tetrads; CH, chains; W, weak. Large colonies are approximately 1 mm; small colonies are about the size of viridans group streptococci. See Table 3.18.1–1, footnote *a*, for other abbreviations and symbols. Tables adapted from references 13, 24, 29, and 35. Also see reference 9.

[b] See reference 8 for guidelines on when DNA target sequencing may be useful for identification. In particular, partial sequencing of the 16S rRNA gene provides excellent identification to the genus and species levels for some of these gram-positive cocci. *Abiotrophia/Granulicatella* can be separated only by molecular identification.

Table 3.18.1–4b Biochemical reactions of PYR-negative, catalase-negative gram-positive cocci

Genus or species	Phenotypic characteristics[a]						
	Gram stain	LAP	NaCl	Van	Arginine	45°C	MRS
Leuconostoc	CH, rods	−	V	R	−	−	+
Weissella confusa	CH, rods	−	V	R	+	+	+
Pediococcus	CL/T	+	V	R		V	−
Streptococcus	CH	+	−	S		V	−
Aerococcus urinae	CL/T	+	+	S		−	−

[a] Van, vancomycin; MRS, gas production in MRS broth. See footnote *a* to Table 3.18.1–4a and Table 3.18.1 for other abbreviations.

Table 3.18.1–5 Catalase-negative, gram-positive rods that can grow aerobically[a]

Organism(s)[b]	H_2S	Vancomycin	Hemolysis	Hippurate	Motility	Nitrate	Esculin	Gram stain morphology
Weissella spp.	−	R	Alpha	−	−	NA	+	Small, short GPR; gas in MRS broth (29)
Erysipelothrix rhusiopathiae	+	R	Alpha	−	−	−	−	Has two cell forms; the long-chaining form can be confused with *Lactobacillus,* and the short form can be confused with *Actinomyces* or even *Enterococcus* (PYR positive)
Lactobacillus spp.	−	R	Alpha	−	−	V	V	Most lactobacilli are long regular-chaining rods. Some are C shaped.
Arcanobacterium haemolyticum	−	S	Beta	−	−	−	−	Branching, which can be rudimentary; reverse-CAMP positive, lecithinase positive, gelatin negative
Arcanobacterium pyogenes	−	S	Beta	+	−	−	−	Branching, which can be rudimentary; reverse-CAMP negative, lecithinase negative, gelatin positive
Arcanobacterium bernardiae	−	S	V	V	−	−	−	Reverse-CAMP negative, gelatin negative; does not branch; not beta-hemolytic on human blood. Kits can misidentify as *Gardnerella.*
Gardnerella vaginalis	−	S	−	$+^-$	−	−	NA	SPS sensitive; beta-hemolytic on human blood
Bifidobacterium spp.	−	S	−	+	−	−	+	Some are aerotolerant; can look like *Actinomyces* or *Gardnerella;* not beta-hemolytic on human blood
Actinomyces israelii	−	S	−	NA	−	+	+	Branching, which can be rudimentary; anaerobic kits will identify; urease negative
Actinomyces spp.	−	S	−	V	−	V	V	Not all species show branching; *A. naeslundii* and others are urease positive. Some colonies of *A. meyerii-odontolyticus* group turn red after 1 wk.
Aerotolerant *Clostridium*	−	S	−	NA	V	$+^-$	+	Forms spores; medium to large regular rod; grows slowly compared to *Bacillus;* anaerobic kits will identify

[a] Data from references 3, 7, 12, and 33. Once *G. vaginalis*, *Arcanobacterium*, *Weissella*, and *E. rhusiopathiae* are ruled out, either call "Anaerobic gram-positive rod" or do anaerobic identification kit. GPR, gram-positive rods; SPS, sodium polyanethol sulfonate. See footnote *a* to Tables 3.18.1–1 and 3.18.1–2 for other abbreviations and symbols. See Table 3.18.1–9 for catalase-positive *Actinomyces.*

[b] See reference 8 for guidelines on when DNA target sequencing may be useful for identification. In particular, partial sequencing of the 16S rRNA gene and/or the *rpoB* gene for some genera provides excellent identification of most of these gram-positive rods.

Table 3.18.1–6 Catalase-positive, usually yellow- or pink-pigmented gram-positive rods[a]

Organism(s)[b]	Motility	Fermentation	Nitrate	Urease	Esculin	Gelatin	Glucose	Comment
Cellulosimicrobium/Cellulomonas (Oerskovia) spp.	+	+	+/V	−	+	+	+	Subsurface or surface hyphae/pseudohyphae may be present.
Microbacterium (Aureobacterium) spp.	+⁻	V	V	V	+⁻	V	+	
Exiguobacterium acetylicum	+	+	V	−	+	V	+	Golden yellow to orange
Leifsonia aquatica	V	−	V	−	V	V	+	Previously called "*Corynebacterium aquaticum*"
Corynebacterium falsenii	−	W	V	W	V	NA	W	Yellow after 72 h
Corynebacterium lipophiloflavum	−	+	−	W	−	−	−	Lipophilic, rarely isolated
Corynebacterium mucifaciens	−	−	−	−	−	−	+	Slightly to deep yellow mucoid colonies, CAMP negative
Rhodococcus equi	−	−	V	+	−	−	−	Mucoid in 48 h, usually pink after 4–7 days, can be yellowish, CAMP positive. Can be acid fast. Important pathogen.

[a] If motile and yellow or positive for esculin and/or gelatin, report as "Motile coryneform, not *Corynebacterium* spp." If of clinical significance, use kit (e.g., Coryne API or RapID Coryne) or send to reference laboratory. Other yellow *Corynebacterium* spp. *(C. aurimucosum, C. falsenii,* and *C. sanguinis)* are rarely isolated or associated with human disease. Data are from references 17, 18, 39, and 42; also see section 6. See footnote *a* to Tables 3.18.1–1, 3.18.1–2, and 3.18.1–3 for abbreviations and symbols.

[b] See reference 8 for guidelines on when DNA target sequencing may be useful for identification. In particular, partial sequencing of the 16S rRNA gene and/or the *rpoB* gene for some genera provides excellent identification of most of these gram-positive rods.

Table 3.18.1–7 Large, regular catalase-positive, gram-positive rods that usually produce spores and are usually motile[a]

Organism(s)[b]	Diam of cell usually above 1 μm	Motility[c]	Beta-hemolysis	Lecithinase	Penicillin	Large colony at 24 h	Sticky, tenacious colony at 24 h
B. anthracis	+	−	−	+	S[b]	+	+
B. cereus	+	+	+	+	R	+	−
Bacillus thuringiensis	+	+, V	+	+	R	+	−
Bacillus mycoides	+	−	W	+	R	+	−
Bacillus megaterium	+	+, V	V	−	S	+	−
Other *Bacillus* spp. and related groups[d]	−	+, V	−⁺	−	V	V	V

[a] *B. cereus, B. thuringiensis* (insect pathogen used in horticulture), *B. mycoides* (rhizoids or hairy projections in agar), and *B. anthracis* are included in the *B. cereus* group. If organism is motile with spores, penicillin resistant, hemolytic, with cells greater than 1 μm in diameter, and/or lecithinase positive, report as "*Bacillus cereus* group, not *B. anthracis.*" Otherwise, if organism is motile, with spores, but nonhemolytic and/or lecithinase negative, report as "*Bacillus,* not *B. anthracis* or *B. cereus.*" Data are from references 25 and 45. Spores can be induced by growing on urea, bile-esculin agar, or an agar plate with vancomycin disk or at 45°C. Spores can be proved by heating broth culture to 80°C for 10 min and subculturing to BAP. Viable colonies indicate that spores survived the heating. See Table 3.18.1–1, footnote *a,* for other abbreviations and symbols.

[b] See reference 8 for guidelines on when DNA target sequencing may be useful for identification. In particular, partial sequencing of the 16S rRNA gene and/or the *rpoB* gene for some genera provides excellent identification of most of these gram-positive rods.

[c] Submit any nonmotile, spore-forming strain to designated higher reference laboratory to rule out *B. anthracis,* regardless of the penicillin susceptibility.

[d] *Kurthia* (diameter, 0.8 to 1.2 μm) organisms are motile and nonhemolytic and do not produce spores (17).

Table 3.18.1–8 Urease-positive *Corynebacterium* spp. of clinical importance[a]

Organism[b]	Nitrate	Urease	Pyrazinamidase	Glucose	Sucrose	Lipophilism	CAMP reaction
C. glucuronolyticum	V	V	+	+	+	−	+
C. pseudotuberculosis[c]	V	+	−	+	V	−	Reverse +
C. ulcerans[c]	−	+	−	+	−	−	Reverse +
C. pseudodiphtheriticum[d]	−	+	+	−	−	−	−
C. riegelii[e]	−	+	V	−	−	−	−
C. urealyticum[f]	−	+	+	−	−	+	−
C. amycolatum	V	V	+	+	V	−	−
CDC group F1	V	+	+	+	+	+	−

[a] Data are from references 17 and 18. Other urease-positive or -variable species of less clinical significance include *C. durum, C. falsenii, C. singulare, C. sundsvallense,* and *C. thomssenii,* which are CAMP and reverse-CAMP negative, are not lypophilic, and ferment glucose. See Table 3.18.1–1, footnote *a*, for abbreviations and symbols.

[b] See reference 8 for guidelines on when DNA target sequencing may be useful for identification. In particular, partial sequencing of the 16S rRNA gene and/or the *rpoB* gene for some genera provides excellent identification of most of these gram-positive rods.

[c] Submit to reference laboratory for diphtheria toxin testing. *C. pseudotuberculosis* is associated with sheep handlers.

[d] Respiratory pathogen; not able to acidify maltose, ribose, or trehalose.

[e] *C. riegelii* is rarely isolated but has been found in urine and other body sites (17). It is able to acidify maltose.

[f] Urinary pathogen; multiresistant to antimicrobials.

Table 3.18.1–9 Catalase-positive, urease-negative, gram-positive rods, excluding *Corynebacterium* spp. and yellow- or pink-pigmented rods[a]

Organism(s)[b]	Fermentation	Nitrate	Esculin	Gelatin	Glucose	CAMP	Gram stain or colony appearance
Actinomyces neuii	+	V	−	−	+	+	Nonhemolytic, slight branching
Actinomyces viscosus	+	+	−	NA	+	−	
Propionibacterium avidium/granulosum	+	−	V	V	+	+	Beta-hemolytic, branching
Turicella otitidis	−	−	−	−	−	+	Large rod, branching; ear pathogen CAMP-positive *C. auris* and *C. afermentans* have similar reactions
Brevibacterium spp.	−	V	−	+	−	−	Some yellowish, distinct odor
Dermabacter hominis	+	−	+	+	+	−	Coccoid rods, distinct odor Lysine +, arginine −, ornithine +
Rothia spp.	+	+	+	V	+	−	Some branching, some black pigmented; if sticky, refer to Tables 3.18.1–1 and 3.18.1–4a for *R. mucilaginosa* (previously *Stomatococcus mucilaginosus*) (9).

[a] Usually irregular rods. Identify only if clinically significant, but all can be pathogens (17, 18). The important tests are fermentation (use Andrade's base or cysteine Trypticase agar), CAMP, and Gram stain, with careful reading of Gram stain morphology. Coryneform identification kits can be helpful. For abbreviations and symbols, see Table 3.18.1–1, footnote *a.*

[b] See reference 8 for guidelines on when DNA target sequencing may be useful for identification. In particular, partial sequencing of the 16S rRNA gene and/or the *rpoB* gene for some genera provides excellent identification of most of these gram-positive rods. Molecular identification is a more accurate method for identification and separation of the heterogeneity within *Actinomyces* spp. (19, 30).

Table 3.18.1–10 Urease-negative *Corynebacterium* spp. of clinical importance[a]

Organism(s)[b]	Nitrate	Urease	Pyrazinamidase	Alkaline phosphatase	Glucose	Maltose	Sucrose	Lipophilism	CAMP	Comment(s)
C. accolens	+	−	V	−	+	−	V	+	−	
CDC group G[c]	V	−	+	+	+	V	V	+	−	Fructose positive
C. jeikeium[c]	−	−	+	+	+	V	−	+	−	Fructose negative
C. afermentans	−	−	V	+	−	−	−	V	V	One subspecies is lipophilic.
C. macginleyi	+	−	−	+	+	−	+	+	−	Found in eye specimens.
C. diphtheriae[d]	+	−	−	−	+	+	−	−	−	*C. diphtheriae/belfanti* is nitrate negative; *C. diphtheriae/intermedius* is lipophilic.
C. propinquum	+	−	V	V	−	−	−	−	−	
C. amycolatum[c]	V	V	+	+	+	V	V	−	−	Dry colony, O/129 R, a common species of human resident microbiota; can be misidentified as *C. xerosis.*
C. minutissimum	−	−	+	+	+	+	V	−	−	O/129 S, DNase positive, PYR positive
C. striatum	+	−	+	+	+	−	V	−	V	O/129 S
C. xerosis	V	−	+	+	+	+	+	−	−	Creamy colony, O/129 S, LAP positive

[a] Other species that are rare are not listed (*see* references 17 and 18), including some CAMP test-positive species. Some *Corynebacterium* organisms have black-pigmented colonies. For identification of species in this table, the combination of CAMP test, lipophilism, O/129 disk, and commercial kits for corynebacteria should be used if identification is clinically important. For abbreviations and symbols, see footnote *a* to Tables 3.18.1–1 and 3.18.1–9.

[b] See reference 8 for guidelines on when DNA target sequencing may be useful for identification. In particular, partial sequencing of the 16S rRNA gene and/or the *rpoB* gene for some genera provides excellent identification of most *Corynebacterium* spp. (23).

[c] Multiresistant to antimicrobials.

[d] Submit to reference laboratory for diphtheria toxin testing.

REFERENCES

1. **Arbique, H. J. C., C. Poyart, P. Trieu-Cuot, G. Quesne, M. G. S. Carvalho, A. G. Steigerwalt, R. E. Morey, D. Jackson, R. J. Davidson, and R. R. Facklam.** 2004. Accuracy of phenotypic and genotypic testing for identification of *Streptococcus pneumoniae* and description of *Streptococcus pseudopneumoniae* sp. nov. *J. Clin. Microbiol.* **42:**4686–4696.
2. **Bannerman, T., and S. J. Peacock.** 2007. *Staphylococcus, Micrococcus,* and other catalase-positive cocci that grow aerobically, p. 390–411. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
3. **Bille, J.** 2007. *Listeria* and *Erysipelothrix,* p. 474–484. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
4. **Centers for Disease Control and Prevention.** 2001. *Basic Protocols for Level A Laboratories for the Presumptive Identification of* Bacillus anthracis. Centers for Disease Control and Prevention, Atlanta, GA. http://www.cdc.gov.
5. **Chuard, C., and L. B. Reller.** 1998. Bile-esculin test for presumptive identification of enterococci and streptococci: effects of bile concentration, inoculation technique, and incubation time. *J. Clin. Microbiol.* **36:**1135–1136.
6. **Clarridge, J. E., III, J. S. M. Attorri, Q. Zhang, and J. Bartell.** 2001. 16S ribosomal DNA sequence analysis distinguishes biotypes of *Streptococcus bovis: Streptococcus bovis* biotype II/2 is a separate genospecies and the predominant clinical isolate in adult males. *J. Clin. Microbiol.* **39:**1549–1552.
7. **Clarridge, J. E., III, and Q. Zhang.** 2002. Genotypic diversity of clinical *Actinomyces* species: phenotype, source, and disease correlation among genospecies. *J. Clin. Microbiol.* **40:**3442–3448.
8. **Clinical and Laboratory Standards Institute.** 2007. *Interpretive Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing.* Approved guideline MM18-1A. Clinical and Laboratory Standards Institute, Wayne, PA.

REFERENCES *(continued)*

9. **Collins, M. D., R. A. Hutson, V. Baverud, and E. Falsen.** 2000. Characterization of a *Rothia*-like organism from a mouse: description of *Rothia nasimurium* sp. nov. and reclassification of *Stomatococcus mucilaginosus* as *Rothia mucilaginosa* comb. nov. *Int. J. Syst. Evol. Microbiol.* **50:**1247–1251.
10. **Coykendall, A. L.** 1989. Classification and identification of the viridans streptococci. *Clin. Microbiol. Rev.* **2:**315–328.
11. **Daley, P., D. L. Church, D. B. Gregson, and S. Elsayed.** 2005. Species-level molecular identification of invasive "*Streptococcus milleri*" group clinical isolates by nucleic acid sequencing in a centralized regional microbiology laboratory. *J. Clin. Microbiol.* **43:**2987–2988.
12. **Dunbar, S. A., and J. E. Clarridge III.** 2000. Potential errors in the recognition of *Erysipelothrix rhusiopathiae*. *J. Clin. Microbiol.* **38:**1302–1304.
13. **Facklam, R., and J. A. Elliott.** 1995. Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clin. Microbiol. Rev.* **8:**479–495.
14. **Facklam, R.** 2002. What happened to the streptococci: overview of taxonomic and nomenclature changes. *Clin. Microbiol. Rev.* **15:**613–630.
15. **Facklam, R. R., and M. D. Collins.** 1989. Identification of *Enterococcus* species isolated from human infections by a conventional test scheme. *J. Clin. Microbiol.* **27:**731–734.
16. **Falk, D., and S. J. Guering.** 1983. Differentiation of *Staphylococcus* and *Micrococcus* spp. with the Taxo A bacitracin disk. *J. Clin. Microbiol.* **18:**719–721.
17. **Funke, G., and K. A. Bernard.** 2007. Coryneform gram-positive rods, p. 485–514. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
18. **Funke, G., A. von Graevenitz, J. E. Clarridge III, and K. Bernard.** 1997. Clinical microbiology of coryneform bacteria. *Clin. Microbiol. Rev.* **10:**125–159.
19. **Hall, V., G. L. O'Neill, J. T. Magee, and B. J. Duerden.** 2001. Identification of clinical isolates of *Actinomyces* species by amplified 16S ribosomal DNA restriction analysis. *J. Clin. Microbiol.* **39:**3555–3562.
20. **Hébert, G. A.** 1990. Hemolysins and other characteristics that help differentiate and biotype *Staphylococcus lugdunensis* and *Staphylococcus schleiferi*. *J. Clin. Microbiol.* **28:**2425–2431.
21. **Hébert, G. A., C. G. Crowder, G. A. Hancock, W. R. Jarvis, and C. Thornsberry.** 1988. Characteristics of coagulase-negative staphylococci that help differentiate these species and other members of the family *Micrococcaceae*. *J. Clin. Microbiol.* **26:**1939–1949.
22. **Kawamura, Y., X.-G. Hou, Y. Todome, F. Sultana, K. Hirose, S.-E. Shu, T. Ezaki, and H. Ohkuni.** 1998. *Streptococcus peroris* sp. nov. and *Streptococcus infantis* sp. nov., new members of the *Streptococcus mitis* group, isolated from human clinical specimens. *Int. J. Syst. Bacteriol.* **48:**921–927.
23. **Khamis, A., D. Raoult, and B. La Scola.** 2004. *rpoB* gene sequencing for identification of *Corynebacterium* species. *J. Clin. Microbiol.* **42:**3925–3931.
24. **LaClaire, L. L., and R. R. Facklam.** 2000. Comparison of three commercial rapid identification systems for the unusual gram-positive cocci *Dolosigranulum pigrum*, *Ignavigranum ruoffiae*, and *Facklamia* species. *J. Clin. Microbiol.* **38:**2037–2042.
25. **Logan, N.A., T. Popovic, and A. Hoffmaster.** 2007. *Bacillus* and other aerobic endospore-forming bacteria, p. 455–473. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
26. **Mahoudeau, I., X. Delabranche, G. Prevost, H. Monteil, and Y. Piemont.** 1997. Frequency of isolation of *Staphylococcus intermedius* from humans. *J. Clin. Microbiol.* **35:**2153–2154.
27. **Manero, A., and A. R. Blanch.** 1999. Identification of *Enterococcus* spp. with a biochemical key. *Appl. Environ. Microbiol.* **65:**4425–4430.
28. **Murray, B. E.** 1990. The life and times of the *Enterococcus*. *Clin. Microbiol. Rev.* **3:**46–65.
29. **Olano, A., J. Chua, S. Schroeder, A. Minari, M. La Salvia, and G. Hall.** 2001. *Weissella confusa* (basonym: *Lactobacillus confusus*) bacteremia: a case report. *J. Clin. Microbiol.* **39:**1604–1607.
30. **Patel, J. B., R. J. Wallace, Jr., B. A. Brown-Elliott, T. Taylor, C. Imperatrice, D. G. B. Leonard, R. W. Wilson, L. Mann, K. C. Jost, and I. Nachamkin.** 2004. Sequence-based identification of aerobic actinomycetes. *J. Clin. Microbiol.* **42:**2530–2540.
31. **Patel, R., K. E. Piper, M. S. Rouse, J. R. Uhl, F. R. Cockerill III, and J. M. Steckelberg.** 2000. Frequency of isolation of *Staphylococcus lugdunensis* among staphylococcal isolates causing endocarditis: a 20-year experience. *J. Clin. Microbiol.* **38:**4262–4263.
32. **Petti, C. A., C. R. Polage, and P. Schreckenberger.** 2005. The role of 16S rRNA gene sequencing in identification of microorganisms misidentified by conventional methods. *J. Clin. Microbiol.* **43:**6123–6125.
33. **Reimer, L. G., and L. B. Reller.** 1985. Use of a sodium polyanetholesulfate disk for the identification of *Gardnerella vaginalis*. *J. Clin. Microbiol.* **21:**146–149.

REFERENCES *(continued)*

34. **Ruoff, K.** 2007. *Aerococcus, Abiotrophia,* and other aerobic catalase-negative, gram-positive cocci, p. 443–454. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
35. **Ruoff, K. L.** 2002. Miscellaneous catalase–negative, gram-positive cocci: emerging opportunists. *J. Clin. Microbiol.* **40:**1129–1133.
36. **Ruoff, K. L., L. de la Maza, M. J. Murtagh, J. D. Spargo, and M. J. Ferraro.** 1990. Species identities of enterococci isolated from clinical specimens. *J. Clin. Microbiol.* **28:**435–437.
37. **Ruoff, K. L., S. I. Miller, C. V. Garner, M. J. Ferraro, and S. B. Calderwood.** 1989. Bacteremia with *Streptococcus bovis* and *Streptococcus salivarius:* clinical correlates of more accurate identification of isolates. *J. Clin. Microbiol.* **27:**305–308.
38. **Sacchi, C. T., A. M. Whitney, L. W. Mayer, R. Morey. A. Steigerwalt, A. Boras. R. S. Weyant, and T. Popovic.** 2002. Sequencing of 16S rRNA gene: a rapid tool for identification of *Bacillus anthracis. Emerg. Infect. Dis.* **8:**1117–1123.
39. **Schumann, P., N. Weiss, and E. Stackebrandt.** 2001. Reclassification of *Cellulomonas cellulans* (Stackebrandt and Keddie 1986) as *Cellulosimicrobium cellulans* gen. nov., comb. nov. *Int. J. Syst. Evol. Microbiol.* **51:**1007–1010.
40. **Shuttleworth, R., R. J. Behme, A. McNabb, and W. D. Colby.** 1997. Human isolates of *Staphylococcus caprae:* association with bone and joint infections. *J. Clin. Microbiol.* **35:**2537–2541.
41. **Spellerberg, B., and C. Brandt.** 2007. *Streptococcus*, p. 412–429. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
42. **Takeuchi, M., and K. Hatano.** 1998. Union of the genera *Microbacterium* Orla-Jensen and *Aureobacterium* Collins et al. in a redefined genus *Microbacterium. Int. J. Syst. Bacteriol.* **48:**739–747.
43. **Teixeira, L. M., M. G. Carvalho, V. L. Merquior, A. G. Steigerwalt, D. J. Brenner, and R. R. Facklam.** 1997. Phenotypic and genotypic characterization of *Vagococcus fluvialis*, including strains isolated from human sources. *J. Clin. Microbiol.* **35:**2778–2781.
44. **Teixeiria, L. M., M. G. S. Carvalho, and R. R. Facklam.** 2007. *Enterococcus*, p. 430–442. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
45. **Turnbull, P. C. B., and J. M. Kramer.** 1991. *Bacillus*, p. 296–303. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), *Manual of Clinical Microbiology*, 5th ed. American Society for Microbiology, Washington, DC.
46. **Whiley, R. A., and D. Beighton.** 1998. Current classification of the oral streptococci. *Oral Microbiol. Immunol.* **13:**195–216.

3.18.2 Identification of Gram-Negative Bacteria

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The gram-negative bacteria are a heterogeneous group of organisms consisting of numerous families, genera, and species, many of which cause serious disease and endotoxic shock. The accurate, rapid identification of gram-negative rods and diplococci provides timely, meaningful treatment and diagnosis of disease. Lack of identification or recognition of a serious pathogen can have long-term effects on the cure and spread of disease. The figures and tables that follow are designed to rapidly determine the agent of infection and to provide guidance in order to avoid misidentification of serious pathogens.

The algorithms presented include a combination of rapid tests for identification, some of which are based on Clinical and Laboratory Standards Institute (formerly NCCLS) guidelines for rapid identification (9) and others of which are based on established references on identification of gram-negative rods (12, 17, 22, 27, 31; G. L. Gilardi, unpublished identification tables). The use of commercial automated and manual multitest systems (kits) is encouraged when colony morphology and a few rapid tests do not provide a definitive identification. Commercial kit systems do an excellent job of identifying most pathogens with minimal work, and they should be a part of any laboratory's identification system. Deciding when to use a commercial system and which system to use for identification is key to success in providing the identification in a timely manner. In addition, knowing the limitations of the databases of the kit systems can be helpful in deciding when to pursue the identification further using the tests listed in the tables that follow. Commercial kit systems are presented in Tables 3.16–1 and 3.16–2 and are also reviewed elsewhere (11, 22). These references should be consulted when deciding which kits to purchase and what are the limitations of the databases. In the algorithms presented here, specific manufacturers' kits are not presented. Because of the increasing microbial diversity and emergence of common pathogens having rare or unique phenotypic characteristics and the identification of new pathogens with incompletely defined phenotypes, more laboratories are relying on a combination of phenotypic and genotypic methods to report an accurate identification of many bacteria, so indications where molecular identification using DNA target sequencing may be useful have been included (9, 24).

The great majority of gram-negative strains found in clinical specimens are either *Escherichia coli* or *Pseudomonas aeruginosa.* The identification schemes that follow include rapid identification of these two pathogens, when they produce typical colony morphologies and reactions. The figures also show the identification of many of the gram-negative rod agents of bioterrorism in less than 2 h (refer to section 16). The figures and tables are designed to prevent misidentifications of agents of bioterrorism, as well as many other gram-negative rods which cause life-threatening infections.

The figures that follow include rapid tests (oxidase, indole, and catalase) that lead the user to the appropriate table or definitive identification, when combined with colony morphology. Most are performed in 2 min. The figures are designed to do the minimum amount of work to arrive at a good identification or lead to the appropriate table or kit, without doing tests that are not helpful. For that reason, the figures may not include all of the options at each step, because the observance of one colony morphology may lead to performing a certain test, while the lack of that morphology may suggest a different test. The figures send the reader to tables which include many organisms that may not be clinically significant. These organisms are listed to avoid misidentifications and to provide biochemical reactions for identification, should they be isolated from a significant site. Finally, the reader is referred to other texts (22, 31) for a more extensive list of tests when the minimal tests indicate what microorganism is suspected but other testing might be helpful to confirm the identification.

An example of using the figures may be helpful. If a nonpigmented respiratory isolate did not grow on MAC but grew at 48 h on BAP, Fig. 3.18.2–3 indicates performing Gram stain, indole, catalase, and oxidase. If the catalase is positive and the oxidase and indole are negative, the figure indicates testing with a polymyxin B disk and consulting Table 3.18.2–5. In Table 3.18.2–5 there are only five microorganisms listed that are oxidase negative or variable, and only one of those is a respiratory coccobacillus. In our case, the Gram stain showed the coccobacillary morphology. A positive rapid (2-h) urea test will complete a preliminary identification of *Bordetella parapertussis.* Inoculation to Mueller-Hinton (MH) agar with

a polymyxin B disk for observation of the brown pigment and susceptibility to polymyxin B would be confirmatory and rule out the rare possibility of *Burkholderia mallei*, the agent of glanders (potential bioterrorism agent). However, the report would have already been communicated to the caregiver for appropriate treatment and isolation techniques. Thus, *B. parapertussis* was identified to the species level in less than 2 h with three <1-min tests, a Gram stain, and one 2-h biochemical test. To have performed a commercial identification system on this isolate would have delayed the final identification and resulted in needless cost and erroneous results, since this pathogen is not in the databases of commonly used identification systems. The user is encouraged to follow the charts to arrive at appropriate testing and rapid reporting, often without the need for using more expensive commercial kits.

II. MICROORGANISMS

A. Gram-negative rods or cocci as determined by Gram stain

B. Assumed gram-negative rods because of colony morphology or growth on MAC or EMB

III. REAGENTS AND SUPPLIES

◩ **NOTE:** Procedures for methods for biochemical tests are listed in procedures 3.17.1 through 3.17.48. To the extent that tests are available in kits, it is not necessary to stock the separate tests.

A. Minimum useful for most laboratories

1. Catalase (3 and 30%) (procedure 3.17.10)
2. Indole (spot, Kovács', and Ehrlich's) (procedure 3.17.23)
3. Oxidase test reagent (procedure 3.17.39)
4. Polymyxin B (300-U) (or colistin [10-µg]) and penicillin (10-U) disks (procedure 3.17.4)
5. MH agar
6. Butyrate disks (procedure 3.17.7)
7. δ-Aminolevulinic acid (ALA) test (procedure 3.17.3)
8. Pyrrolidonyl-β-naphthylamide (PYR) (procedure 3.17.41)
9. Triple sugar iron agar (TSI) or Kligler's iron agar (KIA) (procedure 3.17.25)
10. Urea—rapid method preferred (procedure 3.17.48)
11. Motility medium—wet mount may suffice (procedure 3.17.31)
12. Kit for *Neisseria* identification (*see* procedure 3.9.3 for options)
13. Kit(s) for identification of enteric and nonfermenting, gram-negative rods and fastidious other gram-negative rods (Tables 3.16–1 and 3.16–2)

B. Desired for large-volume and reference laboratories and for those laboratories that provide service to cystic fibrosis patients

1. 4-Methylumbelliferyl-β-D-glucuronide (MUG) (procedure 3.17.34)
2. Andrade's glucose broth with rabbit serum (procedure 3.17.9)
3. MH agar with 4% salt (procedure 3.17.43)
4. O/129 150-µg disks (procedure 3.17.36)
5. Acetamide or F and P agar for *Pseudomonas* (procedures 3.17.1 and 3.17.17)
6. Gelatin (procedure 3.17.18)
7. Lysine, ornithine, arginine decarboxylase-dihydrolase broth (Møller's) (procedure 3.17.15)
8. OF medium (procedure 3.17.9)
9. Nitrate medium (procedure 3.17.35)
10. DNase (procedure 3.17.16)
11. Esculin (procedure 3.17.5)
12. Phenylalanine deaminase (PDA) (procedure 3.17.40)
13. Sugar fermentation media or commercial kit with sugar fermentation tests for fastidious microorganisms (procedure 3.17.9)

ANALYTICAL CONSIDERATIONS

IV. PROCEDURE

A. Observe colony morphology on BAP, CHOC, and MAC or EMB.

B. Perform oxidase from BAP or CHOC.

◩ **NOTE:** To avoid misidentifications, do not skip this step. Omit only for obvious spreading *Proteus* or satelliting colonies.

IV. PROCEDURE *(continued)*

C. If microorganism is not growing on MAC, perform Gram stain and catalase test.

◩ **NOTE:** With rare exceptions, organisms that grow on MAC are catalase-positive, gram-negative rods.

D. Follow Fig. 3.18.2–1 if the organism is a diplococcus.

E. Proceed to Fig. 3.18.2–2 if the organism is a gram-negative rod that does not grow on BAP.

F. Perform spot indole test on gram-negative rods that are growing on BAP. For organisms that grow poorly on MAC, performing the indole test with Kovács' reagent may be necessary. For organisms that are yellow, perform the tube indole test with Ehrlich's method.

◩ **NOTE:** *To avoid misidentifications, do not skip this step.*

G. Proceed to Fig. 3.18.2–3 for microorganisms that grow on BAP but do not grow or grow poorly on MAC.

1. In order to determine if a gram-negative rod ferments glucose, inoculate TSI or KIA.

◩ **NOTE:** Many organisms that do not grow on MAC will not show fermentation in OF media, which leads to erroneous identifications. Users may skip this step if they have the experience to recognize that a specific colony morphology represents a suspected nonfermenting rod. For example, many technologists can recognize colonies of *P. aeruginosa* or *Stenotrophomonas maltophilia* and go directly to Fig. 3.18.2–6.

2. To see if a nonfermenting rod is an oxidizer, inoculate OF medium or use a commercial test system designed to identify nonfermenting rods.

H. Follow Fig. 3.18.2–4 to identify organisms that grow on MAC and are either oxidase negative or oxidase positive and indole positive.

I. Go to Fig. 3.18.2–5 and 3.18.2–6 sequentially to identify oxidase-positive, indole-negative rods or oxidase-negative, nonfermenting rods that grow on MAC.

J. Observe plates at 48 h for colonies that were not present the first day of incubation. This step is particularly important for respiratory specimens.

POSTANALYTICAL CONSIDERATIONS

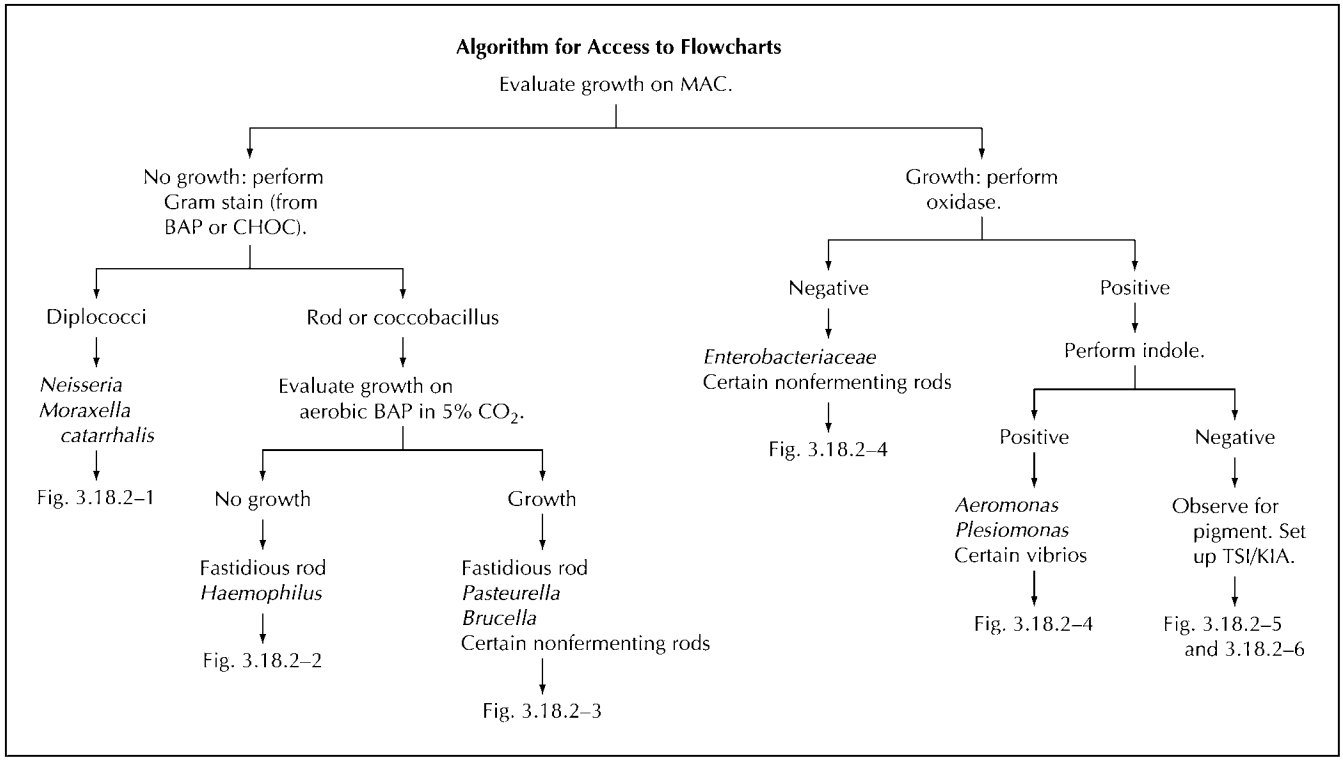
V. REPORTING AND INTERPRETATION OF RESULTS

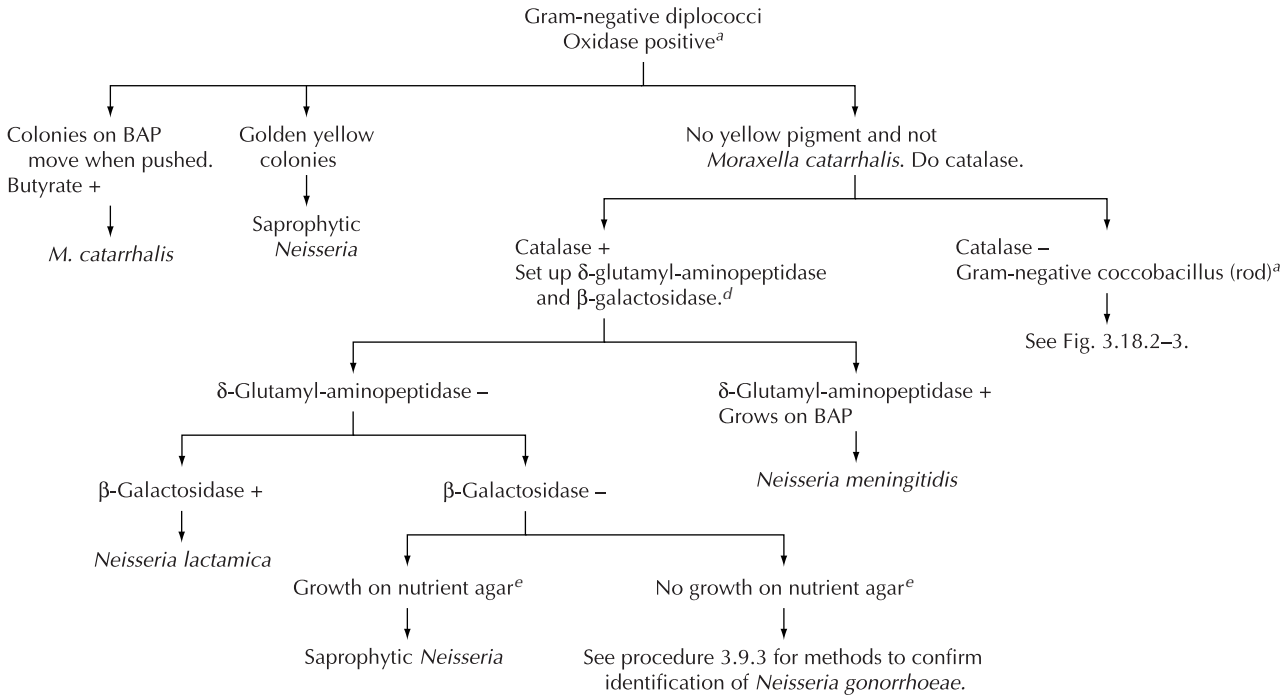
A. Follow tables and kit identifications to report genus and species as appropriate without delay.

B. Significant organisms are noted in the flowcharts to alert the user. Less significant microorganisms can be reported with minimal testing (e.g., yellow pseudomonad).

VI. LIMITATIONS

Because many tests are not 100% positive or negative for a particular strain, it is desirable that more than one positive reaction be used in the confirmation of an identification.


Algorithm for Access to Flowcharts
Evaluate growth on MAC.
No growth: perform Gram stain (from BAP or CHOC).
Growth: perform oxidase.
Diplococci
Rod or coccobacillus
Negative
Positive
Neisseria
Moraxella
catarrhalis
Fig. 3.18.2–1
Evaluate growth on aerobic BAP in 5% CO_2.
Enterobacteriaceae
Certain nonfermenting rods
Fig. 3.18.2–4
Perform indole.
No growth
Growth
Positive
Negative
Fastidious rod
Haemophilus
Fig. 3.18.2–2
Fastidious rod
Pasteurella
Brucella
Certain nonfermenting rods
Fig. 3.18.2–3
Aeromonas
Plesiomonas
Certain vibrios
Fig. 3.18.2–4
Observe for pigment. Set up TSI/KIA.
Fig. 3.18.2–5 and 3.18.2–6



[a] All gram-negative diplococci that grow aerobically are oxidase positive. When there is a question regarding whether an isolate is a coccus or coccobacillus, add a 10-U penicillin disk to the agar plate after streaking with the isolate. After incubation, prepare a Gram stain from growth around the disk. *Neisseria* and *M. catarrhalis* retain the coccoid forms, but other *Moraxella* and *Acinetobacter* organisms demonstrate long rods and filaments (5).

[b] See reference 9 for guidelines on when DNA target sequencing may be useful for identification. In particular, partial sequencing of the 16S rRNA gene provides excellent identification for some genera of oxidase-positive, gram-negative diplococci (25).

[c] Plates should be tape-sealed when *N. meningitidis* is suspected from all types of clinical specimens. All further work should be done in a BSL 2 cabinet. Laboratory staff at risk of acquiring *N. meningitidis* infection should receive meningococcal vaccine (*see* CDC adult immunization schedule [http://www.cdc.gov]).

[d] Sources of δ-glutamyl-aminopeptidase and β-galactosidase include Gonochek, Neisseria Screen, BactiCard, RapID NH, and NET. See procedure 3.9.3 for vendors.

[e] Nutrient agar includes MH agar and TSA.

Figure 3.18.2–1 Identification scheme for gram-negative diplococci; also see Table 3.18.2–1.

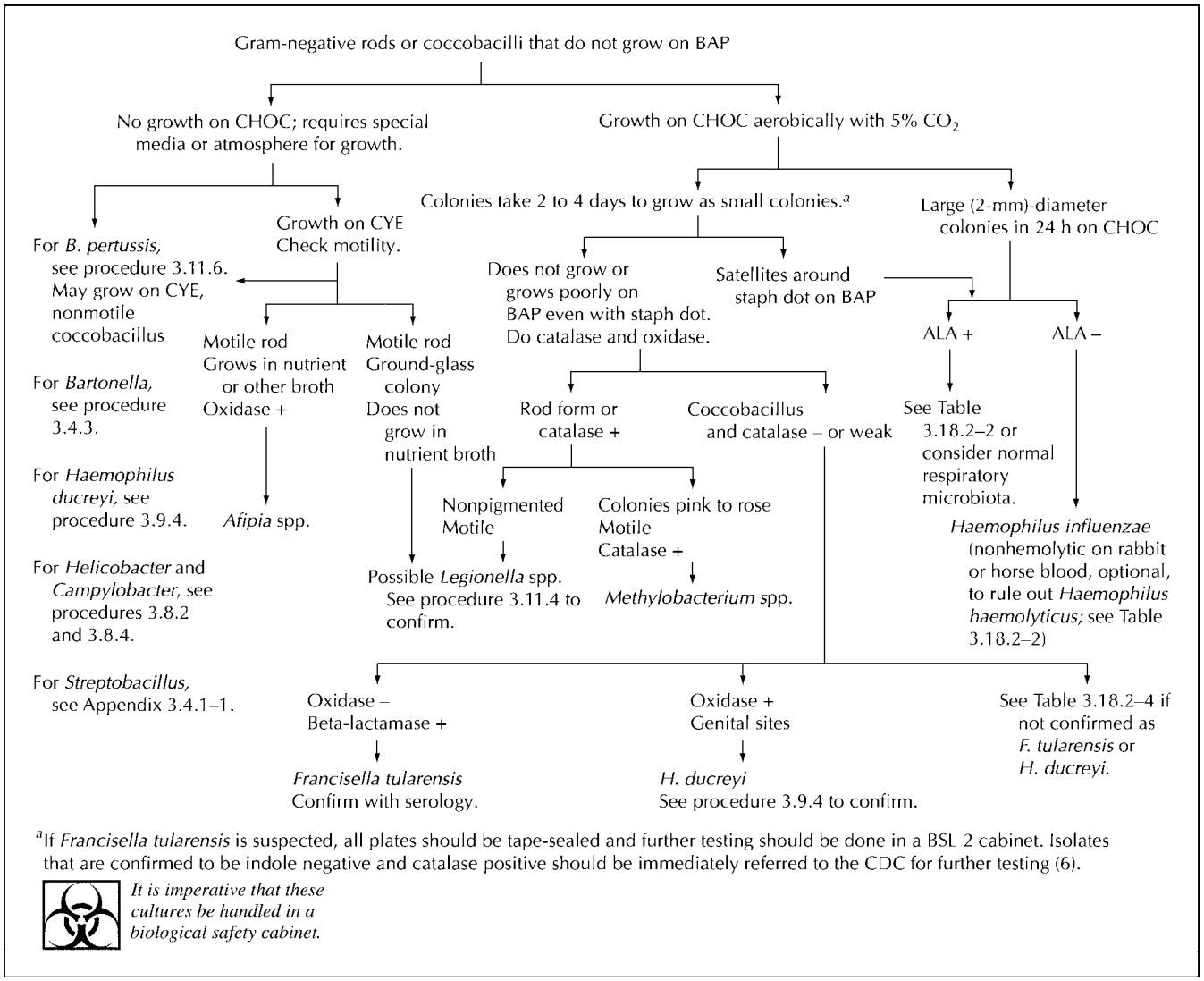

Gram-negative rods or coccobacilli that do not grow on BAP
No growth on CHOC; requires special media or atmosphere for growth.
Growth on CHOC aerobically with 5% CO2
For B. pertussis, see procedure 3.11.6. May grow on CYE, nonmotile coccobacillus
Growth on CYE Check motility.
Colonies take 2 to 4 days to grow as small colonies.a
Large (2-mm)-diameter colonies in 24 h on CHOC
For Bartonella, see procedure 3.4.3.
Motile rod Grows in nutrient or other broth Oxidase +
Motile rod Ground-glass colony Does not grow in nutrient broth
Does not grow or grows poorly on BAP even with staph dot. Do catalase and oxidase.
Satellites around staph dot on BAP
ALA +
ALA –
For Haemophilus ducreyi, see procedure 3.9.4.
Afipia spp.
Rod form or catalase +
Coccobacillus and catalase – or weak
See Table 3.18.2–2 or consider normal respiratory microbiota.
Nonpigmented Motile
Colonies pink to rose Motile Catalase +
Haemophilus influenzae (nonhemolytic on rabbit or horse blood, optional, to rule out Haemophilus haemolyticus; see Table 3.18.2–2)
For Helicobacter and Campylobacter, see procedures 3.8.2 and 3.8.4.
Possible Legionella spp. See procedure 3.11.4 to confirm.
Methylobacterium spp.
For Streptobacillus, see Appendix 3.4.1–1.
Oxidase – Beta-lactamase +
Oxidase + Genital sites
See Table 3.18.2–4 if not confirmed as F. tularensis or H. ducreyi.
Francisella tularensis Confirm with serology.
H. ducreyi See procedure 3.9.4 to confirm.
aIf Francisella tularensis is suspected, all plates should be tape-sealed and further testing should be done in a BSL 2 cabinet. Isolates that are confirmed to be indole negative and catalase positive should be immediately referred to the CDC for further testing (6).
It is imperative that these cultures be handled in a biological safety cabinet.

Figure 3.18.2–2 Identification scheme for gram-negative rods that do not grow on BAP aerobically in 5% CO_2. CYE, charcoal-yeast extract agar.

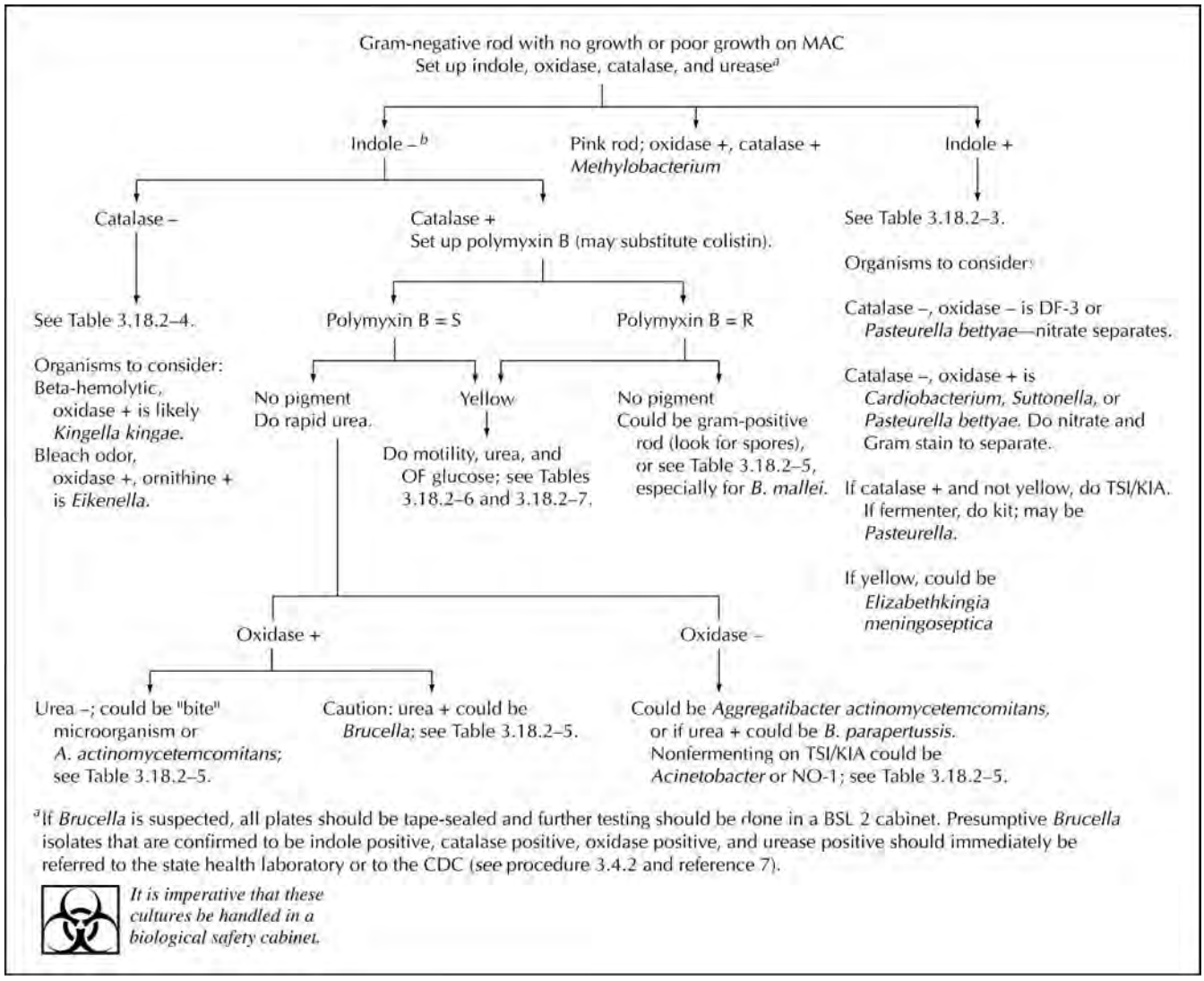
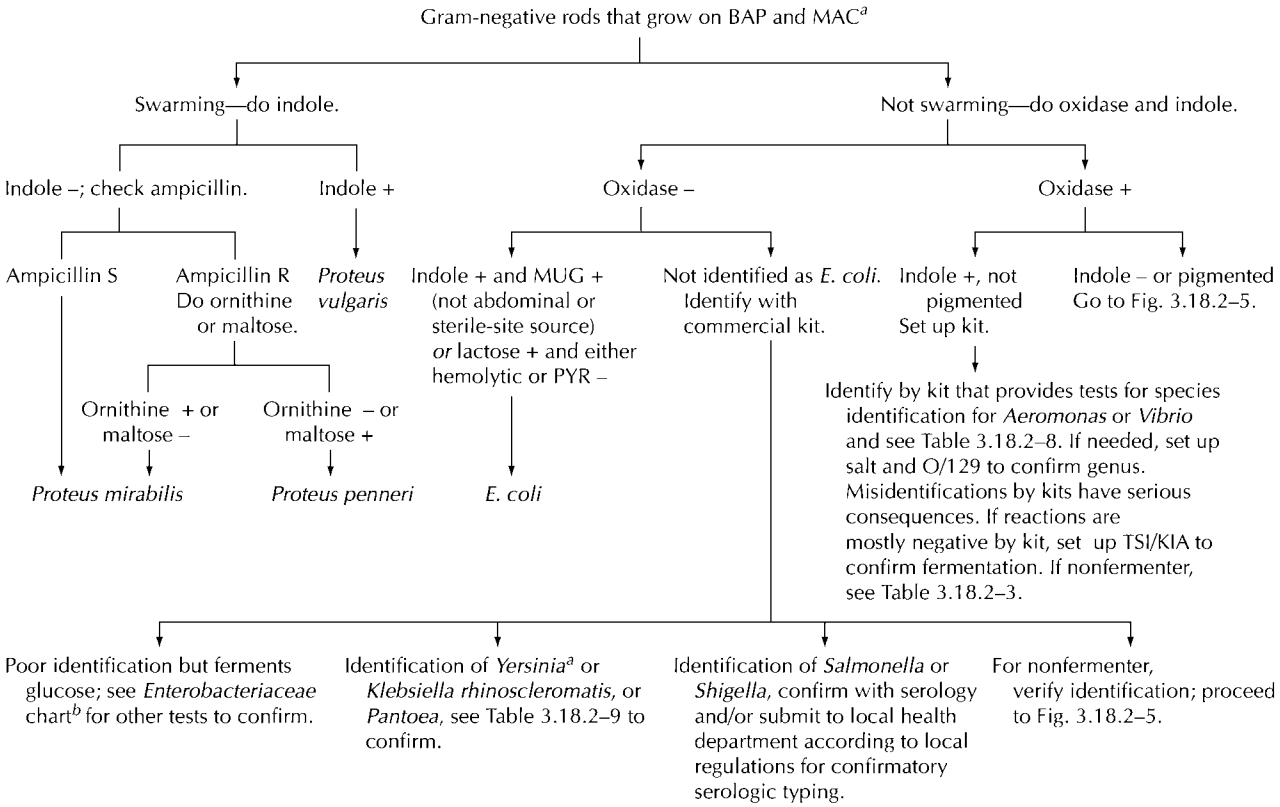

Gram-negative rod with no growth or poor growth on MAC
Set up indole, oxidase, catalase, and urease[a]
Indole –[b]
Pink rod; oxidase +, catalase +
Methylobacterium
Indole +
Catalase –
Catalase +
Set up polymyxin B (may substitute colistin).
See Table 3.18.2–3.
Organisms to consider:
Catalase –, oxidase – is DF-3 or Pasteurella bettyae—nitrate separates.
Catalase –, oxidase + is Cardiobacterium, Suttonella, or Pasteurella bettyae. Do nitrate and Gram stain to separate.
If catalase + and not yellow, do TSI/KIA. If fermenter, do kit; may be Pasteurella.
If yellow, could be Elizabethkingia meningoseptica
See Table 3.18.2–4.
Organisms to consider:
Beta-hemolytic, oxidase + is likely Kingella kingae.
Bleach odor, oxidase +, ornithine + is Eikenella.
Polymyxin B = S
Polymyxin B = R
No pigment
Do rapid urea.
Yellow
Do motility, urea, and OF glucose; see Tables 3.18.2–6 and 3.18.2–7.
No pigment
Could be gram-positive rod (look for spores), or see Table 3.18.2–5, especially for B. mallei.
Oxidase +
Oxidase –
Urea –; could be "bite" microorganism or A. actinomycetemcomitans; see Table 3.18.2–5.
Caution: urea + could be Brucella; see Table 3.18.2–5.
Could be Aggregatibacter actinomycetemcomitans, or if urea + could be B. parapertussis. Nonfermenting on TSI/KIA could be Acinetobacter or NO-1; see Table 3.18.2–5.
[a]If Brucella is suspected, all plates should be tape-sealed and further testing should be done in a BSL 2 cabinet. Presumptive Brucella isolates that are confirmed to be indole positive, catalase positive, oxidase positive, and urease positive should immediately be referred to the state health laboratory or to the CDC (see procedure 3.4.2 and reference 7).
It is imperative that these cultures be handled in a biological safety cabinet.

Figure 3.18.2–3 Identification scheme for gram-negative rods that grow on BAP with 5% CO_2 but do not grow well on MAC in 48 h.


Gram-negative rods that grow on BAP and MAC[a]
Swarming—do indole.
Not swarming—do oxidase and indole.
Indole –; check ampicillin.
Indole +
Oxidase –
Oxidase +
Ampicillin S
Ampicillin R
Do ornithine or maltose.
Proteus vulgaris
Indole + and MUG + (not abdominal or sterile-site source) or lactose + and either hemolytic or PYR –
Not identified as E. coli. Identify with commercial kit.
Indole +, not pigmented Set up kit.
Indole – or pigmented Go to Fig. 3.18.2–5.
Ornithine + or maltose –
Ornithine – or maltose +
Proteus mirabilis
Proteus penneri
E. coli
Identify by kit that provides tests for species identification for Aeromonas or Vibrio and see Table 3.18.2–8. If needed, set up salt and O/129 to confirm genus. Misidentifications by kits have serious consequences. If reactions are mostly negative by kit, set up TSI/KIA to confirm fermentation. If nonfermenter, see Table 3.18.2–3.
Poor identification but ferments glucose; see Enterobacteriaceae chart[b] for other tests to confirm.
Identification of Yersinia[a] or Klebsiella rhinoscleromatis, or Pantoea, see Table 3.18.2–9 to confirm.
Identification of Salmonella or Shigella, confirm with serology and/or submit to local health department according to local regulations for confirmatory serologic typing.
For nonfermenter, verify identification; proceed to Fig. 3.18.2–5.

[a]Gram-negative rods that grow on BAP, MAC, and CHOC that are non-lactose fermenters on MAC/EMB and demonstrate bipolar staining are suspicious of *Yersinia pestis* (plague). If *Y. pestis* is suspected, all plates should be tape-sealed and further testing should be done in a BSL 2 cabinet. Presumptive *Yersinia* isolates that are confirmed to be indole negative, catalase positive, oxidase negative, and urease negative should immediately be referred to the state health laboratory or to the CDC (8).

It is imperative that these cultures be handled in a biological safety cabinet.

[b]Reference 22, p. 652–655, and reference 12.

Figure 3.18.2–4 Identification scheme for gram-negative rods that grow on BAP and MAC.

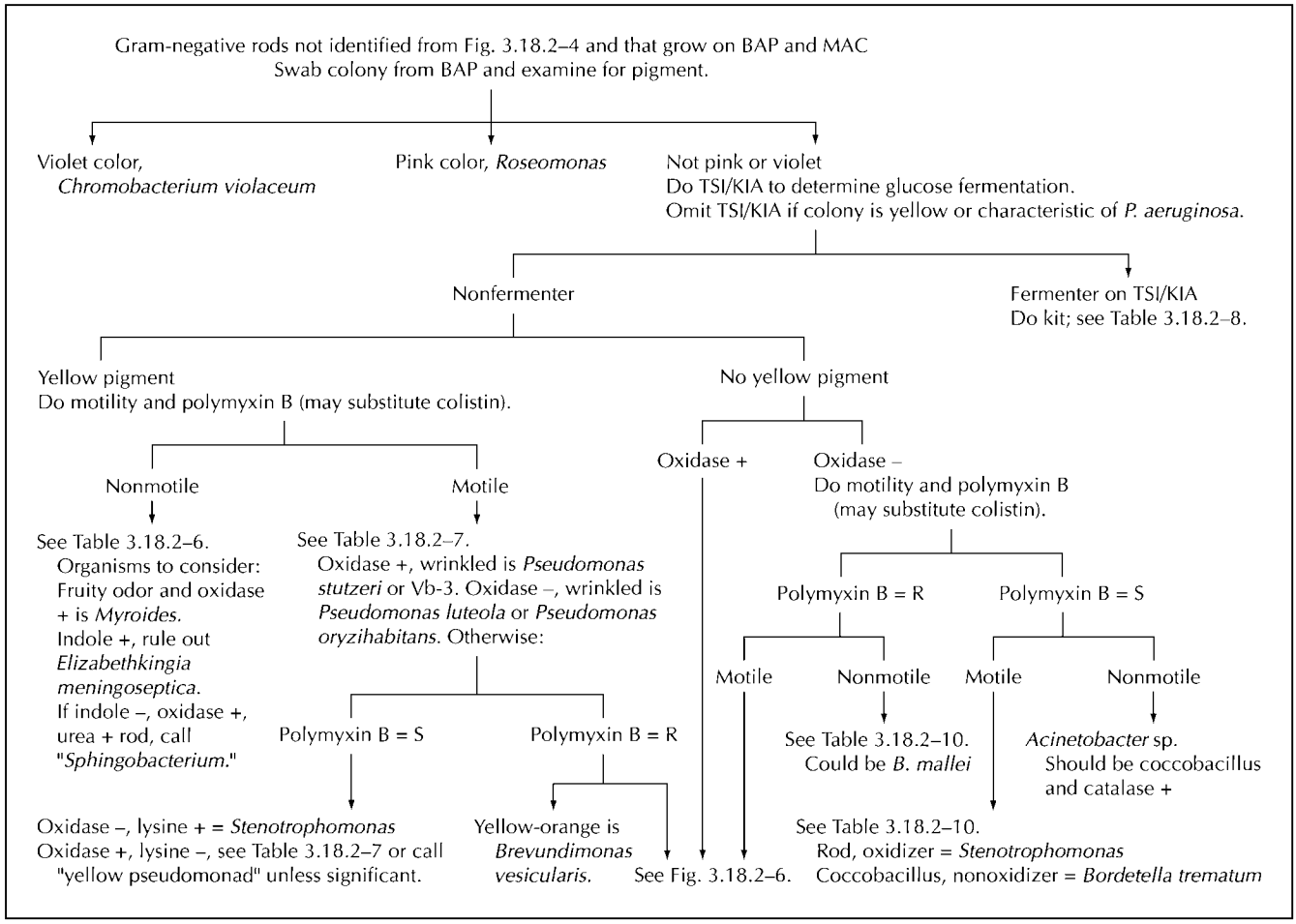

Gram-negative rods not identified from Fig. 3.18.2–4 and that grow on BAP and MAC
Swab colony from BAP and examine for pigment.
Violet color, Chromobacterium violaceum
Pink color, Roseomonas
Not pink or violet
Do TSI/KIA to determine glucose fermentation.
Omit TSI/KIA if colony is yellow or characteristic of P. aeruginosa.
Nonfermenter
Fermenter on TSI/KIA
Do kit; see Table 3.18.2–8.
Yellow pigment
Do motility and polymyxin B (may substitute colistin).
No yellow pigment
Nonmotile
Motile
Oxidase +
Oxidase –
Do motility and polymyxin B (may substitute colistin).
See Table 3.18.2–6.
Organisms to consider: Fruity odor and oxidase + is Myroides. Indole +, rule out Elizabethkingia meningoseptica. If indole –, oxidase +, urea + rod, call "Sphingobacterium."
See Table 3.18.2–7.
Oxidase +, wrinkled is Pseudomonas stutzeri or Vb-3. Oxidase –, wrinkled is Pseudomonas luteola or Pseudomonas oryzihabitans. Otherwise:
Polymyxin B = R
Polymyxin B = S
Motile
Nonmotile
Motile
Nonmotile
Polymyxin B = S
Polymyxin B = R
See Table 3.18.2–10.
Could be B. mallei
Acinetobacter sp.
Should be coccobacillus and catalase +
Oxidase –, lysine + = Stenotrophomonas
Oxidase +, lysine –, see Table 3.18.2–7 or call "yellow pseudomonad" unless significant.
Yellow-orange is Brevundimonas vesicularis.
See Fig. 3.18.2–6.
See Table 3.18.2–10.
Rod, oxidizer = Stenotrophomonas
Coccobacillus, nonoxidizer = Bordetella trematum

Figure 3.18.2–5 Identification scheme for gram-negative rods that grow well on BAP and MAC and are not identified from Fig. 3.18.2–4.

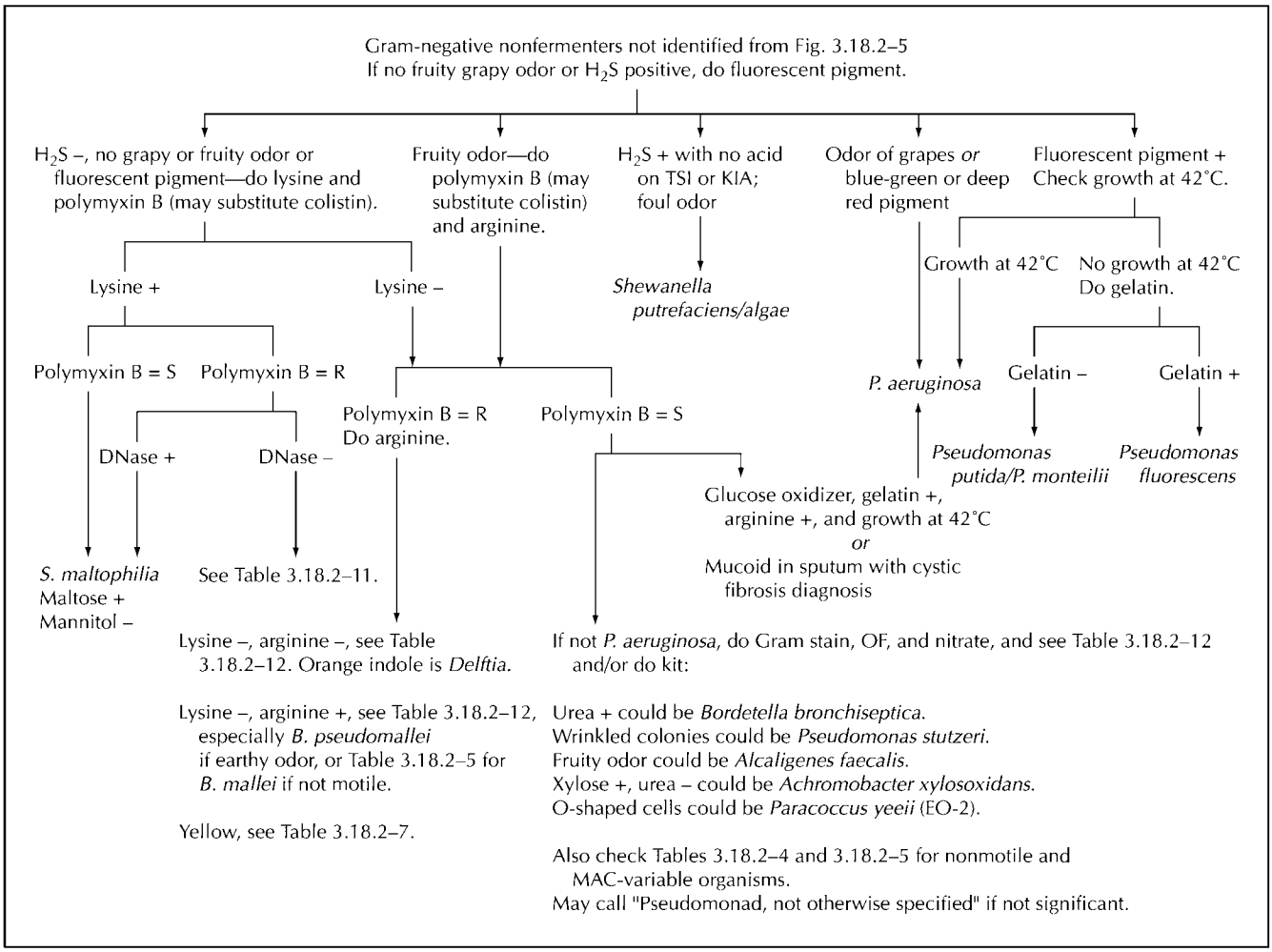

Gram-negative nonfermenters not identified from Fig. 3.18.2–5
If no fruity grapy odor or H_2S positive, do fluorescent pigment.
H_2S –, no grapy or fruity odor or fluorescent pigment—do lysine and polymyxin B (may substitute colistin).
Fruity odor—do polymyxin B (may substitute colistin) and arginine.
H_2S + with no acid on TSI or KIA; foul odor
Odor of grapes *or* blue-green or deep red pigment
Fluorescent pigment + Check growth at 42°C.
Lysine +
Lysine –
Shewanella putrefaciens/algae
Growth at 42°C
No growth at 42°C Do gelatin.
Polymyxin B = S
Polymyxin B = R
P. aeruginosa
Gelatin –
Gelatin +
Polymyxin B = R Do arginine.
Polymyxin B = S
DNase +
DNase –
Pseudomonas putida/P. monteilii
Pseudomonas fluorescens
Glucose oxidizer, gelatin +, arginine +, and growth at 42°C
or
Mucoid in sputum with cystic fibrosis diagnosis
S. maltophilia Maltose + Mannitol –
See Table 3.18.2–11.
Lysine –, arginine –, see Table 3.18.2–12. Orange indole is *Delftia.*
If not *P. aeruginosa*, do Gram stain, OF, and nitrate, and see Table 3.18.2–12 and/or do kit:
Lysine –, arginine +, see Table 3.18.2–12, especially *B. pseudomallei* if earthy odor, or Table 3.18.2–5 for *B. mallei* if not motile.
Urea + could be *Bordetella bronchiseptica.*
Wrinkled colonies could be *Pseudomonas stutzeri.*
Fruity odor could be *Alcaligenes faecalis.*
Xylose +, urea – could be *Achromobacter xylosoxidans.*
O-shaped cells could be *Paracoccus yeeii* (EO-2).
Yellow, see Table 3.18.2–7.
Also check Tables 3.18.2–4 and 3.18.2–5 for nonmotile and MAC-variable organisms.
May call "Pseudomonad, not otherwise specified" if not significant.

Figure 3.18.2–6 Identification scheme for gram-negative rods that grow on BAP and MAC and are not identified by Fig. 3.18.2–4 and 3.18.2–5.

Table 3.18.2–1 Biochemical reactions of *Neisseria* and related oxidase-positive diplococcus and rods that may grow on Thayer-Martin or similar selective agar[a,c]

Organism(s)[b]	Superoxol, 30% H_2O_2	Growth on basic agar media at 35°C[e]	Colistin 10-μg disk	Glucose	Maltose	Lactose, ONPG, or BGAL	Sucrose	PRO	GLUT	Butyrate[f]
Neisseria gonorrhoeae	4+	−	R	+⁻	−	−	−	+	−	−
Neisseria meningitidis	2–4+	V	R	+	+⁻	−	−	V	+	−
Neisseria lactamica	2+	+	R	+	+	+	−	+	−	−
Neisseria cinerea[d]	2+	+	V	V	−	−	−	+	−	−
Neisseria flavescens[d]	2+	+	S	−	−	−	−	+	−	−
Neisseria elongata[d]	V	+	S	−	−	−	−	V	−	−
Neisseria polysaccharea[g]	1–3+	V	R	+	+	−	−	+	−	−
Other nonpathogenic neisseriae[d]	2+	+	V	+	+	−	V	V	V[d]	−
Moraxella catarrhalis	2–4+	+	V	−	−	−	−	V	−	+
Kingella denitrificans	−	+	R	+	−	−	−	+	−	−

[a] Abbreviations for tests: PRO, prolyl iminopeptidase; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; BGAL, β-galactosidase; GLUT, ∂-glutamyl-aminopeptidase. Reactions are from package inserts, the website http://www.CDC.gov/ncidod/dastlr/gcdir/neident/index.html, and references 10, 18, and 32. Polymyxin B can be substituted for colistin; alternatively, resistance to these agents can be determined by growth or lack of growth on Thayer-Martin or other selective agar with colistin or polymyxin B. Results for sugars are in cysteine Trypticase agar.

[b] See reference 9 for guidelines on when DNA target sequencing may be useful for identification. In particular, partial sequencing of the 16S rRNA gene provides excellent identification for some genera of oxidase-positive, gram-negative diplococci (25)

[c] Abbreviations and symbols for results: +, greater than 90% of strains positive in 48 h; −, greater than 90% of strains negative; V, results are between 90 and 10% positive; +⁻, most strains are positive but a few are known to be negative, resulting in critical misidentifications if other tests are not also performed; R, 90% of strains resistant or no zone around disk; S, 90% of strains susceptible or zone around disk.

[d] Does not usually grow on selective media for *N. gonorrhoeae*. *N. subflava* and *N. flavescens* colonies are yellow; *N. subflava* is the only species other than *N. meningitidis* to be GLUT positive.

[e] Nutrient or MH agar or TSA without blood at 35°C.

[f] See procedure 3.17.7. Do not read after time period in package insert, as this delay may result in false-positive reactions. Many *Moraxella* spp. and *Acinetobacter* spp. are butyrate positive. Isolate must be a diplococcus for identification of *M. catarrhalis* to be accurate.

[g] *N. polysaccharea* strains were previously identified as nontypeable strains of *N. meningitidis*. Strains of *N. polysaccharea* may also have been misidentified previously as *N. subflava* because their ability to produce polysaccharide from sucrose was not determined (http://www.cdc.gov).

Table 3.18.2–2 Biochemical reactions of *Haemophilus* and *Aggregatibacter* species that satellite on BAP[a]

Organism[b]	Satellite (V factor)	ALA	Lactose[d]	Urea	Indole	Ornithine	Hemolysis[c]	Catalase
H. influenzae	+	−	−	V	V	V	−	+
H. haemolyticus	+[c]	−	−	+	V	−	+	+
H. parahaemolyticus	+[c]	+	−	+	−	−	+	+
H. parainfluenzae	+	+	−	V	V	V	−	V
A. paraphrophilus	+	+	+	−	−	−	−	−

[a] Data for table from reference 31, p. 218–219. See footnote *c* of Table 3.18.2–1 for abbreviations and symbols. *Haemophilus aphrophilus* and *Haemophilus paraphrophilus* have been reclassified as a single species on the basis of multilocus sequence analysis (23), *Aggregatibacter aphrophilus*, which includes V factor-dependent and V factor-independent isolates.

[b] See reference 9 for guidelines on when DNA target sequencing may be useful for identification. In particular, partial sequencing of the 16S rRNA gene provides excellent identification for some genera of oxidase-positive, gram-negative diplococci (25).

[c] Hemolysis demonstrated on horse, sheep, or rabbit blood agar. Note that hemolytic strains may grow on BAP without staphylococcal dot even though they require V factor.

[d] Test is performed in 1% lactose in phenol red broth base (BD Diagnostic Systems) supplemented with hemin and NAD (10 mg of each per liter; Sigma Chemical Co.) (reference 22, p. 628).

Table 3.18.2–3 Differential biochemical reactions for indole-positive, gram-negative rods that grow poorly on MAC in 48 h[a]

Organism[b]	MAC	Oxidase	Catalase	Nitrate	Glucose	Mannitol	Maltose	Urea	Other differential trait(s)
Balneatrix alpica[d]	−	+	+	+	O	(+)	+	−	Yellow, esculin −, motile, polymyxin B = S
Bergeyella zoohelcum	−	+	+	−	n-o	−	−	+	Arginine +; rod from animal bites; polymyxin B = R
Bisgaard's taxon 16	V	V	+	+	F	−	+	−	Decarboxylase −
Cardiobacterium hominis	−	+	−	−	F[s]	+ or (+)	+ or (+)	−	Pleiomorphic rod; sorbitol +, alkaline phosphatase −
Cardiobacterium valvarum[h]	No data	+	−	−	V	−	V	−	Pleiomorphic rod, barely grows on CHOC, may pit agar, indole V
CDC group II-g	+	+	+	−	n-o	−	−	−	Reduces nitrite
CDC groups II-c, II-e, II-h, II-i	−	+	+	−	O	−	V	−	Rarely isolated
Chryseobacterium gleum/Chryseobacterium indologenes[e]	V	+	+	V	O	−	+	V	Deep yellow, esculin +, polymyxin B = R
Dysgonomonas capnocytophagoides (DF-3)[c]	−	−	−	−	F[s]	−	+	−	Small rod, acid on slant and in butt of TSI, fecal pathogen
Elizabethkingia meningoseptica[e]	V	+	+	−	O	+	+	−	Yellow V, esculin +, polymyxin B = R
Pasteurella bettyae	V	V	−	+	F, gas	−	−	−	Rod, genital sites, acid on slant and in butt of TSI
Pasteurella canis	−	+	+	+	F	−	−	−	Ornithine +
Pasteurella dagmatis	−	+	+	+	F, gas	−	+	+	Decarboxylase −[f]
Pasteurella multocida	−	+	+	+	F	+	−	−	Ornithine +; from cat bites
Pasteurella stomatis	−	+	+	+	F	−	−	−	Decarboxylase −
Suttonella indologenes	−	+	V	−	F	−	+ or (+)	−	Plump rod; sorbitol −, alkaline phosphatase +; rare
Weeksella virosa	−	+	+	−	n-o	−	−	−	Light yellow, decarboxylase −, polymyxin B = S

[a] See reference 26. Indole is done with Kovács at 48 h directly on plate. For yellow colonies, Ehrlich's method may be needed. CDC group II-g grows on MAC but is a non-glucose oxidizer. See Table 3.18.2–8 for indole-positive, fermenting rods that grow on MAC. Data are from reference 31. (+), greater than 90% of strains positive in 3 to 7 days; F, glucose fermenting in TSI or KIA or Andrade's glucose broth or other sugar fermentation medium; n-o, nonoxidizer in glucose OF medium and no reaction in fermentation medium; O, oxidizer in glucose OF medium; F[s], addition of rabbit serum to Andrade's or other glucose fermentation medium may be required to demonstrate fermentation; gas, gas from either glucose or nitrate, depending on the test. Results for carbohydrates are in OF medium for oxidizers and Andrade's or rapid sugar agar for fermenters. See footnote *c* to Table 3.18.2–1 for other abbreviations and symbols.

[b] See reference 9 for guidelines on when DNA target sequencing may be useful for identification. In particular, 16S rRNA gene partial sequencing separates *P. multocida* and *P. canis*, the two species not commonly isolated. *C. hominis* may also be distinguished from *C. valvarum* (13).

[c] Three species are now recognized in the *Dysgonomonas* genus, including *D. capnocytophagoides* (formerly CDC DF-3), *D. gadei*, and *D. mossii* (15, 20). All resemble *Capnocytophaga* in their growth characteristics.

[d] All strains except *Balneatrix alpica* are nonmotile.

[e] See Table 3.18.2–6 to separate from *Empedobacter brevis*, which is yellow and indole positive and grows on MAC.

[f] Decarboxylase − indicates negative reaction for lysine, arginine, and ornithine.

[g] Primarily animal pathogens; rarely isolated from humans.

[h] See reference 29. *C. valvarum* is a newly described species that may cause endocarditis (4). Some isolates may be indole negative.

Table 3.18.2–4 Gram-negative rods that grow on BAP but are catalase negative or weak, with poor growth on MAC in 48 h[a]

Organism[b]	MAC	Catalase	Oxidase	Nitrate	Indole	Glucose	Xylose	Mannitol	Sucrose	Maltose	Urea	Other differential trait(s)
Aggregatibacter aphrophilus	−	−	V	+	−	F, gas	−	−	+	+	−	Tiny rod, acid on slant and in butt of TSI/KIA, esculin −
Bordetella holmesii	V	V	−	−	−	n-o	−	−	−	−	−	Coccoid rod, brown pigment on MH agar, nonhemolytic
Brevundimonas vesicularis	V	V	+	−	−	O	V	−	−	+	−	Half the strains are yellow, motile, polymyxin B = R
Capnocytophaga ochracea (DF-1) and related species	−	−	−	V	−	F	−	−	+	V	−	Gliding motility, esculin V, tapered ends, yellow
Cardiobacterium hominis	−	−	+	−	+	F[s]	−	(+)	V	V	−	Slender rod; sorbitol +; alkaline phosphatase −
Dysgonomonas capnocytophagoides (DF-3)	−	−	−	−	V	F[s]	V	−	V	V	−	Coccobacilli, acid on slant and in butt of TSI/KIA, esculin V
Eikenella corrodens	−	−	+	+	−	n-o	−	−	−	−	−	Ornithine + is definitive; odor of bleach, can be yellow
Kingella denitrificans	−	−	+	+, gas	−	F	−	−	−	−	−	Grows on Thayer-Martin; polymyxin B = R
Kingella kingae	−	−	+	−	−	F	−	−	−	+	−	Beta-hemolytic, nitrite V
Leptotrichia	−	−	−	−	−	F	−	−	+	+	−	Long filamentous rods, grows poorly
Moraxella bovis	−	V	+	−	−	n-o	−	−	−	−	−	Beta-hemolytic
Neisseria elongata	−	V	+	V	−	n-o	−	−	−	−	−	Often nitrite +; polymyxin B = S
Paracoccus yeeii (EO-2), *Psychrobacter* spp.	V	V	+	+	−	O	+	−	−	V	V	Coccoid but large cells with vacuoles and mucoid colony is *Paracoccus yeeii*
Pasteurella bettyae	V	−	V	+	+	F, gas	−	−	−	−	−	Rod, genital sites, acid on slant and in butt of TSI
Simonsiella muelleri	−	V	+	V	−	F	−	−	−	+	−	"Roll of coins" smear, beta-hemolytic, rarely isolated
Streptobacilllus moniliformis	−	−	−	−	−	F	−	−	−	+	−	Needs 20% ascitic fluid, blood, or serum to grow; pleomorphic forms; arginine +
Suttonella indologenes	−	V	+	−	+	F	−	−	+	V	−	Plump rod; sorbitol −, alkaline phosphatase +; rarely isolated

[a] All are nonmotile except *B. vesicularis*. Data are from references 28 and 31. See footnote *c* to Table 3.18.2–1 and footnote *a* to Table 3.18.2–3 for abbreviations and symbols.

[b] See reference 9 for guidelines on when DNA target sequencing may be useful for identification.

Table 3.18.2–5 Biochemical differentiation of non-yellow-pigmented, gram-negative rods that are catalase-positive and indole-negative but do not grow well on MAC[a]

Organism(s)[b]	MAC	Oxidase	Urea[c]	Nitrate	Glucose	Arginine	Other differential trait(s)
Aggregatibacter actinomycetemcomitans	−	V	−	+	F, gas V	−	Tiny rod; esculin −; acid slant and butt in TSI; sucrose fermentation −.
Avibacterium gallinarum	V	+	−	+	F	−	Rod, acid slant and butt in TSI; sucrose +
Bordetella parapertussis	−	−	+	−	n-o	NA	Coccobacilli, brown pigment on MH agar, beta-hemolytic
Brucella[c]	V	+	+	+	O	NA	Coccoid tiny cells; work in safety cabinet
Burkholderia mallei	V	V	V	+	O	+	Coccobacilli, hazardous; resistant to polymyxin B; citrate −
Capnocytophaga canimorsus/cynodegmi (DF-2)	−	+	−	−	F[s]	+	Rod; gliding motility, ONPG +; from dog and cat bites
CDC group EF-4b	V	+	−	+	O	−	Reduces nitrite with no gas; no reaction in TSI; from dog and cat bites
CDC group EF-4a	V	+	−	+, gas	F	V	May reduce nitrite with no gas; 73% acid in butt of TSI; from dog and cat bites
CDC group EO-5	−	−	W	−	O	−	Coccobacilli; 20% are yellow
CDC group NO-1	V	−	−, W	+	n-o	−	Rod; from dog and cat bites. *Acinetobacter* organisms that fail to grow on MAC are coccoid and nitrate −.
Methylobacterium spp.	V	+	V	V	n-o, O	NA	Vacuolated rod pink in 72 h; grows faster on CHOC; motile
Moraxella spp.	V	+	−	V	n-o	NA	Coccoid, thick cells
Neisseria weaveri	V	+	−	−	n-o	−	Rod; PDA V, nitrite +; Gilardi rod group 1 similar, except nitrite − and PDA +
Oligella ureolytica	V	+	+	+, gas V	n-o	NA	Coccoid, PDA +, motile
Oligella urethralis	V	+	−	−	n-o	NA	Coccoid, PDA +, nitrite +
Paracoccus yeeii (EO-2)	V	+	V	+	O	NA	Coccoid but large cells with vacuoles; mucoid colony
Pasteurella haemolytica	V	+	−	+	F	−	Acid slant and butt in TSI; beta-hemolytic
Psychrobacter immobilis	V	+	V	V	n-o, O	NA	Coccoid; may have rose-like odor
Psychrobacter phenylpyruvicus	V	+	+	V	n-o	NA	Coccoid, PDA +, nitrite −, 6.5% salt +

[a] For indole-positive strains, see Table 3.18.2–3. All strains nonmotile, except as noted, but even with those, motility is difficult to demonstrate. See Table 3.18.2–4 for catalase-variable rods. Data are from references 28 and 31. W, weak reaction; NA, not applicable or available. See footnote *c* to Table 3.18.2–1 and footnote *a* to Table 3.18.2–3 for other abbreviations and symbols.

[b] See reference 9 for guidelines on when DNA target sequencing may be useful for identification.

[c] If *Brucella* is suspected, all plates should be tape-sealed and further testing should be done in a BSL 2 cabinet. Presumptive *Brucella* isolates that are confirmed to be indole positive, catalase positive, oxidase positive, and urease positive should immediately be referred to the state health laboratory or to the CDC (*see* procedure 3.4.2 and reference 7).

Table 3.18.2–6 Biochemical characteristics of the nonmotile, yellow, nonfermenting, gram-negative rods that are catalase positive[a]

Organism(s)[b]	MAC	Oxidase	Yellow	Indole	Urea	Polymyxin B	Nitrate	Glucose	Mannitol	Sucrose	Maltose	Esculin	DNase	Other differential trait(s)
Chryseobacterium gleum and *Chryseobacterium indologenes*	V	+	Bright	+	V	R	V	O	−	V	+	+	−	
Elizabethkingia meningoseptica	(+)	+	Pale	+	−	R	−	O	+	−	+	+	+	
Empedobacter brevis	+	+	Pale	+	−	R	−	O, n-o	−	−	+	−	+	
EO-3, EO-4 (EO-5)	V	+	+	−	V	NA	−	O	V	−	V	−	NA	Coccoid; EO-5 is oxidase negative—see Table 3.18.2–5
Myroides odoratus/odoratimimus	V	+	Green	−	+	R	−	n-o	−	−	−	−	+	Fruity odor
Sphingobacterium mizutaii	−	+	Pale	−	−	R	−	O	−	+	+	+	−	
Sphingobacterium multivorum	+	+	Pale	−	+	R	−	O	−	+	+	+	V	
Sphingobacterium spiritivorum	V	+	Pale	−	+	R	−	O	+	+	+	+	+	
Sphingobacterium thalpophilum	+	+	Pale	−	+	R	+	O	−	+	+	+	+	Grows at 42°C
Weeksella virosa	−	+	Pale	+	−	S	−	n-o	−	−	−	−	−	Penicillin = S

[a] For indole reaction, Ehrlich's method may be needed. See Table 3.18.2–4 for yellow-pigmented, catalase-negative *Eikenella* and *Capnocytophaga.* Data are from references 26, 28, and 31. See footnote *c* to Table 3.18.2–1 and footnote *a* to Tables 3.18.2–3 and 3.18.2–5 for abbreviations and symbols.

[b] See reference 9 for guidelines on when DNA target sequencing may be useful for identification.

Table 3.18.2–7 Biochemical differentiation of the motile, yellow, non-glucose-fermenting, gram-negative rods[a]

Organism(s)[b]	MAC	Oxidase	Yellow	Polymyxin B	Urea	Nitrate	Glucose	Mannitol	Sucrose	Maltose	Esculin	Arginine	Other differential traits
Acidovorax spp.	V	+	+⁻	NA	+⁻	+	O	V	−	−	−	V	Rarely isolated
Agrobacterium yellow group	−⁺	+	+	V	(+)	−	O, n-o	−	(+)	+	V	−	Catalase +
Balneatrix alpica	−	+	Pale	S	−	+	O	(+)	−	+	−	−	Indole +, PDA +
Brevundimonas vesicularis	V	+	Orange	R	−	−	W	−	−	+	+	−	Catalase V, PYR −, vancomycin = S
Burkholderia cepacia complex/*B. gladioli*	+	V	+	R	V	V	O	V	V	V	V	−	Lysine V, DNase − (*see* Table 3.18.2–11)
O-1	−⁺	V	+	NA	V	−	O, n-o	−	−	−	+	−	Rarely isolated
O-2	−	+	Orange	NA	V	V	O, n-o	−	(+)	(+)	V	V	Rarely isolated
Pseudomonas luteola	+	−	+	S	V	V	O	+	V	+	+	V	Wrinkled colonies; PYR +
Pseudomonas mendocina (Vb-2)	+	+	+	S	V	+, gas	O	−	−	−	−	+	Colonies buttery
Pseudomonas oryzihabitans	+	−	+	S	V	−	O	+	V	+	−	−	Wrinkled colonies; PYR +
Pseudomonas stutzeri	+	+	Pale, V	S	V	+, gas	O	V	−	+	−	−	Wrinkled colonies; PYR −
Pseudomonas stutzeri-like (CDC group Vb-3)	+	+	+	S	V	+, gas	O	V	−	+	−	+	Wrinkled colonies
Sphingomonas paucimobilis	−⁺	V	Deep	V	−	−	W	−	+	+	+	−	Motility better by wet mount; vancomycin = S
Stenotrophomonas maltophilia	+	−	+	V	−	V	O	−	V	+	+	−	Lysine +, DNase +

[a] Motility is best at 22°C. All are indole negative except *Balneatrix.* Data are from references 3, 26, 28, and 31. −⁺, most strains are negative but a few are known to be positive. See footnote *c* to Table 3.18.2–1 and footnote *a* to Tables 3.18.2–3 and 3.18.2–5 for other abbreviations and symbols.

[b] See reference 9 for guidelines on when DNA target sequencing may be useful for identification.

Table 3.18.2–8 Characteristics of the common pathogenic oxidase-positive, glucose-fermenting rods that grow on MAC and are not yellow pigmented[a,b]

Organism[f]	Oxidase	Indole	Lactose	Sucrose	Lysine	Arginine	Ornithine	O/129[c]		Growth on[d]:	
								10 μg	150 μg	MH agar	MH agar + 4% salt
V. cholerae	+	+	−	+	+	−	+	S	S	V	V
V. mimicus	+	+	−+	−	+	−	+	S	S	V	V
V. parahaemolyticus	+	+	−	−	+	−	+	R	V	−	+
V. vulnificus	+	+	+−	−+	+	−	V	S	S	+	V
V. alginolyticus	+	+−	−	+	+	−	V	R	V	V	+
V. fluvialis	+	−+	−	+	−	+	−	R	V	−	+
Aeromonas	+	+−	V	V	+−	+−	−+	R	R	+	−
Plesiomonas	+	+	−	−	+	+	+	R, S	S	+	−
Chromobacterium violaceum[e]	V	V	−	V	−	+	−	NA	NA	+ (violet)	−

[a] See references 2 and 16.

[b] 1% salt may be required for sugar and decarboxylase reactions. All strains ferment glucose on TSI or KIA. CDC group II-g grows on MAC and is indole positive but is a non-glucose oxidizer; see Table 3.18.2–3. Data are from references 17, 22, and 31. See footnote *c* to Table 3.18.2–1 and footnote *a* to Tables 3.18.2–5 and 3.18.2–7 for abbreviations and symbols.

[c] Resistant *V. cholerae* strains have been isolated in India. Confirmation of arginine-negative *Aeromonas* may be needed to prevent misidentifications. Testing with O/129 is key to prevention of misidentification of *V. fluvialis* and *V. vulnificus* as *Aeromonas* spp.

[d] Data on file at University of California, San Francisco. The importance of using both media is to get growth on at least one plate in order to observe the O/129 and other disk susceptibility and to observe that *Aeromonas* and *Plesiomonas* do *not* grow with salt added. Some *Vibrio* organisms do not grow on MH agar without salt added, and some do not grow with 4% salt.

[e] The combination of polymyxin B resistance and indole positivity is associated with nonpigmented strains. The combination of sucrose negativity and growth on MH agar separates this organism from *V. fluvialis. C. violaceum* is lysine, maltose, and mannitol negative, which separates it from *Aeromonas.*

[f] See reference 9 for guidelines on when DNA target sequencing may be useful for identification.

Table 3.18.2–9 Differentiation of *Yersinia pestis* from similar bacteria[a]

Organism[c]	Lysine	Ornithine	Urea[b]	Motility at:		Glucose	ONPG	Mannitol	Rhamnose	Other differential trait(s)
				35°C	25°C					
Yersinia pestis	−	−	5%	−	−	+	V	+	1%	VP − at 25°C
Yersinia pseudotuberculosis	−	−	+	−	+−	+	V	+	70%	VP − at 25°C
Yersinia enterocolitica	−	+	+, (+)	−	+	+	+	+	−	VP + at 25°C
Klebsiella rhinoscleromatis	−	−	−	−	−	+	−	+	+	Sucrose +
Pantoea agglomerans group	−	−	V	V	V	+	+	+	+	Citrate +
Shigella spp.	−	V	−	−	−	+	V	V	−+	Serology

[a] Reactions of *Yersinia* are faster at room temperature. Data are from references 12 and 30. See footnote *c* to Table 3.18.2–1 and footnote *a* to Tables 3.18.2–3 and 3.18.2–7 for abbreviations and symbols.

[b] Use rapid urea test method to increase sensitivity. See procedure 3.17.48.

[c] See reference 9 for guidelines on when DNA target sequencing may be useful for identification.

Table 3.18.2–10 Biochemical reactions of non-glucose-fermenting, gram-negative rods that are catalase positive, oxidase negative, or delayed and grow well on MAC within 48 h[a]

Organism(s)[b]	Motility	Pigment	Polymyxin B	Lysine	Glucose	Urea	Nitrate	Arginine	Mannitol	Maltose	Other differential trait(s)
Acinetobacter spp. Saccharolytic	−	−	S	−	O	−	−	V	−	NA	Coccobacilli; most are *A. baumannii*, the only one that grows at 42°C
Asaccharolytic	−	−	S	−	n-o	−	−	V	−	−	Coccobacilli; not all grow on MAC; nitrate negativity and rod shape separates them from NO-1
Bordetella trematum	+	−	NA	−	n-o	−	V	−	−	−	Coccobacilli
Burkholderia gladioli/ Pandoraea	+	Variable yellow	R	−	O (O)	V	V	−	V	V	Rod; see Table 3.18.2–11 for species identification; DNase −
Burkholderia mallei	−	−	R	−	O	V	+	+	V	V	Coccobacilli, hazardous; MAC and oxidase V, citrate −; no growth at 42°C
Pseudomonas oryzihabitans/luteola	+	Yellow	S	−	O	V	V	V	+	+	Wrinkled colonies; PYR +
Roseomonas spp.	V	Pink	R	NA	n-o	+	V	NA	−	−	Coccoid, mucoid, delayed positive oxidase; does not absorb long-wave UV light
Stenotrophomonas maltophilia	+	Variable yellow	V	+	O	−	V	−	−	+	Rod; DNase +, PYR −

[a] Reactions from references 28 and 31. All strains are catalase positive. For oxidase-negative, yellow-pigmented organisms, see Table 3.18.2–7. See footnote *c* to Table 3.18.2–1 and footnote *a* to Tables 3.18.2–3 and 3.18.2–5 for abbreviations and symbols.

[b] See reference 9 for guidelines on when DNA target sequencing may be useful for identification.

Table 3.18.2–11 Characteristics of *Burkholderia cepacia* complex and related polymyxin B-resistant organisms[a,b]

Organism(s)[e]	Catalase	Oxidase[d]	Oxidation of:						Lysine decarboxylase	Ornithine decarboxylase	ONPG	PYR
			Glucose	Maltose	Lactose	Xylose	Sucrose	Adonitol				
B. cepacia genomovars I and III	I is −; III is +	+	+	V	V, (+)	V, (+)	+	+	+	V	+	−
B. multivorans	−	+	+	+	+	+	−	+	V	−	+	−
B. stabilis	−	+	+	+	+	V	−	V, (+)	+	+	−	−
B. vietnamieusis	−	+	+	+	+	V	+	−	+	−	+	−
B. gladioli	+	−	+	−	−	+	−	+	−	−	+	+
Pandoraea	+	V	−, (+)	−	−	−	−	−	−	−	−	NA
Ralstonia pickettii/ R. mannitolytica[c]	+	+	+	V	V	V	−	−	−	−	−	+

[a] See reference 21.

[b] Data are from references 14 and 22. PYR data are from reference 19. All strains are arginine negative. See footnote *c* to Table 3.18.2–1 and footnote *a* to Tables 3.18.2–3 and 3.18.2–5 for abbreviations and symbols.

[c] *Ralstonia mannitolytica,* formerly known as *Ralstonia pickettii* bv. 3, is unique among the *Ralstonia* organisms in that it is mannitol positive. *R. mannitolytica* is nitrate negative. *R. pickettii* is nitrate positive.

[d] Oxidase reactions may be slow, up to 30 s.

[e] See reference 9 for guidelines on when DNA target sequencing may be useful for identification.

Table 3.18.2–12 Biochemical reactions of nonyellow gram-negative rods that are oxidase positive and grow well on MAC within 48 h[a,b]

Organism(s)	Polymyxin B	Glucose	Nitrate	Arginine	Urea[c]	PYR	Xylose	Mannitol	Sucrose	Maltose	Other differential trait(s)
Achromobacter xylosoxidans subsp. *denitrificans*	S	n-o	+, gas	−	−	+	−	−	−	−	*Achromobacter piechaudii* is similar but nitrite − and no gas from nitrate.
Achromobacter xylosoxidans subsp. *xylosoxidans*	V	O	+, gas V	V	−	+	+	−	−	−	Oxidizes xylose better than glucose
Alcaligenes faecalis[d]	S	n-o	−	−	−	−	−	−	−	−	Fruity odor; nitrite +, acetamide +
Bordetella avium/hinzii	S	n-o	−	V	−+	+	−	−	−	−	*B. avium* is beta-hemolytic, nitrite −
Bordetella bronchiseptica	S	n-o	+	−	+	−	−	−	−	−	Grows on SS agar
Brevundimonas diminuta	R	n-o/O	−	−	V	−	−	−	−	−	Brown on MH agar
Burkholderia cepacia complex, *Ralstonia, Pandoraea*	R	O (O)	V	−	V	V	V	V	V	V	See Table 3.18.2–11 for species identification.
Burkholderia pseudomallei[c]	R	O	+, gas	+	V	−	+	+	V	+	White opaque colonies with sheen, then wrinkled, not beta-hemolytic, earthy odor; lactose +
CDC group Ic	NA	O	+	+	V	NA	−	−	−	+	
Comamonas spp.	S	n-o	+	−	−	+−	−	−	−	−	
Delftia acidovorans	R	n-o	+	−	−	+	−	+	−	−	Orange color of colony with Kovács' indole
Ochrobactrum anthropi and unnamed *Achromobacter* groups B, E, and F	V	O	V, gas V	V	+	+	+	V	V	V	PDA +; see reference 11, p. 760
OFBA-1	S	NA	+, gas	+	−	NA	NA	NA	NA	NA	Beta-hemolytic; turns OF base +
Oligella ureolytica	NA	n-o	+, gas V	NA	+	NA	−	−	−	−	PDA +
Paracoccus yeeii (EO-2)	S	O	+	−	V	NA	+	−	−	−	Coccoid but large cells with vacuoles; mucoid colonies
Pseudomonas alcaligenes, Pseudomonas CDC group 1, *Pseudomonas pseudoalcaligenes*	S	n-o	V	V	−	−	W	−	−	−	*P. pseudoalcaligenes* is nitrate + but no gas and 42°C +; *P. alcaligenes* is nitrate V and 42°C −; *Pseudomonas* CDC group 1 is nitrate + with gas.
Pseudomonas fluorescent group	S	O	V	+	V	V	+	V	V	−	See Fig. 3.18.2–6 for species separation.
Pseudomonas stutzeri	S	O	+, gas	−	V	−	+	V	−	+	Wrinkled colonies
Ralstonia paucula (IVc-2)	V	n-o	−	NA	+	+	−	−	−	−	No growth on SS agar
Rhizobium radiobacter	V	O	V	−	+	+	+	+	+	+	PDA +; see reference 11, p. 760
Shewanella putrefaciens/algae	S	O	+	−	V	+	−	−	+	+	H_2S +, ornithine +, brown, foul smelling

[a] See reference 21.

[b] All strains are motile and indole negative. Also see Table 3.18.2–5 for nonmotile, gram-negative rods that are MAC variable. Verify that strains are nonfermenting rods using TSI or KIA. For fermenting rods, see Table 3.18.2–8. Data are from references 19, 22, 27, and 31 and from G. L. Gilardi, unpublished identification tables. SS, salmonella-shigella. See footnote *c* to Table 3.18.2–1 and footnote *a* to Tables 3.18.2–3, 3.18.2–5, and 3.18.2–7 for other abbreviations and symbols for reaction key.

[c] *B. mallei* can have similar reactions but is nonmotile, has no odor, and does not produce gas from nitrate.

[d] To separate *Alcaligenes faecalis* from other related nonoxidizers: *Ralstonia gilardii* is nitrite negative; nonyellow *Myroides* is urea and PYR positive but nonmotile and polymyxin B resistant, and Gilardi rod group 1 is nonmotile and PDA positive.

[e] See reference 9 for guidelines on when DNA target sequencing may be useful for identification.

REFERENCES

1. **Abbott, S. L., L. S. Seli, M. Catino, Jr., M. A. Hartley, and J. M. Janda.** 1998. Misidentification of unusual *Aeromonas* species as members of the genus *Vibrio:* a continuing problem. *J. Clin. Microbiol.* **36:**1103–1104.
2. **Abbott, S. L., J. M. Janda, J. A. Johnson, and J. J. Farmer III.** 2007. *Vibrio* and other organisms, p. 723–733. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
3. **Blondel-Hill, E., D. A. Henry, and D. P. Speert.** 2007. *Pseudomonas,* p. 734–748. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
4. **Bothelo, E., F. Gouriet, P.-E. Fournier, V. Roux, G. Habib, F. Thuny, D. Metras, D. Raoult, and J.-P. Casalta.** 2006. Endocarditis caused by *Cardiobacterium valvarum. J. Clin. Microbiol.* **44:**637–658.
5. **Catlin, B. W.** 1975. Cellular elongation under the influence of antibacterial agents: a way to differentiate coccobacilli from cocci. *J. Clin. Microbiol.* **1:**102–105.
6. **Centers for Disease Control and Prevention.** 2001. *Basic Protocols for Level A Laboratories for the Presumptive Identification of* Francisella tularensis. Centers for Disease Control and Prevention, Atlanta, GA. http://www.cdc.gov.
7. **Centers for Disease Control and Prevention.** *Basic Protocols for Level A Laboratories for the Presumptive Identification of* Brucella *spp.* Centers for Disease Control and Prevention, Atlanta, GA. http://www.cdc.gov.
8. **Centers for Disease Control and Prevention.** 2002. *Basic Protocols for Level A Laboratories for the Presumptive Identification of* Yersinia pestis. Centers for Disease Control and Prevention, Atlanta, GA. http://www.cdc.gov.
9. **Clinical and Laboratory Standards Institute.** 2007. *Interpretive Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing.* Approved guideline MM18-1A. Clinical and Laboratory Standards Institute, Wayne, PA.
10. **D'Amato, R. F., L. A. Eriques, K. N. Tomforde, and E. Singerman.** 1978. Rapid identification of *Neisseria gonorrhoeae* and *Neisseria meningitidis* by using enzymatic profiles. *J. Clin. Microbiol.* **7:**77–81.
11. **Evangelista, A. T., A. L. Truant, and P. Bourbeau.** 2002. Rapid systems and instruments for the identification of bacteria, p. 22–49. *In* A. L. Truant (ed.), *Manual of Commercial Methods in Microbiology.* ASM Press, Washington, DC.
12. **Farmer, J. J., III, B. R. Davis, F. W. Hickman-Brenner, A. McWhorter, G. P. Huntley-Carter, M. A. Asbury, C. Riddle, H. G. Wathen-Grady, C. Elias, G. R. Fanning, A. G. Steigerwalt, C. M. O'Hara, G. K. Morris, P. B. Smith, and D. J. Brenner.** 1985. Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. *J. Clin. Microbiol.* **21:**46–76.
13. **Geißdörfer, W., R. Tandler, C. Schlundt, M. Weyand, W. G. Daniel, and C. Schoerner.** 2007. Fatal bioprosthetic aortic valve endocarditis due to *Cardiobacterium valvarum. J. Clin. Microbiol.* **45:**2324–2326.
14. **Henry, D. A., E. Mahenthiralingham, P. Vandamme, T. Coeyne, and D. P. Speert.** 2001. Phenotypic methods for determining genomovar status of the *Burkholderia cepacia* complex. *J. Clin. Microbiol.* **39:**1073–1078.
15. **Hofstad, T., I. Olsen, E. R. Eribe, E. Falsen, M. D. Collins, and P. A. Lawson.** 2000. *Dysgonomonas* gen. nov. to accommodate *Dysgonomonas gadei* sp. nov., an organism isolated from a human gallbladder, and *Dysgonomonas capnocytophagoides* (formerly CDC group DF-3). *Int. J. Syst. Evol. Microbiol.* **50:**2189–2195.
16. **Horneman, A. J., A. Ali, and S. L. Abbott.** 2007. *Aeromonas,* p. 716–722. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
17. **Janda, J. M., C. Powers, R. G. Bryant, and S. L. Abbott.** 1988. Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. *Clin. Microbiol. Rev.* **1:**245–267.
18. **Janda, W. M., and C. A. Gaydos.** 2007. *Neisseria,* p. 601–621. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
19. **Laffineur, K., M. Janssens, J. Charlier, V. Avesani, G. Wauters, and M. Delmée.** 2002. Biochemical and susceptibility tests useful for identification of nonfermenting gram-negative rods. *J. Clin. Microbiol.* **40:**1085–1087.
20. **Lawson, P. A., E. Falsen, E. Inganas, R. S. Weyant, and M. D. Collins.** 2002. *Dysgonomonas mossii* sp. nov., from human sources. *Syst. Appl. Microbiol.* **25:**194–197.
21. **LiPuma, J. L., B. J. Currie, G. D. Lum, and P. A. R. Vandamme.** 2007. *Burkholderia, Stenotrophomonas, Ralstonia, Cupriavidus, Pandoraea, Brevundimonas, Comamonas, Delftia,* and *Acidovorax,* p. 749–769. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
22. **Murray, P. R., E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.).** 2007. *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
23. **Norsko-Lauritsen, N., and M. Killian.** 2006. Reclassification of *Actinobacillus actinomycetemcomitans, Haemophilus aphrophilus, Haemophilus paraphrophilus,* and *Haemophilus segnis* as *Aggregatibacter actinomycetemcomitans* gen. nov., comb. nov., *Aggregatibacter aphrophilus* comb. nov. and *Aggregatibacter segnis* comb. nov., and emended description of *Aggregatibacter aphrophilus* to include V factor-dependent and V factor-independent isolates. *Int. J. Syst. Evol. Microbiol.* **56:**2135–2146.

REFERENCES *(continued)*

24. **Petti, C. A., C. R. Polage, and P. Schreckenberger.** 2005. The role of 16S rRNA gene sequencing in identification of microorganisms misidentified by conventional methods. *J. Clin. Microbiol.* **43:**6123–6125.
25. **Rennie, R. P., C. Brosnikoff, S. Shokopes, L. B. Reller, S. Mirrett, W. Janda, K. Ristow, and A. Krilcich.** 2008. Multicenter evaluation of the new Vitek 2 *Neisseria-Haemophilus* identification card. *J. Clin. Microbiol.* **46:**2681–2685.
26. **Schreckenberger, P., M. I. Daneshvar, and D. G. Hollis.** 2007. *Acinetobacter, Achromobacter, Chryseobacterium, Moraxella,* and other nonfermentative gram-negative rods, p. 770–802. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
27. **Schreckenberger, P. C.** 2000. *Practical Approach to the Identification of Glucose-Nonfermenting Gram-Negative Bacilli*, 2nd ed. University of Illinois at Chicago, Chicago.
28. **Schreckenberger, P. C., and D. Lindquist.** 2007. Algorithms for identification of aerobic gram-negative bacteria, p. 371–376. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
29. **von Graevenitz, A., R. Zbinden, and R. Mutters.** 2007. *Actinobacillus, Capnocytophaga, Eikenella, Kingella, Pasteurella,* and other fastidious or rarely encountered gram-negative rods, p. 621–635. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
30. **Wanger, A.** 2007. *Yersinia*, p. 688–697. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
31. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1995. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria*, 2nd ed. Williams & Wilkins, Baltimore, MD.
32. **Yajko, D. M., A. Chu, and W. K. Hadley.** 1984. Rapid confirmatory identification of *Neisseria gonorrhoeae* with lectins and chromogenic substrates. *J. Clin. Microbiol.* **19:**380–382.

SECTION 4 Anaerobic Bacteriology

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The Anaerobic Bacteriology section in the third edition retains all of the authors from the first and second editions, although parts have been modified. Modifications and updates have been made throughout all procedures, but in particular, significant taxonomic changes have been made where appropriate. Descriptions of specimen collection, transport, and handling, including culture techniques, have remained very similar to those in the previous editions. Updates to the phenotypic identification characteristics have been included if known, and additional references to the use of molecular techniques have been included. Methods for molecular detection or identification have not been included, although pertinent references are given if further information is needed.

4.1 Introduction

Anaerobic bacteria are a significant component of the normal microbiota of the human host. There are anaerobes present on most body surfaces and mucous membranes; they exist in large numbers throughout the entire gastrointestinal tract, from the mouth to the colon, with the exception of the stomach and esophagus; they are found in large numbers in the female genitourinary tract. In most areas, a true symbiotic relationship exists: humans supply the environment for the anaerobes to live and multiply in the presence of food, water, and a "friendly" atmosphere; the bacteria aid in digestion of foodstuffs for metabolism, prevent attachment of more virulent microbes by virtue of their presence in very large numbers, and make up a major component of the innate immunity of the host. In addition, the normal anaerobic microbiota bacteria are important in supplying needed vitamins and cofactors like vitamin K that humans cannot manufacture on their own (6).

Anaerobes can cause infections when they increase in number in areas where they are part of the normal microbiota (endogenous infections) or when they are introduced into a new site in the body or when a non-normal microbiota anaerobic bacterium gains entrance into the host via penetrating wounds as a result of trauma, accidents, or surgical procedures. Anaerobic bacteria can cause a wide variety of infections, including wound infections as a result of trauma or surgery; abscesses of the liver, brain, lung, and other local sites; appendicitis; peritonitis; chronic otitis media and sinusitis; endophthalmitis; bacteremia; endocarditis; myonecrosis; gas gangrene; and dental and oral infections (1, 3). The incidence of anaerobic bacteremia was as high as 20% in the days before prophylactic antimicrobial agents preceded abdominal and genitourinary tract surgeries (2). Today, the incidence is reported as 0.5 to 9%, depending upon the population being studied (2, 4, 8, 9). Anaerobes are rarely found causing urinary tract infections, meningitis, and diarrhea (excepting, of course, *Clostridium difficile*-associated diarrhea).

To optimally recover anaerobic bacteria, appropriate collection, transport, and processing procedures must be followed. Defined methods for the collection of specimens should be produced by the clinical microbiology laboratory and shared with clinical colleagues who actually perform the collection. Proper transport devices for fluids, aspirates, and tissues should be suggested or provided to those who collect the samples, with procedures as to how best to deliver them to the laboratory. Collection of specimens on swabs should be discouraged for anaerobic recovery. Swabs have very small surface areas, are prone to be used in areas where anaerobic normal microbiota organisms predominate and therefore can provide a skewed culture result of what is the true pathogen, and do not provide a good anaerobic atmosphere for adequate transport. If used, they should always be transported in devices made specifically for anaerobic transport. If samples cannot be processed in a short period of time (less than 2 h), incubation at or near room temperature is preferable over refrigeration temperatures. The media for optimal anaerobic bacterial recovery should be fresh media and include added vitamin K and hemin, minimally. For some anaerobes, from specific sites or diseases, more enriched media may be required. Inoculation of broth media in addition to solid media is controversial; however, most literature would at least suggest to laboratorians that they employ it only in certain situations and with full knowledge that contamination can be an issue if used inappropriately (5, 7).

Direct examination of all specimens for anaerobic culture is mandatory. In order to obtain information that will guide culture workup and enhance clinical relevance of the results, correlation of what is isolated with the initial Gram stain results is necessary. If a specimen is from a cerebrospinal shunt or demonstrates no white cells or organisms on Gram smear, the isolation of *Propionibacterium acnes* or an anaerobic gram-positive coccus may be insignificant. If a gram-positive nonspore-forming rod was seen in the polymorphonuclear cells, and the concomitant aerobic culture is not growing anything consistent with this morphology, the isolation of *P. acnes* could be very relevant. Likewise, if a liver abscess is submitted to the laboratory, and on Gram stain, one sees polymorphonuclear cells in abundance, with intracellular gram-negative bacilli and extracellular gram-negative coccobacillary bacteria, when *Klebsiella pneumoniae* and *Bacteroides fragilis* are cultured, both may be considered pathogens in the infection. There are some specimens, such as sputum, that should never be cultured for anaerobes because of the inability to collect such specimens without getting normal oral microbiota anaerobes in the sample. However, a Gram stain of sputum that is loaded with polymorphonuclear cells and a mixed microbiota of gram-positive and gram-negative bacteria, many of which "resemble" anaerobes (fusiforms and branching bacilli), is suggestive to the clinician that an anaerobic pleuropulmonary infection is likely and exact anaerobic identification is not necessary to treat appropriately. Many times, just knowing that anaerobes are present is sufficient; on the other hand, in sterile sites, such as blood or an abscess, complete

identification of the anaerobes present may be deemed appropriate.

This section outlines practical procedures that can be used to establish the presence of clinically important anaerobes. Attempts will be made to suggest practical approaches to the reporting of anaerobic bacteria based upon the site of the infection and whether the specimen is considered from a sterile versus a nonsterile site. Procedures are presented for proper collection and transport; specimen types appropriate for anaerobic culture and those that should be rejected; methods for planting of specimens, incubation, and direct observations; methods for initial characterization and presumptive and confirmatory identification according to conventional biochemical approaches; and use of spot tests for more rapid identification. Also presented is an introduction into newer molecular methods for identification which will be part of the future of anaerobic bacteriology. What may be most important in the overall detection and proper analysis of anaerobic cultures is the communication between the microbiologist and the clinician. It is only when both groups understand what the laboratory is capable of doing in regard to anaerobic isolation, and what is mutually agreed upon as significant, that the results will be meaningful and patient care will be enhanced.

REFERENCES

1. **Finegold, S. M., and W. L. George.** 1989. *Anaerobic Infections in Humans.* Academic Press, Inc., San Diego, CA.
2. **Goldstein, E. J.** 1996. Anaerobic bacteremia. *Clin. Infect. Dis.* **23:**S97–S101.
3. **Jousimies-Somer, H. R., P. Soummanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold.** 2002. *Wadsworth Anaerobic Bacteriology Manual*, 6th ed. Star Publishing Co., Belmont, CA.
4. **Mathur, P., R. Chaudhry, L. Kumar, A. Kapil, and B. Dhawan.** 2002. A study of bacteremia in febrile neutropenic patients at a tertiary-care hospital with special reference to anaerobes. *Med. Oncol.* **19:**267–272.
5. **Meredith, F. T., H. K. Phillips, and L. B. Reller.** 1997. Clinical utility of broth cultures of cerebrospinal fluid from patients at risk for shunt infections. *J. Clin. Microbiol.* **35:**3109–3111.
6. **Mims, C., H. M. Dockrell, R. V. Goering, I. Roitt, D. Wakelin, and M. Zuckerman.** 2004. *Medical Microbiology*, 3rd ed. Mosby, Philadelphia, PA.
7. **Morris, A. J., S. J. Wilson, C. E. Marx, M. L. Wilson, S. Mirrett, and L. B. Reller.** 1995. Clinical impact of bacteria and fungi recovered only from broth cultures. *J. Clin. Microbiol.* **33:**161–165.
8. **Rosenblatt, J. E.** 1997. Can we afford to do anaerobic cultures and identification? A positive point of view. *Clin. Infect. Dis.* **25:**S127–S131.
9. **Zahar, J. R., H. Farhat, E. Chachaty, P. Meshak, S. Antoun, and G. Nitenberg.** 2005. Incidence and clinical significance of anaerobic bacteraemia in cancer patients: a 6-year retrospective study. *Clin. Microbiol. Infect.* **11:**724–729.

4.2 Collection and Transport of Clinical Specimens for Anaerobic Culture

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Proper collection of specimens to avoid contamination with organisms of the normal microbiota and prompt transport to the laboratory for processing are essential. The isolation of anaerobic bacteria from appropriately collected clinical samples and reporting of that information as quickly as possible are extremely important for the clinician to be able to implement appropriate therapeutics. Gram stains are needed to provide a rapid analysis of the appropriateness of the sample and are essential for the correlation to what grows out in the laboratory.

The laboratory director and staff must provide health care personnel with guidelines for the optimal type and amount of clinical specimen that are needed for anaerobic culture, including the appropriate methods for specimen collection. They must stress the need to transport all specimens to the laboratory without delay. Patient care units, clinics, emergency rooms, and satellite facilities should be provided with appropriate collection devices and complete instructions for their use. Regular in-services would be an excellent approach to educate all who collect as to the proper methods. Clinicians and other health care providers should be educated to supply information regarding the specific source of the culture, the diagnosis that is suspected, special status of the patient (e.g., immunosuppression or treatment with antimicrobial agents), or if they suspect an unusual anaerobic organism. Good communication between the clinical microbiology laboratory and the clinical staff will ensure that collection and transport are optimal (5, 7, 8, 10).

II. SPECIMEN

Observe standard precautions.

A. Specimen collection (Table 4.2–1)

1. The best specimen for anaerobic culture is one that is collected at the site of infection, with sterile technique, and far removed from areas that are heavily contaminated with bacteria of the normal microbiota. This usually means aspirates (collected with needle and syringe) or tissue or biopsy samples.
2. Collection of an anaerobic culture with a swab should be discouraged or rejected, especially when there are other means to obtain that sample more effectively. The volume of specimen that can be adequately obtained on a swab is small, reducing the probability that anaerobic pathogens will be recovered. Many organisms have a tendency to adhere to the fibers of the swab, which can also greatly reduce the recovery of anaerobes. When swabs are used, the specimen may be much more superficial than what could be obtained with an aspirate, which, again, could decrease recovery of the pathogen while potentially increasing recovery of bacteria of the normal microbiota (1, 4).
3. There are newer collection devices on the market, including the flocked swab, which may provide a greater surface area for collection, but more data will be needed to determine if they will provide better material than previous swabs (12).

B. Specimen transport (*see* Appendix 4.2–1 for a partial list of suppliers)

1. Transport time depends on the volume and nature of the specimen. Large volumes of purulent material and large pieces of tissue maintain the viability

Table 4.2–1 Acceptable specimens for anaerobic culture

Site	Acceptable specimens	Unacceptable specimens
Head and neck	Abscess aspirate obtained by needle and syringe after surface decontamination Biopsy material surgically obtained	Throat or nasopharyngeal swabs Gingival swabs Superficial material collected with swabs
Lungs	Transtracheal aspirate Material from percutaneous lung puncture Biopsy material surgically obtained Bronchoscopic specimen obtained by protected brush Thoracotomy specimen	Expectorated sputum Induced sputum Endotracheal aspirate Bronchoscopic specimens not specially collected
Central nervous system	Abscess aspirate obtained by needle and syringe Biopsy material surgically obtained	Aerobic swabs
Abdomen	Peritoneal fluid obtained by needle and syringe Abscess aspirate obtained by needle and syringe Bile Biopsy material surgically obtained	Aerobic swabs
Urinary tract	Suprapubic aspirate	Voided urine Catheterized urine
Female genital tract	Culdoscopy specimens Endometrial aspirate obtained by suction or protected collector Abscess aspirate obtained by needle and syringe Biopsy material surgically obtained IUD[a] for *Actinomyces* species	Vaginal or cervical swabs
Bone and joint	Aspirate obtained by needle and syringe Biopsy material surgically obtained	Superficial material collected with swabs
Soft tissue	Aspirate obtained by needle and syringe Biopsy material surgically obtained Aspirate from sinus tract obtained by needle and small plastic catheter Deep aspirate of open-wound margin obtained through decontaminated skin Deep aspirate of surface ulcer obtained through decontaminated skin	Superficial material collected from skin surface or edges of wound

[a] IUD, intrauterine device. Culture of an IUD for *Actinomyces* is controversial. *Actinomyces* spp. are normal organisms of the genitourinary tract microbiota, and their isolation may not always represent infection. If cultures are done, results should always be correlated with pathological tissue findings (2, 3, 9, 13).

II. SPECIMEN *(continued)*

of anaerobes for many hours. Swabs (when necessary) and small volumes of aspirated material, biopsy samples, or curettings should be transported in an anaerobic transport device (Table 4.2–2). Suggested transport times relative to specimen volumes and methods of collection are listed in Table 4.2–3.

2. Avoid extremes of heat or cold. *If delays are unavoidable, hold the specimen at room temperature until processing.*
3. Do not transport material for culture in the needle and syringe. Needle transport is very unsafe because there is always the risk of a needle stick injury, and syringe transport poses a risk because the specimen may be expelled during transport, creating a threat to personnel and the environment (1). Transfer aspirated material to an anaerobic transport vial. Large volumes of purulent material may be transported in a sterile screw-cap tube.

Table 4.2–2 Anaerobic specimen transport devices

Specimen type	Transport system	Commercially available systems[a]
Aspirated material	Vial or tube with anaerobic atmosphere and agar base with indicator system	Port-A-Cul tube or vial (BD) Anaerobic Transport Medium (Anaerobe Systems) A.C.T. transport tube (Remel)
Tissue, biopsy material, or curettings	Bag systems that act by removing molecular oxygen or tube systems that permit material to be added	Bio-Bag (type A) (BD Biosciences) Anaerobic Transport System (Anaerobe Systems) A.C.T. transport tube (Remel) AnaeroGen (Oxoid) AnaeroPouch (Mitsubishi) Venturi Transystem (Copan) Port-A-Cul widemouthed jar (BD Biosciences)
Specimen collected on swabs	Tube with anaerobic atmosphere and agar base with indicator system, or tube with anaerobic atmosphere and reduced transport medium	Anaerobic Transport System (Anaerobe Systems) A.C.T. transport tube (Remel) Port-A-Cul (BD Biosciences) Venturi Transystem Vi-Pak Amies (Copan Diagnostics)

[a] Partial list of suppliers.

II. SPECIMEN *(continued)*

4. Place tissue samples, biopsy samples, or curettings into an anaerobic transport device or a sterile tube or petri dish. Place all of this into a sealable plastic bag (Becton Dickinson [BD], Oxoid, Mitsubishi) that generates an anaerobic atmosphere. Large pieces of tissue can be transported in a widemouthed anaerobic transport device or in a sterile tube or jar.
5. If specimens must be collected by swab, transport swabs in a tube containing anaerobic transport medium (*see* Table 4.2–2).

C. Collection methods

1. Abscess

   Aspirate material with needle and syringe after the surface of intact tissue is disinfected with a povidone-iodine wash that remains on the surface for at least 1 min. When needle use is contraindicated, aspirate material through a flexible plastic catheter or directly into the syringe with no needle.

Table 4.2–3 Suggested transport times for certain specimen volumes and collection methods

Specimen type	Optimal time for transport to laboratory	Additional comment(s)
Aspirated material		
Very small vol (<1.0 ml)	≤10 min	Transport small vol of aspirated material in anaerobic transport vial whenever possible for best possible results.
Small vol (~1.0 ml)	≤30 min	
Large vol (>2.0 ml)	≤2–3 h	Transport large vol of purulent material; large pieces of tissue; or aspirated material, tissue, biopsy material, or curettings in an anaerobic transport medium or container.
In anaerobic transport device	≤2–3 h	These specimens can generally be accepted for anaerobic culture with good results even after a delay of 8–24 h. Include comment regarding transport delay in report when these cultures are processed.
Tissue or biopsy material		
In sterile container	≤30 min	
In anaerobic bag or transport device	≤2–3 h	
Anaerobic swabs		
In tube with moist anaerobic atmosphere	≤1 h	
In anaerobic transport medium	≤2–3 h	

II. SPECIMEN *(continued)*

2. Sinus tract or deep-wound drainage
   Aspirate material with a small flexible plastic catheter and syringe after proper disinfection of the skin surface, or collect curettings of material from deep within the tract or wound.
3. Decubiti and other surface ulcers
   Results on specimens from decubiti and other surface ulcers can be very misleading unless special precautions are utilized. Analysis should be performed only on specimens from punch biopsy, on aspirated material obtained by needle and syringe after thorough and proper disinfection of the surface area, or on small curettings of material from deep tissue at the wound margin. Swabs from decubiti and other surface ulcers are never appropriate for anaerobic culture (6, 10). There has been some work done with biofilms and PCR detection of organisms at sites of decubiti and other wounds that supports the fact that culturing of these samples is fraught with error. The true pathogen may be overgrown with other more rapidly growing organisms, and the predominant organisms might not even be involved in the infection. PCR may prove too sensitive, but studies are corroborating the lack of utility of most cultures from decubiti and other ulcers or wounds (2, 3).
4. Pulmonary specimens
   Collect lung tissue, transtracheal aspirate, percutaneous aspirate, transcutaneous aspirate, and bronchial brushings via double-lumen catheter. The use of shielded catheters to obtain specimens from pulmonary sources is essential to obtain proper specimens; otherwise the laboratory will be working up and identifying normal respiratory microbiota and providing useless information to the physician. Bronchial washings and other respiratory specimens not obtained via double-lumen catheters are not appropriate for anaerobic culture.
5. Female genital tract specimens
   a. Disinfect the cervical opening by swabbing it with povidone-iodine.
   b. Sample the upper genital tract by using a double-lumen collector and self-contained transport system. The Pipelle system obtains cellular material from the uterine wall by suction, and the AccuCulShure uses a double-lumen collector that reduces the potential of contamination (1, 2). Specimens collected by laparoscopy, culdocentesis, or surgery are appropriate for anaerobic culture.
   c. Culture intrauterine devices anaerobically for *Actinomyces* species. There is controversy about whether cultures should be performed anaerobically on intrauterine devices for *Actinomyces* spp. since these anaerobes are part of the bacteria comprising the normal microbiota of the female genital tract. However, if cultures are performed anaerobically, they should be incubated for a minimum of 10 days to recover *Actinomyces* spp., and results should always be correlated with tissue findings in pathology (7, 8, 9, 11, 13).
6. Urinary tract
   Obtain material via suprapubic bladder tap.
7. Other situations
   In some cases, when aspiration or biopsy is not feasible (e.g., animal bite wounds), an anaerobic swab may be used for anaerobic culture. Anaerobic swabs are the least desirable specimen for a number of reasons, including small volume of specimen, greater chance of contamination with normal microbiota, excessive dryness, bacterial adherence to cotton fibers, and poor Gram stain quality. Studies have shown poor recovery of anaerobic organisms from some swab transport systems beyond 24 h (3, 4, 6). If a swab

II. SPECIMEN *(continued)*

must be used, a swab using polyurethane adsorbing material instead of cotton, with two swabs (one for culture and the other for smear), may provide a useful alternative. An aspirate or biopsy sample or even a very small sliver of tissue may often be a better specimen than a swab for anaerobic culture.

ANALYTICAL CONSIDERATIONS

III. PROCESSING SPECIMENS FOR ANAEROBIC CULTURE

A. Visual examination

Gross inspection of the specimen may provide information about the nature and quality of the specimen. Characteristics to note include blood, purulence, necrotic tissue, foul odor, and sulfur granules.

B. Specimen preparation

1. Vortex grossly purulent specimens in the anaerobic transport vial to ensure even distribution of microorganisms.

2. Grind bone or tissue with approximately 1 ml of liquid medium (THIO or chopped meat) to make a thick paste. Grind the materials in an anaerobic chamber, when possible, to minimize aeration.

3. Wring out swabs in 0.5 ml of liquid medium (THIO or chopped meat), and then treat them as a liquid specimen. Alternatively, plant swabs directly onto appropriate media, but this option is less desirable because the loss of organisms on each medium will result in a poorer specimen for Gram stain.

4. Centrifuge large volumes of nonpurulent material. Use the sediment to inoculate the media and to prepare the Gram stain.

C. Inoculation of media

1. Media for anaerobic culture (*see* procedure 4.3 for more information)

a. Brucella agar with 5% sheep blood supplemented with vitamin K and hemin for the isolation of most organisms.

Include QC information on reagent container and in QC records.

b. Phenylethyl alcohol (PEA)-sheep blood agar for the inhibition of enteric and certain other facultatively anaerobic gram-negative bacilli that may overgrow the anaerobes. PEA also reduces the spreading or swarming characteristic of some anaerobes.

c. Kanamycin-vancomycin-laked blood agar for the selection of pigmented *Prevotella* and other *Bacteroides* spp.

d. *Bacteroides* bile esculin agar for the selection and presumptive identification of *Bacteroides fragilis* group organisms and *Bilophila wadsworthia. Fusobacterium mortiferum/varium* group organisms may also occasionally grow on this medium.

e. Chopped meat broth or THIO (supplemented with vitamin K and hemin).

f. Freshly prepared or prereduced anaerobically sterilized (PRAS) media are preferred. PRAS media have a prolonged shelf life and are superior to commercial media that have been reduced 24 h before use (1, 2). Further descriptions of media used for anaerobic culture are given in procedure 4.3.

Observe standard precautions.

2. Inoculation procedure

a. Transfer the prepared specimen onto the appropriate aerobic and anaerobic media, liquid medium, and a slide for Gram stain. Use 1 drop of purulent material per plate, 2 or 3 drops of nonpurulent material per plate, 0.5 to 1.0 ml of specimen on the bottom of the liquid medium, and 1 drop of specimen spread evenly on a glass slide.

b. When swabs are used, inoculate the nonselective plates first. If two swabs are available, use one for medium inoculation and one for Gram stain preparation.

IV. PROCESSING SPECIMENS FOR ANAEROBIC CULTURE *(continued)*

3. Incubation

When inoculated media cannot be placed immediately into an anaerobic atmosphere, it would be best to batch process the specimens so that multiple specimens will be inoculated and placed into the anaerobic environment at once. Holding the clinical specimen in an appropriate transport device and batch processing the inoculation to media is preferred to processing specimens one at a time and leaving them in holding jars until time permits to place them at once in an anaerobic environment (either bags, jars, or an anaerobic chamber; *see* procedure 4.5). The viability of anaerobes can be maintained for hours in a good anaerobic specimen collection device.

IV. MICROSCOPIC EXAMINATION

A. A direct smear can be gently heat fixed or fixed in absolute methanol for 1 min and then stained by standard Gram stain procedure and reagents. Alternatively, use basic fuchsin in place of safranin to enhance the staining of some gram-negative anaerobes.

B. Gram stain reveals the types and relative numbers of microorganisms and host cells present and serves as a QC measure for the adequacy of anaerobic techniques. Correlation of specimen type with bacterial morphology on the Gram stain can provide the clinician with rapid presumptive information about the identity of the bacteria present. (*See* procedures 4.10, 4.11, and 4.12 for additional Gram stain clues.)

1. Large gram-positive rods with boxcar-shaped cells and no spores usually indicate *Clostridium perfringens.* Some *C. perfringens* cells may appear gram negative but may have the same morphology as the gram-positive cells within the same microscopic field. (*See* procedure 4.11 on anaerobic gram-positive bacilli and additional Gram stain clues.)

2. Gram-negative coccobacillary forms suggest pigmenting *Prevotella* group or *Porphyromonas* group.

3. Thin gram-negative bacilli with tapered ends suggest *Fusobacterium nucleatum.*

4. Pleomorphic pale-staining gram-negative bacilli suggest *Bacteroides* spp.

5. Very small gram-negative cocci suggest *Veillonella* spp.

6. See procedures 4.10 and 4.11 for additional Gram stain clues.

C. Results of the Gram stain may indicate the need for additional media or special stains. Aspirated material from a lung nodule, for example, may reveal long, thin, branching gram-positive bacilli. These bacilli suggest the possibility of *Actinomyces* or *Nocardia* spp. The addition of a modified Kinyoun acid-fast stain may quickly provide useful information for the clinician.

D. Techniques such as phase-contrast microscopy or dark-field examination have been used to demonstrate spirochetes.

REFERENCES

1. **Citron, D. M., Y. A. Warren, M. K. Hudspeth, and E. J. C. Goldstein.** 2000. Survival of aerobic and anaerobic bacteria in purulent clinical specimens maintained in the Copan Venturi Transystem and Becton Dickinson Port-A-Cul transport systems. *J. Clin. Microbiol.* **38:**892–894.
2. **Dowd, S. E., Y. Sun, P. R. Secor, D. D. Rhoads, B. M. Wolcott, G. A. James, and R. D. Wolcott.** 2008. Survey of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosome shotgun sequencing. *BMC Microbiol.* **8:**43.
3. **Dowd, S. E., R. D. Wolcott, Y. Sun, T. McKeehan, E. Smith, and D. Rhoads.** 2008. Polymicrobial nature of chronic diabetic foot ulcer biofilm infections determined using bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP). *PLoS ONE* **10:**e3326.
4. **Hindiyeh, M., V. Acevedo, and K. C. Carroll.** 2001. Comparison of three transport systems (Starplex StarSwab II, the new Copan ViPak Amies agar gel collection and transport swabs, and BBL Port-A-Cul) for maintenance of anaerobic and fastidious aerobic organisms. *J. Clin. Microbiol.* **39:**377–380.

REFERENCES *(continued)*

5. **Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold.** 2002. *Wadsworth Anaerobic Bacteriology Manual*, 6th ed. Star Publishing Co., Belmont, CA.
6. **Kessler, L., Y. Piemont, F. Ortega, O. Lesens, C. Boeri, C. Averous, R. Meyer, Y. Hansmann, D. Christmann, J. Gaudias, and M. Pinget.** 2006. Comparison of microbiological results of needle puncture vs. superficial swab in infected diabetic foot ulcer with osteomyelitis. *Diabet. Med.* **23:**99–102.
7. **Miller, J. M.** 1999. *A Guide to Specimen Management in Clinical Microbiology*, 2nd ed. ASM Press, Washington, DC.
8. **Miller, J. M., K. Krisher, and H. T. Holmes.** 2007. General principles of specimen collection and handling, p. 43–54. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
9. **Quercia, R., F. Bani Sadr, A. Cortez, G. Arlet, and G. Pialoux.** 2006. Genital tract actinomycosis caused by *Actinomyces israelii*. *Med. Mal. Infect.* **36:**393–395.
10. **Senneville, E., H. Melliez, E. Beltrand, L. Legout, M. Valette, M. Cazaubiel, M. Cordonnier, M. Caillaux, Y. Yazdanpanah, and Y. Mouton.** 2005. Culture of percutaneous bone biopsy specimens for diagnosis of diabetic foot osteomyelitis: concordance with ulcer swab cultures. *Clin. Infect. Dis.* **42:**57–62.
11. **Thomson, R. B.** 2007. Specimen collection, transport, and processing: bacteriology, p. 291–233. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
12. **Van Horn, K. G., C. D. Audette, K. A. Tucker, and D. Sebeck.** 2008. Comparison of 3 swab transport systems for direct release and recovery of aerobic and anaerobic bacteria. *Diagn. Microbiol. Infect. Dis.* **62:**471–473.
13. **Westhoff, C.** 2007. Intrauterine devices and colonization or infection with Actinomyces. *Contraception* **75:**S48–S50.

SUPPLEMENTAL READING

Finegold, S. M., and W. L. George. 1989. *Anaerobic Infections in Humans*. Academic Press, Inc., San Diego, CA.

Forbes, B. A., D. F. Sahm, and A. S. Weissfeld (ed.). 2007. *Bailey and Scott's Diagnostic Bacteriology*, 12th ed. Mosby Elsevier, St. Louis, MO.

Mangels, J. I. 1994. Anaerobic transport systems: are they necessary? *Clin. Microbiol. Newsl.* **16:**101–104.

APPENDIX 4.2–1

Sources of Supplies

BD Diagnostic Services
7 Loveton Circle
Sparks, MD 21152
http://www.bd.com/ds/productCenter/CT-PortACul.com

Transport for anaerobe swabs	Catalog no.
Port-A-Cul tube	221606
Port-A-Cul tube, sterile pack	221607
Transport for fluids or tissues	**Catalog no.**
Port-A-Cul vial	221608
Port-A-Cul fluid transport kit	221625
Port-A-Cul vial, sterile pack	221609
Port-A-Cul transport jar	221602
Environmental chambers	**Catalog no.**
Bio-Bag, type A	261215

Anaerobe Systems
15906 Concord Circle
Morgan Hill, CA 95037
http://www.anaerobesystems.com

Transport media for fluids, tissues, or swabs	Catalog no.
Anaerobe Transport Medium	AS-911
Anaerobe Transport Medium, surgery pack	AS-914
Wide Mouth Anaerobic Transport Medium, 8 ml	AS-919

APPENDIX 4.2–1 *(continued)*

Copan Diagnostics, Inc.
26055 Jefferson Ave.
Murrieta, CA 92562
http://www.copanusa.com

Transport systems for swabs	Catalog no.
Venturi Transystem Amies gel Vi-Pak, flushed with nitrogen	018C
E Swab with flocked swabs	480C and 481C

Hardy Diagnostics
1430 W. McCoy Ln.
Santa Maria, CA 93455
http://www.hardydiagnostics.com

Fluid, tissue, or swab system	Catalog no.
Anaerobic Transport Medium	AS-911
Anaerobic Transport, sterile surgery pack	AS-914
Anaerobic Transport System, sterile pack, widemouthed jar	AS-915

ThermoFisher Scientific
Remel Products
12076 Santa Fe Dr.
P.O. Box 14428
Lenexa, KS 66215
http://www.remel.com

Fluid, tissue, or swab system	Catalog no.
A.C.T. tube system	R12401
A.C.T. II, sterile pack tube system	R12402
A.C.T. Dual	R124022

4.3 Culture Media for Anaerobes

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The choice of media for use in the anaerobic bacteriology laboratory is important for the success of anaerobic bacteriology. The media must contain appropriate nutrients and supplements needed by clinically significant anaerobes. A combination of enriched, nonselective, selective, and differential media should be used for the initial processing, isolation, and presumptive identification of anaerobic bacteria from clinical specimens (Fig. 4.3–1 and Tables 4.3–1 and 4.3–2) (1–3). Anaerobes have a wide range of nutritional needs; most, however, require hemin and vitamin K. Some studies suggest that freshly prepared, properly stored, highly enriched media are essential for recovery of anaerobes (4, 8), while another study has shown that prereduced anaerobically sterilized (PRAS) media best support the growth of anaerobes (7). Recent studies have suggested that using media containing oxyrase may be another alternative (9, 10). Media that have been exposed to air contain oxidized products that may delay or inhibit the growth of many anaerobes. The ideal media for use in anaerobic bacteriology, therefore, are those that have had limited exposure to oxygen.

II. SPECIMEN

The proper specimen for primary culture of anaerobes should be free of normal microbial contamination. Such contamination must be avoided to ensure proper interpretation of results and to prevent the identification of organisms that are not clinically significant. An aspirated fluid or tissue is best (*see* procedure 4.2).

III. MATERIALS

Include QC information on reagent container and in QC records.

A. The following may be employed. These media are available in dehydrated form and are prepared according to the manufacturer's directions. See Appendix 4.3–1. Commercially prepared enriched primary agar media are available from suppliers (*see* Appendix 4.3–2).

1. Enriched all-purpose medium for primary growth, e.g., brucella, CDC, Columbia, or Schaedler agar (1–3, 5)

2. Kanamycin-vancomycin-laked blood agar (KVLB or LKV) (2, 3, 5)

3. Phenylethyl alcohol agar (PEA) or Columbia nalidixic acid agar (1, 4, 9)

4. *Bacteroides* bile esculin agar (BBE) (2, 3, 5)

5. Cycloserine-cefoxitin fructose agar (CCFA) (2, 3, 5)

6. Egg yolk agar (EYA) (1–3, 5)

7. Broths

a. THIO (thioglycolate) supplemented with vitamin K_1 (menadione) (0.1 μg/ml), hemin (5 μg/ml), and a marble chip or sodium bicarbonate (1 μg/ml)

b. Chopped meat-carbohydrate or chopped meat-glucose

B. See Table 4.3–1 and Appendix 4.3–1 to this procedure for composition, purpose, interpretation, limitations, and recommended QC organisms for each medium.

C. If these media are purchased, they should be as fresh as possible to ensure their ability to cultivate anaerobes. PRAS media will provide a longer shelf life and will not have developed harmful oxidized products (7). Media containing oxyrase (9, 10) may be another alternative.

D. If media are prepared in the laboratory, they should be as fresh as possible (24 to 48 h) when used (1, 4, 8).

E. A scheme for the recommended primary processing of clinical specimens for anaerobes is given in Fig. 4.3–1 and Table 4.3–2, and their general use is shown in Table 4.3–1.

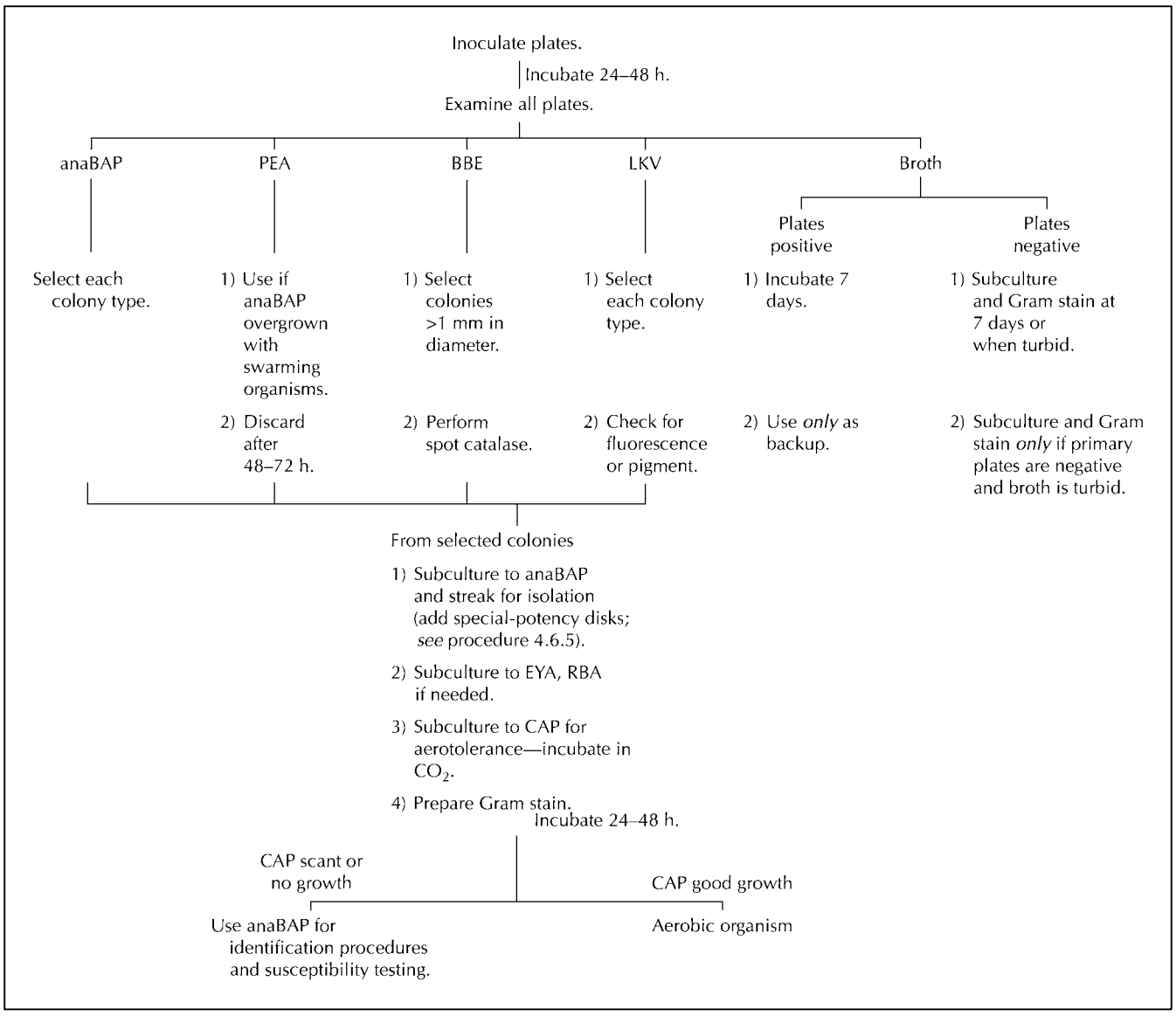

Inoculate plates.
Incubate 24–48 h.
Examine all plates.
anaBAP
PEA
BBE
LKV
Broth
Plates positive
Plates negative
Select each colony type.
1) Use if anaBAP overgrown with swarming organisms.
2) Discard after 48–72 h.
1) Select colonies >1 mm in diameter.
2) Perform spot catalase.
1) Select each colony type.
2) Check for fluorescence or pigment.
1) Incubate 7 days.
2) Use *only* as backup.
1) Subculture and Gram stain at 7 days or when turbid.
2) Subculture and Gram stain *only* if primary plates are negative and broth is turbid.
From selected colonies
1) Subculture to anaBAP and streak for isolation (add special-potency disks; *see* procedure 4.6.5).
2) Subculture to EYA, RBA if needed.
3) Subculture to CAP for aerotolerance—incubate in CO_2.
4) Prepare Gram stain.
Incubate 24–48 h.
CAP scant or no growth
CAP good growth
Use anaBAP for identification procedures and susceptibility testing.
Aerobic organism

Figure 4.3–1 Flowchart for processing primary anaerobic culture plates. anaBAP, anaerobic blood agar plate; CAP, chocolate agar plates; RBA, rabbit blood agar.

ANALYTICAL CONSIDERATIONS

IV. PROCEDURE

Observe standard precautions.

A. Inoculate a properly obtained clinical specimen onto the medium, and streak to obtain isolated colonies. The primary enriched medium may be inoculated directly with clinical material or from a broth that has been previously inoculated with clinical material. Immediately incubate anaerobically at 35°C. (*See* procedure 4.5 for anaerobic incubation techniques.)

B. Examine initially at 24 h if incubating plates in an anaerobic chamber or at 48 h if incubating plates in an anaerobic jar or anaerobic pouch. Additional periods of incubation may be necessary to recover some anaerobes (Table 4.3–1).

Table 4.3–1 Anaerobic media and their uses

Medium[a]	Purpose	Interpretation	Limitations	QC organisms
BHI blood agar Brucella blood agar CDC anaerobe agar Columbia blood agar TSA blood agar Schaedler blood agar	General all-purpose enriched primary media[b] that allow growth of all clinically significant anaerobes	Observe initially at 48 h, unless incubation system used can be opened earlier.	You must perform Gram stain and aerotolerance test. Aerobes will grow on medium.	*Clostridium perfringens* ATCC 13124 (growth) *Bacteroides fragilis* ATCC 25285 (growth) *Fusobacterium nucleatum* ATCC 25586 (growth) *Peptostreptococcus anaerobius* ATCC 27337 (growth) (Also consider *Porphyromonas levii* ATCC 29147 [growth and pigment]) *Fusobacterium necrophorum* ATCC 25286 (growth)
KVLB or LKV	Rapid isolation and selection of *Bacteroides* species and *Prevotella* spp. and earlier detection of pigment-producing strains of *Prevotella* spp.	Growth is presumptive; *Bacteroides* spp., *Prevotella* spp., or *Fusobacterium mortiferum.* Note: *Porphyromonas* spp. will not grow.	You must perform Gram stain and aerotolerance test. Yeast cells and other kanamycin-resistant organisms may grow on this medium.	*Bacteroides fragilis* ATCC 25285 (growth) *Clostridium perfringens* ATCC 13124 (no growth) *Escherichia coli* ATCC 25922 (no growth)
PEA	Inhibits facultative gram-negative rods and inhibits *Proteus* from swarming. Also prevents certain clostridia from swarming. Pigment-producing *Prevotella* and *Porphyromonas* spp. may pigment first on PEA.	Most gram-positive and gram-negative anaerobes will grow on PEA. Growth may be considered presumptive evidence of anaerobic organisms, but further testing is required.	Examine at 24–48 h. Additional time may be necessary for some slower-growing anaerobes. You must perform Gram stain and aerotolerance test.	*Bacteroides fragilis* ATCC 25285 (growth) *Proteus mirabilis* ATCC 12453 (inhibition of swarming and growth) *Escherichia coli* ATCC 25922 (no growth)
BBE	Rapid selection, isolation, and presumptive identification of *Bacteroides fragilis* group. Also allows growth and presumptive identification of *Bilophila wadsworthia.*	Supports growth of bile-resistant *Bacteroides fragilis* group organisms, which grow and form brown to black colonies. *Bilophila wadsworthia* produces small, transparent colonies with a black dot in the center (“fish eyes”).	Some strains of *Fusobacterium mortiferum, Klebsiella pneumoniae,* enterococci, and yeasts may grow to limited extents on this medium. Some anaerobic organisms that should grow on BBE may be inhibited, so inoculate nonselective medium also. You must perform Gram stain and aerotolerance test. *Bacteroides vulgatus* may not form black colonies or discolor medium.	*Bacteroides fragilis* ATCC 25285 (growth, black colonies) *Fusobacterium necrophorum* ATCC 25286 (no growth) *Escherichia coli* ATCC 25922 (no growth)

(continued)

Table 4.3–1 *(continued)*

Medium[a]	Purpose	Interpretation	Limitations	QC organisms
CCFA	Selective and differential medium for recovery and presumptive identification of *Clostridium difficile*	*Clostridium difficile* produces yellow ground glass colonies, and original pink agar turns yellow in vicinity of colonies.	Colonial morphology and Gram stain should be consistent with those of *Clostridium difficile*. Additional identification methods should be used.	*Clostridium difficile* ATCC 9689 (growth) *Bacteroides fragilis* ATCC 25285 (no growth) *Escherichia coli* ATCC 25922 (no growth)
EYA	For use when *Clostridium* spp. are suspected or when proteolytic enzyme (lecithinase or lipase) may be useful for identification of anaerobic isolate	Positive lecithinase reaction is indicated by opaque zone (white) in medium around bacterial growth. Positive lipase is indicated by iridescent sheen on surface of bacterial growth of agar. Proteolysis is indicated by total clearing around bacterial growth.	Lipase is observed under oblique light. It is best not to use all the plate so that lecithinase activity can be compared with portion of EYA acting as negative control.	*Clostridium perfringens* ATCC 13124 (positive lecithinase) *Fusobacterium necrophorum* ATCC 25286 (positive lipase) *Bacteroides fragilis* ATCC 25285 (growth but no proteolytic activity-negative control)
THIO supplemented with vitamin K_1, hemin, and marble chip or sodium bicarbonate Chopped meat-carbohydrate Chopped meat-glucose	General enrichment broth media that support growth of most anaerobes; provide backup source of culture material if anaerobic environment fails, for enrichment for small numbers, or when growth is inhibited.	Incubate at 35°C until there is growth on primary plates. If primary plates have no growth, incubate liquid medium for at least 7 days and subculture.	Examine and Gram stain broth only if plating medium reveals no growth or if chamber, jar, or pouch is not functioning. Never rely on broth cultures exclusively for isolation of anaerobes. Some anaerobes may be inhibited by metabolic products or acids produced from more rapidly growing facultative anaerobes.	*Bacteroides fragilis* ATCC 25285 (growth) *Clostridium perfringens* ATCC 13124 (growth) *Peptostreptococcus anaerobius* ATCC 27337 (growth) *Escherichia coli* ATCC 25922 (growth)

[a] See Appendix 4.3–1 for formulas of commonly used media.

[b] All media for anaerobes should contain vitamin K and hemin.

POSTANALYTICAL CONSIDERATIONS

V. LIMITATIONS OF THE PROCEDURE

A. The clinical specimen must be obtained properly and transported to the laboratory in a suitable anaerobic transport container (*see* procedure 4.2).

B. The microbiologist must be able to verify QC of the medium and determine whether the anaerobic environment (chamber, jar, or anaerobic pouch) is indeed anaerobic.

C. The microbiologist must perform aerotolerance testing on each isolate recovered from the primary enriched medium to ensure that the organism is an anaerobe (*see* procedure 4.4).

Table 4.3–2 Recommended primary medium setup

Medium[a]	Organisms inhibited	Organisms that grow
Brucella blood agar	None	All
PEA	Facultative anaerobic organisms; prevents swarming of *Clostridium* and *Proteus* spp.	Almost all anaerobes; a few organisms occasionally do not grow
BBE	Most aerobes and anaerobes, except for *Bacteroides fragilis* group, *Bilophila wadsworthia,* and some *Fusobacterium* spp.	*Bacteroides fragilis* group, *Bilophila wadsworthia,* some *Fusobacterium* spp.
LKV	All gram-positive and gram-negative facultative anaerobes and many gram-positive and gram-negative anaerobes, including most *Fusobacterium* spp., *Bacteroides ureolyticus* group, and *Porphyromonas* spp.	*Bacteroides* spp., some *Fusobacterium* spp., and pigmented and nonpigmented *Prevotella* spp.
Broth		
Enriched THIO	None	All
Chopped meat-carbohydrate	None	All
Chopped meat-glucose	None	All

[a] These and all media for isolation of anaerobes should contain vitamin K_1 and hemin.

REFERENCES

1. **Dowell, V. R., Jr., G. L. Lombard, F. S. Thompson, and A. Y. Armfield.** 1977. *Media for Isolation, Characterization, and Identification of Obligately Anaerobic Bacteria.* CDC laboratory manual. Center for Disease Control, Atlanta, GA.
2. **Finegold, S. M., P. T. Sugihara, and V. L. Sutter.** 1971. Use of selective media for isolation of anaerobes, p. 99–108. *In* D. A. Shapton and R. G. Board (ed.), *Isolation of Anaerobes.* Academic Press, Inc., London, England.
3. **Forbes, B. A., D. F. Sahm, and A. S. Weissfeld (ed.).** 2007. *Bailey and Scott's Diagnostic Microbiology,* 12th ed. Mosby Elsevier, St. Louis, MO.
4. **Hanson, C. W., and W. J. Martin.** 1976. Evaluation of enrichment, storage, and age of blood agar medium in relation to its ability to support growth of anaerobic bacteria. *J. Clin. Microbiol.* **4:**394–399.
5. **Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold.** 2002. *Wadsworth Anaerobic Bacteriology Manual*, 6th ed. Star Publishing Co., Belmont, CA.
6. **Livingston, S. J., S. D. Kominos, and R. B. Yee.** 1978. New medium for selection and presumptive identification of the *Bacteroides fragilis* group. *J. Clin. Microbiol.* **7:**448–453.
7. **Mangels, J. I., and B. P. Douglas.** 1989. Comparison of four commercial brucella agar media for growth of anaerobic organisms. *J. Clin. Microbiol.* **27:**2268–2271.
8. **Murray, P. R.** 1978. Growth of clinical isolates of anaerobic bacteria on agar media: effects of media composition, storage conditions, and reduction under anaerobic conditions. *J. Clin. Microbiol.* **8:**708–714.
9. **Thurston, M., D. Maida, and C. Gannon.** 2000. Oxyrase cell-membrane preparations simplify cultivation of anaerobic bacteria. *Lab. Med.* **31:**509–512.
10. **Wiggs, L., J. Cavallaro, and M. Miller.** 1998. Evaluation of oxyrase OxyPlate anaerobe incubation system, abstr. C-449. *Abstr. 98th Gen. Meet. Am. Soc. Microbiol.* American Society for Microbiology, Washington, DC.

APPENDIX 4.3–1

Formulas of Media for Anaerobes

General all-purpose media

A. BHI blood agar

BHI agar (BBL, Difco, Inolex)	26.0 g
agar	2.5 g
distilled water	1,000 ml

Suspend the ingredients in the water, and dissolve them by boiling. Autoclave for 15 min at 121°C and 15 lb/in^2, cool to about 45°C, and aseptically add 5% defibrinated animal blood. Mix well. Dispense into petri dishes. Final pH is approximately 7.4.

B. Brucella formulation (BBL, Difco, Inolex)

Polypeptone	23.0 g
glucose	1.0 g
yeast extract	2.0 g
sodium chloride	5.0 g
hemin (1% solution)	10.0 ml
vitamin K_1 (1% solution)	1.0 ml
L-cystine	0.4 g
agar	15.0 g
distilled water	1,000.0 ml

Suspend the ingredients in the water, and dissolve them by boiling. Autoclave for 15 min at 121°C and 15 lb/in^2, cool to about 45°C, and aseptically add 5% defibrinated animal blood. Mix well. Dispense into petri dishes. Final pH is 7.0 $\pm$ 0.2.

C. CDC anaerobe agar

TSA (BBL)	40.0 g
agar	5.0 g
yeast extract	5.0 g
hemin	5.0 mg
L-cystine	400.0 mg
vitamin K_1 stock solution	1.0 ml
distilled water	1,000.0 ml

Suspend the ingredients in the water, and dissolve them by boiling. Autoclave for 15 min at 121°C and 15 lb/in^2, cool to about 45°C, and aseptically add 5% defibrinated animal blood. Mix well. Dispense into petri dishes. Final pH is 7.5.

D. Columbia agar base

Polypeptone (BBL) or Pantone (Difco)	10.0 g
Biosate (BBL) or Bitone (Difco)	10.0 g
Myosate (BBL) or tryptic digest of beef heart	3.0 g
cornstarch	1.0 g
sodium chloride	5.0 g
agar	13.5 g
distilled or demineralized water	1,000.0 ml

Suspend the ingredients in the water, and dissolve them by boiling. Autoclave for 15 min at 121°C and 15 lb/in^2, cool to about 45°C, and aseptically add 5% defibrinated animal blood. Mix well. Dispense into petri dishes. Final pH is 7.3 $\pm$ 0.2.

E. TSA

Trypticase	15.0 g
Phytone	5.0 g
sodium chloride	5.0 g
agar	15.0 g
distilled water	1,000.0 ml

Suspend the ingredients in the water, and dissolve them by boiling. Autoclave for 15 min at 121°C and 15 lb/in^2, cool to about 45°C, and aseptically add 5% defibrinated animal blood. Mix well. Dispense into petri dishes. Final pH is 7.3.

APPENDIX 4.3–1 *(continued)*

F. Schaedler blood agar (per liter of deionized water) (BBL, Difco, Inolex)

casein peptone	5.70 g
soy peptone	1.00 g
meat peptone	2.50 g
yeast extract	3.00 g
glucose	2.80 g
hemin	0.01 g
L-cystine	0.40 g
Tris	3.00 g
sodium chloride	5.70 g
dipotassium phosphate	0.80 g
vitamin K_1	0.01 g
agar	15.00 g
sheep blood	50.00 ml

Suspend the ingredients in the water, and dissolve them by boiling. Autoclave for 15 min at 121°C and 15 lb/in^2, cool to about 45°C, and aseptically add 5% defibrinated animal blood. Mix well. Dispense into petri dishes. Final pH is 7.6 ± 0.2 at 25°C.

KVLB or LKV

Trypticase peptone	23.0 g
glucose	1.0 g
yeast extract	2.0 g
sodium chloride	5.0 g
hemin (1% solution)	10.0 ml
vitamin K_1 (1% solution)	1.0 ml
L-cystine	0.5 g
agar	15.0 g
kanamycin	100.0 mg
vancomycin	7.5 mg
sheep blood (laked)	45.5 ml
distilled water	1,000.0 ml

Treat as described for Schaedler blood agar. Mix well. Dispense into petri dishes. Final pH is 7.0 ± 0.2.

PEA (BBL, Difco, Inolex)

Trypticase peptone	23.0 g
glucose	1.0 g
yeast extract	2.0 g
sodium chloride	5.0 g
hemin (1% solution)	10.0 ml
vitamin K_1 (1% solution)	1.0 ml
L-cystine	0.4 g
agar	15.0 g
sheep blood	50.0 ml
phenylethyl alcohol	2.7 ml
distilled water	1,000.0 ml

Treat as described for Schaedler blood agar. Mix well. Dispense into petri dishes. Final pH is 7.0 ± 0.2.

BBE (BBL, Difco)

TSA	40.0 g
oxgall	20.0 g
esculin	1.0 g
ferric ammonium citrate	0.5 g
hemin solution (5 mg/ml)	2.0 ml
gentamicin solution (40 mg/ml)	2.5 ml
distilled water	1,000.0 ml

Adjust pH to 7.0, heat to dissolve, dispense into 100-ml bottles, autoclave at 121°C for 15 min, and cool to 50°C. Mix well. Dispense into petri dishes. Final pH is 7.0 ± 0.2.

APPENDIX 4.3–1 *(continued)*

***Clostridium difficile* agar (BBL, Difco)**

casein peptone	18.0 g
glucose	1.0 g
BHI-meat peptone	8.0 g
dipotassium phosphate	2.5 g
sodium chloride	5.5 g
yeast extract	2.0 g
fructose	6.0 g
neutral red	30.0 mg
cycloserine	500.0 mg
cefoxitin	16.0 mg
agar	15.0 g

Treat as described for Schaedler blood agar. Mix well. Dispense into petri dishes. Final pH is 7.4 ± 0.2 at 25°C.

EYA (BBL, Difco)

casein meat peptone	40.0 g
glucose	2.0 g
disodium phosphate	5.0 g
monopotassium phosphate	1.0 g
sodium chloride	2.0 g
magnesium sulfate	0.1 g
hemin	5.0 mg
egg yolk enrichment	100.0 ml
agar	20.0 g

Treat as described for Schaedler blood agar. Add the egg yolk enrichment to the autoclaved base. Mix, and then dispense into petri dishes. Final pH is 7.4 ± 0.2 at 25°C.

Liquid broths

A. THIO medium without indicator

peptone	20.0 g
L-cystine	0.25 g
glucose	6.0 g
sodium chloride	2.5 g
sodium thioglycolate	0.5 g
sodium sulfite	0.1 g
agar	0.7 g
distilled water	1,000.0 ml

Dispense 10 ml per tube. Autoclave for 15 min at 121°C and 15 lb/in^2. Final pH is 7.2.

B. Enriched THIO

Enriched THIO is prepared by adding to freshly prepared autoclaved medium (or to previously prepared medium that has been boiled for 10 min and then cooled) vitamin K_1 solution (0.1 μg/ml), sodium bicarbonate (1 mg/ml), and hemin (5 μg/ml). Rabbit or horse serum (10%) or Fildes enrichment (5%) may also be added.

C. Chopped-meat–glucose broth

lean ground beef	500.0 g
distilled water	1,000.0 ml
sodium hydroxide (1 N)	25.0 ml
Trypticase	30.0 g
yeast extract	5.0 g
K_2HPO_4	5.0 g
L-cystine	0.5 g
hemin (1% solution)	0.5 ml
vitamin K_1 (1% alcohol solution)	0.1 ml
glucose	3.0 g

Dispense 5 ml into screw-cap tubes. Autoclave for 15 min at 121°C and 15 lb/in^2.

D. Chopped-meat–carbohydrate broth

To the above chopped-meat–glucose formula add the following:

maltose	1.0 g
cellobiose	1.0 g
starch	1.0 g

Dispense 5 ml into screw-cap tubes. Autoclave for 15 min at 121°C and 15 lb/in^2.

APPENDIX 4.3–2

Partial List of Suppliers of Media for Anaerobes

Anaerobe Systems
15906 Concord Circle
Morgan Hill, CA 95037
(408) 782-7557
http://www.anaerobesystems.com

BD Biosciences
7 Loveton Circle
Sparks, MD 21152
(410) 316-4000
http://www.bd.com/microbiology

Hardy Diagnostics
1430 W. McCoy Ln.
Santa Maria, CA 93455
(805) 346-2766
http://www.hardydiagnostics.com

PML Microbiologicals, Inc.
27120 Southwest 95th Ave.
P.O. Box 570
Wilsonville, OR 97070
http://www.pmlmicro.com

Oxyrase, Inc.
P.O. Box 1345
Mansfield, OH 44901
(419) 589-8800
http://www.oxyrase.com

Remel, Inc.
P.O. Box 14428
12076 Santa Fe Dr.
Lenexa, KS 66215
(800) 255-6730
http://www.remelinc.com

4.4 Examination of Primary Culture Plates for Anaerobic Bacteria

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The goal of processing primary culture plates is to isolate significant anaerobic organisms present in the original specimen for identification and when susceptibility testing is indicated. The original Gram stain of the specimen is critical. At that time all morphological types should be carefully described and recorded. When evaluating culture plates, all morphotypes observed in the original Gram stain should match.

It is important to remember that anaerobic culture media permit facultatively anaerobic bacteria to grow. All bacterial isolates must be subjected to aerotolerance testing before being designated anaerobes (*see* Fig. 4.4–1).

Special precautions must be taken to avoid exposure of culture plates to oxygen during examination. Even a 10-min exposure will kill some oxygen-sensitive anaerobes (*Fusobacterium* spp., *Porphyromonas* spp., and anaerobic cocci). In addition, reduced culture media will become oxidized when exposed to air, making the media unsuitable for isolation of fastidious anaerobes.

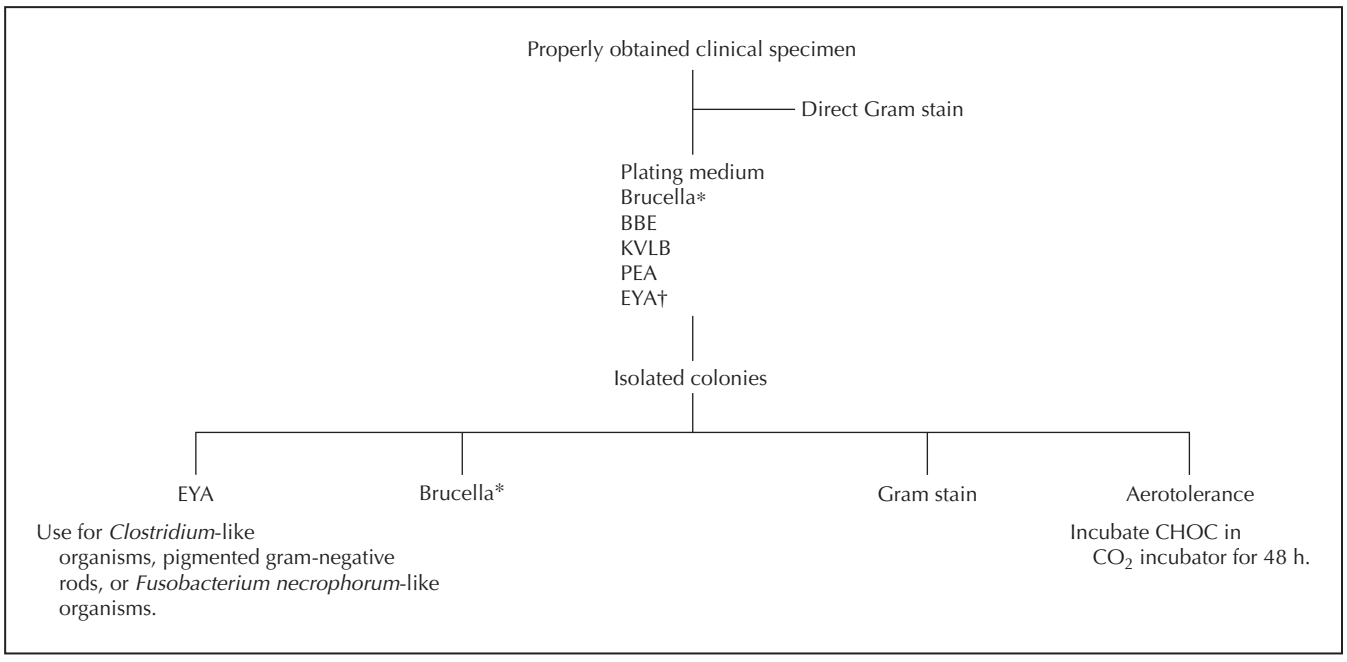


Figure 4.4–1 Procedure for the examination of primary culture plates for anaerobes. Symbols: *, use any other suitable enriched primary media that contain vitamin K and hemin and that allow good growth of anaerobes; †, use EYA if clostridia are suspected from Gram stain or from nature of clinical specimen. KVLB, kanamycin-vancomycin-laked blood agar.

II. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

1. Gram stain reagents
2. Absolute methanol stored in a screw-cap amber bottle
3. Special-potency antimicrobial agent disks (Anaerobe Systems, Becton Dickinson [BD], Hardy, PML, Remel) (*see* procedure 4.6 for details of disks to add and a list of manufacturers)
   colistin ..... 10 μg
   kanamycin ..... 1,000 μg
   vancomycin .....5 μg
4. Nitrate disk (Anaerobe Systems, BD, Hardy, PML, Remel)
   Nitrate A and nitrate B reagents, zinc powder (*see* procedure 4.6 for details)
5. Sodium polyanethol sulfonate disk (Anaerobe Systems, BD, Hardy, PML, Remel) (*see* procedure 4.6 for details)
6. 15% H_2O_2
7. *p*-Dimethylaminocinnamaldehyde (Anaerobe Systems, BD, Hardy, PML, Remel) (*see* procedure 4.6 for details)

B. Supplies

1. Clean glass microscope slides
2. Inoculating loops (sterile wooden applicator sticks may be substituted for loops). Platinum loops should be used in place of Nichrome loops.
3. Anaerobic BAP (anaBAP) (*see* procedure 4.3 for manufacturers)
4. CHOC plates (CAP) for aerotolerance testing
5. Egg yolk agar (EYA) plates for lipase and lecithinase testing
6. Rabbit blood agar (RBA) for enhancement of pigment production (optional)
7. Selective media: *Bacteroides* bile esculin (BBE), laked blood-kanamycin-vancomycin (LKV), and phenylethyl alcohol agar (PEA)
8. Other media for special circumstances are available from Anaerobe Systems, BD, Hardy, PML, or Remel.
9. Equipment (*see* procedure 4.5 for incubation techniques). The choice of one of the three anaerobic incubation systems listed depends upon volume and space limitations.
   a. Anaerobic bags or pouches (*see* procedure 4.5)
   b. Anaerobic jars (*see* procedure 4.5)
   c. Anaerobic chambers (Coy, Forma, Sheldon, Toucan) (*see* procedure 4.5)
   d. Stereoscopic microscope (7× to 15×) or hand lens (8×)
   e. UV light, 366-nm wavelength (Wood's lamp)

III. QUALITY CONTROL

Include QC information on reagent container and in QC records.

QC of media, reagents, disks, equipment, appropriate organisms, etc., is discussed in procedure 4.3 and elsewhere. Abbreviations used are also listed in that procedure.

ANALYTICAL CONSIDERATIONS

IV. PROCEDURE

See Fig. 4.4–1 for a flowchart of a procedure for the examination of primary culture plates for anaerobes.

A. Perform the initial plate examination 24 h after plate inoculation if an anaerobic chamber is used. Delay this first examination until 48 h after inoculation if anaerobic jars or bags are used.

B. Carefully examine the anaBAP with a stereoscopic microscope or hand lens. Record a detailed description of each colony type, noting such characteristics as pitting, swarming, hemolysis, pigment, "greening" of the medium, etc. These colony characteristics can provide valuable clues to the identity of the isolates when used in conjunction with rapid identification tests and Gram stain (*see* Table 4.4–1 for anaerobic organism clues and use of supplemental media).

C. Select a single, well-isolated colony of each morphological type observed. Touch each colony with a loop or sterile stick, subculture it onto anaBAP and CAP, and make a smear for Gram stain.

Table 4.4–1 Anaerobic organism clues from primary culture plates, and use of supplemental media

Colony morphology	Possible identification	Supplemental medium
Agar pitting	*Bacteroides ureolyticus* group	
Black or tan pigmentation	*Porphyromonas* spp. or pigmented *Prevotella* spp.	EYA for lipase (*Prevotella intermedia*)
Brick red fluorescence	*Porphyromonas* spp. or pigmented *Prevotella* spp. (*Porphyromonas gingivalis* does not fluoresce)	EYA for lipase (*Prevotella intermedia*)
Chartreuse fluorescence (gram-negative rod)	*Fusobacterium* spp.	EYA for lipase
Chartreuse fluorescence (gram-positive rod)	*Clostridium difficile* or *Clostridium innocuum*	CCFA
Double zone of beta hemolysis	*Clostridium perfringens*	EYA for lecithinase
"Fried egg"	*Fusobacterium necrophorum, Fusobacterium varium*	EYA for lipase, BBE for bile growth
"Greening" of medium	*Fusobacterium* spp.	EYA for lipase
Large with irregular margin	*Clostridium* spp.	EYA for proteolytic activity
"Medusa-head"	*Clostridium septicum*	PEA
"Molar tooth"	*Actinomyces* spp.	
Pink to red colony (gram-positive rod)	*Actinomyces odontolyticus*	
Speckled or "breadcrumb"	*Fusobacterium nucleatum*	
Swarming growth	*Clostridium septicum, Clostridium sordellii Clostridium tetani*	PEA to prevent swarming

IV. PROCEDURE *(continued)*

1. anaBAP
   a. Streak the subcultured organism in four quadrants to obtain isolated colonies.
   b. If the organism is gram negative, add special-potency kanamycin, vancomycin, and colistin antimicrobial agent disks to first quadrant of this plate. Add a nitrate disk to the second or third quadrant.
   c. If the organism is a gram-positive coccus, add nitrate and sodium polyanethol sulfonate disks (from Anaerobe Systems, BD, Hardy, PML, or Remel) only. The addition of special-potency antimicrobial agent disks does not help in further characterization of gram-positive organisms.
   d. If the organism is a gram-positive rod, add a nitrate disk only. The addition of special-potency antimicrobial agent disks does not help in further characterization of gram-positive organisms.
   e. Incubate anaerobically at 35 to 37°C for 24 to 48 h. Table 4.4–2 outlines results to expect with special-potency antimicrobial agent disks. Refer to procedure 4.10 for further details on identification of gram-negative organisms and procedure 4.11 for further details on identification of gram-positive organisms.

Include QC information on reagent container and in QC records.

2. CHOC
   a. Divide this plate into quadrants, and subculture four organisms onto each plate.
   b. Incubate at 35 to 37°C in a 5% CO_2 environment for 24 to 48 h to detect slow-growing aerobic organisms such as *Capnocytophaga*, *Actinobacillus*, and *Eikenella* spp.

Table 4.4–2 Special-potency antimicrobial agent disks for the identification of anaerobic bacteria[a]

Organisms	Response[b] to:		
	Kanamycin, 1,000 μg	Vancomycin, 5 μg	Colistin, 10 μg
Gram-positive organisms	V	S[c]	R
Bacteroides fragilis group	R	R	R
Bacteroides ureolyticus group	S	R	S
Fusobacterium spp.	S	R	S
Porphyromonas spp.	R	S[d]	R
Prevotella spp.	R	R	V
Gram-negative cocci	S	R	S

[a] Adapted with permission from H. R. Jousimies-Somer, P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold, *Wadsworth-KTL Anaerobic Bacteriology Manual*, 6th ed., 2002, Star Publishing Co., Belmont, CA.

[b] R, resistant; S, susceptible; V, variable.

[c] Exceptions: rare strains of *Lactobacillus* spp. and *Clostridium* spp. may be vancomycin resistant.

[d] *Porphyromonas* spp. are vancomycin susceptible but fluoresce or are pigmented.

IV. PROCEDURE *(continued)*

c. Use only CHOC for aerotolerance testing. *Haemophilus* spp. will grow anaerobically on BAP and therefore will be mistaken for anaerobic gram-negative rods if CHOC is not used.

d. Some authorities have suggested performing aerotolerance testing in multiple environments (ambient air, CO_2, microaerophilic environment) on problem isolates. This is not necessary for identification of the most commonly isolated anaerobes but may be considered for isolates when their exact atmospheric requirement is difficult to determine.

3. Gram stain

a. Air dry the smear. It is preferred not to heat fix the slide as heat can distort the morphology of many anaerobes. Fix smears by flooding the slide with absolute methanol. Methanol should also be used when fixing smears prepared from colonies.

b. Drain the methanol from the slide after 1 min, and then immediately begin your normal Gram stain procedure. Complete the Gram stain according to standard procedure.

Include QC information on reagent container and in QC records.

D. Examine selective media such as PEA, BBE, and LKV (*see* procedure 4.3 for media, manufacturers, and usage and Table 4.3–1 for QC organisms to use on these media).

1. Pick any colonies on PEA that are different from the colonies isolated on the anaBAP. The PEA plate may be used in place of the anaBAP if the culture is overgrown with swarming *Clostridium* spp., *Proteus* spp., or other organisms. PEA may also provide earlier detection of pigmented anaerobic organisms (*see* procedure 4.6).

2. Pick all the different colonies growing on BBE that are >1 mm in diameter. Record the esculin hydrolysis reaction (black = positive), and perform a spot catalase test on each colony type.

3. Pick all colony types isolated on LKV. Check organisms for pigment or fluorescence by exposing the plate to UV (366-nm) light. Subculture organisms onto RBA to enhance pigment production (optional).

4. Process organisms isolated from selective media using the schema described for isolates from the anaBAP. Subculture onto anaBAP and CHOC, and add appropriate disks according to Gram stain reaction.

E. Use of supplemental media (EYA, RBA, BBE, etc.) can aid in the rapid identification of anaerobic organisms (*see* Table 4.4–1). These media provide evidence of lipase and/or lecithinase production (EYA), pigmentation (RBA), abil-

IV. PROCEDURE *(continued)*

ity to grow in the presence of 20% bile (BBE), and other useful characteristics. Divide the supplemental plates into quadrants, and subculture four organisms to a plate. Use Table 4.4–1 to select supplemental media on the basis of colony morphology and to provide anaerobic organism clues.

F. Broth culture

1. No growth on original plates

a. Examine the broth culture daily for evidence of growth.

b. Prepare a Gram stain, and subculture onto anaBAP, PEA, BBE, LKV, and CAP as soon as growth is apparent. Use the addition of broth *only* as a backup; i.e., subculture only when anaerobic systems fail or when primary plates are negative but the broth is turbid.

c. Incubate negative broth cultures for 7 days, examine visually, and discard appropriately.

d. Refer to procedure 4.3 for medium and broth usage (Fig. 4.3–1).

2. Growth on original plates

If there is growth on the primary plates, subculture of the backup broth is generally not helpful and can lead to needless duplication. If, on the other hand, *Actinomyces* is suspected, the backup broth can be occasionally helpful to recover this slow-growing organism, which may not grow readily on solid media.

3. Subsequent plate examination

a. Incubate primary anaBAP for 5 to 7 days. Examine the primary plates at 24- to 48-h intervals, depending on the type of anaerobic environment employed. Isolate and perform aerotolerance tests on any new colony types that appear.

b. Pigmented *Prevotella* spp., *Porphyromonas* spp., and *Actinomyces* spp. commonly appear after 2 to 3 days of incubation. Examine the primary anaBAP for fluorescent and pigmented organisms. Use the stereoscope to check for the characteristic "molar tooth" colonies of *Actinomyces* spp.

c. *Bilophila wadsworthia* commonly appears after 3 to 4 days of incubation, generally first observed from the BBE medium. This organism appears as small, translucent colonies with a black center and can resemble "fish eyes."

d. Discard PEA and BBE plates after 4 days of incubation. These media lose their selective properties upon incubation because of evaporation and antibiotic degradation. Secondary growth will occur as these media lose selectivity.

POSTANALYTICAL CONSIDERATIONS

V. REPORTING RESULTS

Appendix 4.4–1 is an example of a worksheet that can be used on computer or on paper, depending upon what the laboratory utilizes for workup.

SUPPLEMENTAL READING

Dowell, V. R., Jr. 1989. *Procedures for Isolation and Characterization of Anaerobic Bacteria.* Centers for Disease Control, Atlanta, GA.

Engelkirk, P. G., J. Duben-Engelkirk, and V. R. Dowell, Jr. 1992. *Principles and Practice of Clinical Anaerobic Bacteriology.* Star Publishing Co., Belmont, CA.

Forbes, B. A., D. F. Sahm, and A. S. Weissfeld (ed.). 2007. *Bailey and Scott's Diagnostic Bacteriology,* 12th ed. Mosby Elsevier, St. Louis, MO.

Holdeman, L. V., E. P. Cato, and W. E. C. Moore. 1977. *Anaerobe Laboratory Manual,* 4th ed., p. 2–4, 122, 149. Virginia Polytechnic Institute and State University, Blacksburg.

Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold. 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual,* 6th ed. Star Publishing Co., Belmont, CA.

Mangels, J. I., M. E. Cox, and L. H. Lindberg. 1984. Methanol fixation—an alternative to heat fixation of smears before staining. *Diagn. Microbiol. Infect. Dis.* **2:**129–137.

Murray, P. R., E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.). 2007. *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.

APPENDIX 4.4–1

Example of a Possible Computer Screen To Use for Workup of Anaerobe Isolates

SAMPLE #_____ DATE_____ SPEC. TYPE_____

1º GRAM STAIN_____

_____

_____

ISOLATE #_____ FINAL ID_____

FROM: anaBAP_____ BBE____ LKV_____ PEA_____ AEROTOL. RESULT_____

COLONY MORPH_____ GRAM_____

C 10_____ K 1000_____ V 5_____

IND____ CAT____ SPS____ NIT____ ESC____ BILE GR_____ LIP____

LEC___ PRO___ UREA___ FLUOR___ HEMO___ PIG___ PIT___ OTHER TEST___

_____

ISOLATE #_____ FINAL ID_____

FROM: anaBAP_____ BBE____ LKV_____ PEA_____ AEROTOL. RESULT_____

COLONY MORPH_____ GRAM_____

C 10_____ K 1000_____ V 5_____

IND____ CAT____ SPS____ NIT____ ESC____ BILE GR_____ LIP____

LEC___ PRO___ UREA___ FLUOR___ HEMO___ PIG___ PIT___ OTHER TEST___

_____

ISOLATE #_____ FINAL ID_____

FROM: anaBAP_____ BBE____ LKV_____ PEA_____ AEROTOL. RESULT_____

COLONY MORPH_____ GRAM_____

C 10_____ K 1000 _____ V 5 _____

IND____ CAT____ SPS____ NIT____ ESC____ BILE GR_____ LIP____

LEC___ PRO___ UREA___ FLUOR___ HEMO___ PIG___ PIT___ OTHER TEST___

_____

4.5 Incubation Techniques for Anaerobic Bacteriology Specimens

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

This procedure describes the various environmental methods and incubation conditions used for anaerobic bacteriology specimens once a properly selected, collected, and transported specimen arrives in the laboratory. Ideally, the specimen is processed immediately on arrival in the laboratory and incubated under anaerobic conditions to prevent further exposure to oxygen. Refer to procedure 4.2 for collection procedures, to procedure 4.3 for anaerobic media, and to procedure 4.4 for processing and inoculation techniques.

In general, growing cultures must not be exposed to oxygen until after 48 h of incubation, since anaerobes are most sensitive to oxygen during the log phase of growth. One of the obvious advantages of using an anaerobic chamber is that technologists can inspect inoculated media without removing them from the anaerobic environment of the chamber at any time.

II. SPECIMENS

It is imperative that these cultures be handled in a biological safety cabinet.

Observe standard precautions.

All primary bacteriology specimens should be collected, processed, inoculated, and incubated according to the techniques described in section 2 of this handbook and in procedures 4.2 to 4.4. When preparing specimens for culture, observe standard precautions at all times. All steps in the processing of a specimen should be conducted either in an anaerobic chamber or in a laminar-flow safety hood.

A good transport system and a good specimen (fluid or tissue) permit the laboratory to batch inoculate and incubate plates at convenient times throughout the day without jeopardizing the recovery of anaerobes.

ANALYTICAL CONSIDERATIONS

III. INCUBATION OF SPECIMENS

Observe standard precautions.

Immediately place inoculated plates into an anaerobic environment, and incubate them at 35 to 37°C for 48 h. If you are using an anaerobic chamber, inoculated plates may be examined at 24 h. Some anaerobes require a longer incubation depending on their growth requirements. *Monitor the incubator temperature daily.*

Alternatively, some laboratories may use a holding system (a jar, box, or small chamber) to store uninoculated plates or inoculated plates under near-anaerobic conditions until they are placed into an anaerobic chamber or jar. The holding system is constantly flushed with a light flow of oxygen-free gas (nitrogen is preferred, since carbon dioxide may change the pH of the medium). Inoculated plates should not remain in the holding jar for extended periods (i.e., not longer than 1 h) at room temperature. The holding jar should remain as anaerobic as possible, and care should be taken to minimize convection currents whenever freshly inoculated plates are added to the jar. These holding systems are often used to collect a sufficient number of inoculated plates before the final anaerobic system used in the laboratory is set up. However, a holding jar may not be needed if the laboratory batch inoculates specimens periodically throughout the day.

IV. INCUBATION SYSTEMS

Include QC information on reagent container and in QC records.

The choice of incubation systems is influenced by cost, the number of anaerobic cultures performed, and space limitations. The anaerobic environment is monitored with a methylene blue strip or resazurin indicator (Becton Dickinson, Hardy, PML, Remel). These indicators, initially blue and pink (respectively), become colorless with low concentrations of oxygen. *Monitor the indicator strip daily*. Alternatively, monitor the anaerobic system with biological indicators by incubating a plate freshly inoculated with a fastidious anaerobe such as *Fusobacterium nucleatum* ATCC 25586, *Porphyromonas levii* ATCC 29147, or *Clostridium novyi* type B ATCC 25758. The most common choices of anaerobic incubation systems are the following.

A. Anaerobic chamber

See Appendix 4.5–1 at the end of this procedure for manufacturers.

1. Anaerobiosis is maintained in a gastight box or chamber by a gas mixture containing 80 to 90% nitrogen (N_2), 5% hydrogen (H_2), and 5 to 10% carbon dioxide (CO_2) and by using a palladium catalyst. Usually the systems have a positive pressure inside the chamber. The catalyst reduces oxygen to water, thus removing atmospheric oxygen from the device. Carbon dioxide is included because many anaerobes require it for growth.
2. Add anaerobic indicator or biological indicator to chamber interior. Place indicator in empty petri dish to prevent drying.
3. Humidity is controlled by the absorption of water formed in the catalytic conversion with silica gel crystals. In other chambers, humidity is controlled with a "cold spot" that condenses excess humidity and allows the water formed to be removed through a drain.
4. Change catalyst daily.
5. Plates are incubated at 35 to 37°C and can be examined at any time within the chamber (generally at 24 to 48 h).

B. Anaerobic bag or pouch

See Appendix 4.5–1 for manufacturers.

1. Pouch systems exist that create anaerobic conditions with a sachet that absorbs atmospheric oxygen without the generation of hydrogen, without the addition of water, and without requiring a catalyst. A methylene blue or resazurin indicator strip is used to monitor anaerobic conditions.

   ◩ **NOTE:** The resulting carbon dioxide level produced in these systems is generally higher than 10%.
2. Other bag or pouch systems generate gas to create anaerobic conditions. An envelope or ampoule containing reagents either is crushed or has water added to it to begin a chemical reaction that provides an atmosphere of 80 to 90% N_2, 5% H_2, and 5 to 10% CO_2. A new catalyst should be used *each time* to convert hydrogen and oxygen to water. A resazurin or methylene blue indicator strip is used to monitor anaerobic conditions.
3. Activate the generating envelope, ampoule, or sachet, add an anaerobic indicator, and seal the bag or pouch. Incubate the bag at 35 to 37°C in a standard incubator for 48 h. This prevents exposure of smaller colonies to oxygen.
4. Remove the plates from the bag to examine them and work up the organisms as quickly as possible.
5. Reseal bag and pouches and use a new generating envelope, ampoule, or sachet.

C. Anaerobic jars

See Appendix 4.5–1 for a list of manufacturers.

1. Place inoculated plates into a self-contained jar containing a catalyst; a gas-generating system (usually an envelope, ampoule, or sachet) providing an

IV. INCUBATION SYSTEMS *(continued)*

atmosphere of 80 to 90% N_2, 5% H_2, and 5 to 10% CO_2; and an anaerobic indicator. If a sachet is employed, hydrogen is not produced and a catalyst is not required (*see* item IV.B above). Add an anaerobic indicator to the jar.

2. Close the jar, and incubate it at 35 to 37°C in a standard incubator.
3. Incubate the plates for 48 h before opening the jar. This prevents exposure of smaller colonies to oxygen.
4. The catalyst, composed of palladium-coated alumina pellets, should be fresh or rejuvenated each time prior to use unless a catalyst is included in the gas pack envelope or a waterless anaerobic generating system is used.
5. Remove the plates from the bag or jar to examine them and work up the organisms as quickly as possible.
6. Reseal jar and use a new generating envelope, ampoule, or sachet.

D. Anoxomat Mark II

The Anoxomat is a system for culturing anaerobes and microaerophilic bacteria rapidly and automatically in an anaerobic jar. The Anoxomat uses precise pressure measurements to control the evacuation and refilling of the anaerobic jar. An embedded computer guides the process. The evacuated air is replaced by up to three different gases (usually the standard anaerobic gas mixture, plus CO_2, and an H_2 mixture). The use of gas connections depends on the gas composition the customer requires in the jars.

POSTANALYTICAL CONSIDERATIONS

V. PROCEDURE NOTES

A. A clinical laboratory that receives very few requests for anaerobic culture (one per day) and/or receives a rare anaerobic specimen after normal laboratory hours may consider the use of anaerobic bags or pouches. Bags and pouches are convenient and easy to use, but they are the most expensive way of producing an anaerobic environment (about $6.00 per bag).

B. A clinical laboratory that receives perhaps three or four specimens per day for anaerobic culture may consider the use of anaerobic jars. Anaerobic jars may be most economically employed if the laboratory batches the processing of specimens at convenient times rather than using one jar for one specimen. If the laboratory receives a specimen at odd times after jars have been closed, perhaps the new specimen may be incubated in a pouch or bag and then after 48 h included in an anaerobic jar.

C. For a laboratory that may receive perhaps five or more specimens per day, the most economical way of producing an anaerobic atmosphere is by using an anaerobic chamber. The laboratory would need to consider the initial expense and the space required for the chamber. The ability to examine cultures at 24 h and report the presence of anaerobes earlier using an anaerobic chamber may also be a patient care benefit for the hospital. Many laboratories, however, find that jars work well for them even if they may receive 5 to 10 specimens for anaerobic culture per day. These decisions depend upon the daily operation of the laboratory and financial considerations.

SUPPLEMENTAL READING

Engelkirk, P. G., J. Duben-Engelkirk, and V. R. Dowell, Jr. 1992. *Principles and Practice of Clinical Anaerobic Bacteriology.* Star Publishing Co., Belmont, CA.

Forbes, B. A., D. F. Sahm, and A. S. Weissfeld (ed.). 2007. *Bailey and Scott's Diagnostic Bacteriology*, 12th ed. Mosby Elsevier, St. Louis, MO.

Isenberg, H. D. (ed.). 1992. *Clinical Microbiology Procedures Handbook.* American Society for Microbiology, Washington, DC.

Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold. 2002. *Wadsworth Anaerobic Bacteriology Manual*, 6th ed. Star Publishing Co., Belmont, CA.

Shahin, M., W. Jamal, T. Verghese, and V. O. Rotimi. 2003. Comparative evaluation of anoxomat and conventional anaerobic GasPak jar systems for the isolation of anaerobic bacteria. *Med. Princ. Pract.* **12:**81–86.

APPENDIX 4.5–1

Partial List of Manufacturers

Anaerobic bags or pouches

BD Biosciences
7 Loveton Circle
Sparks, MD 21152
http://www.bd.com/microbiology

Oxoid
800 Proctor Ave.
Ogdenburg, NY 13669
http://www.oxoid.com

Mitsubishi Gas Chemical America, Inc.
520 Madison Ave., 17th Floor
New York, NY 10022
http://www.mgc-a.com

Anaerobic jars

BD Biosciences
7 Loveton Circle
Sparks, MD 21152
http://www.bd.com/microbiology

Hardy Diagnostics
1430 W. McCoy Ln.
Santa Maria, CA 93455
http://www.hardydiagnostics.com

PML Microbiologicals, Inc.
27120 Southwest 95th Ave.
P.O. Box 570
Wilsonville, OR 97070
http://www.pmlmicro.com

Remel, Inc.
P.O. Box 14428
12076 Santa Fe Dr.
Lenexa, KS 66215
http://www.remelinc.com

Anaerobic chambers

Coy Laboratory Products
14500 Coy Dr.
Grass Lake, MI 49240
http://www.coylab.com

Forma Scientific, Inc.
Millcreek Rd.
P.O. Box 649
Marietta, OH 45750
http://www.forma.com

Sheldon Manufacturing, Inc.
300 North 26th Ave.
Cornelius, OR 97113
http://www.shellab.com

Toucan Technologies, Inc.
1158 Altadena Dr.
Cincinnati, OH 45230
http://www.toucantek.net

Anoxomat
Mart Microbiology B.V.
P.O. Box 165
7130 AD Lichtenvoorde
The Netherlands
www.anoxomat.com

4.6.1 Introduction

Rapid disk, spot tests, and other methods described in this procedure provide a cost-effective system for the identification of anaerobes. Many of the tests described cost less than $0.25 each to perform (*see* Appendix 4.6–1 (turn to 4.6.13) for a summary of tests used for the rapid identification of anaerobes).

Depending upon the source, the type of isolate recovered, the type of patient, and the needs of the physician, the tests described in this procedure are adequate to permit a rapid presumptive identification of the isolate which may be sufficient in some situations for patient care.

4.6.2 Spot Indole Test

I. PRINCIPLE

The indole test is important in the grouping and identification of anaerobic bacteria. Indole is split from tryptophan by certain organisms possessing the enzyme tryptophanase. The indole test specifically detects indole and is based on the formation of a colored adduct complex when indole reacts with the aldehyde group of the test reagent (*p*-dimethyl-aminocinnamaldehyde [DMACA]). To perform the test, the growth medium must contain tryptophan (BAP or egg yolk agar [EYA]).

II. SPECIMEN

A. The specimen for the spot indole test consists of a 24- to 48-h pure culture on an agar medium that contains sufficient tryptophan, such as brucella blood agar or EYA.

B. Do not use a plate that also contains a nitrate disk, because the nitrate disk may cause a false-negative indole result.

C. Since the enzyme that degrades tryptophan diffuses into the agar, only pure cultures of the test organism can be present. For example, a few colonies of a common contaminant, *Propionibacterium acnes*, or another indole-producing organism can cause an erroneous result.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

Perform and record QC as required. Include expiration date on label.

1. Prepare DMACA as follows.
DMACA (Sigma-Aldrich) ….1 g
hydrochloric acid
(10%, vol/vol) ……100 ml
  a. DMACA is carcinogenic, so use gloves, and work in a fume hood when preparing the reagent.
  b. Dissolve DMACA in the hydrochloric acid solution in a glass flask.
  c. Store refrigerated (4 to 6°C) in a dark bottle, and label "Indole reagent DMACA," with the date of preparation and an expiration date of 2 months.

2. DMACA is also available commercially (Anaerobe Systems, Becton Dickinson [BD], Hardy, PML, Remel; *see* Appendix 4.6–2 for addresses).

B. Supplies

1. Filter paper
2. Clean empty petri dish or glass slide
3. Wooden sticks or bacteriological loops

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Check the spot indole reagent when it is prepared and monthly thereafter.

B. Test *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 as described below under item V. The results of the test should show the following.

1. *E. coli:* indole positive
2. *P. aeruginosa:* indole negative

C. Record the results on a QC log (*see* Appendix 4.6–3 for a sample QC sheet).

V. PROCEDURE

A. Place a piece of filter paper (no. 1 Whatman) in petri dish cover, or place a small piece of filter paper on the surface of a glass slide.

B. Moisten paper with reagent. Paper should be saturated but not dripping wet.

C. Remove several colonies from agar with a wooden stick or loop, and rub them on the filter paper. When testing anaerobic bacteria, it is best to use a heavy inoculum, because the reaction may be fairly weak.

D. You can perform several tests on a single filter paper while it is wet.

Caution: *Do not use a plate that has a nitrate disk on it: a positive nitrate test can interfere with the spot indole test by causing a false-negative result.*

VI. RESULTS

A. Indole positive: development of a blue or green color on the filter paper around the inoculum within 30 s

The dark-pigmented organisms may give a greenish color. In addition, the color development may be masked by the pigment, so examine the filter paper with care.

B. Indole negative: no color change or pinkish color

Color development after 30 s should be disregarded.

SUPPLEMENTAL READING

Engelkirk, P. G., J. Duben-Engelkirk, and V. R. Dowell, Jr. 1992. *Principles and Practice of Clinical Anaerobic Bacteriology*. Star Publishing Co., Belmont, CA.

Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold. 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual,* 6th ed. Star Publishing Co., Belmont, CA.

Lombard, G. L., and V. R. Dowell, Jr. 1983. Comparison of three reagents for detecting indole production by anaerobic bacteria in microtest systems. *J. Clin. Microbiol.* **18:**609–613.

MacFaddin, J. F. 2000. *Biochemical Tests for Identification of Medical Bacteria,* 3rd ed. Lippincott Williams & Wilkins, Philadelphia, PA.

4.6.3 Nitrate Disk Reduction Test

I. PRINCIPLE

Nitrate can be reduced to nitrite and other reduction products by organisms possessing the enzyme nitrate reductase. Certain anaerobic bacteria are capable of reducing nitrate, and testing for it is useful in determining the species of anaerobic organisms and in grouping them. The presence of nitrites can be demonstrated by naphthylamide and sulfanilic acid reagents, which form a red diazonium dye when reacting with nitrite. If the organism is capable of reducing nitrite further, the test for nitrite will give a negative result. Therefore, all negative results must be confirmed by the addition of metallic zinc. Zinc catalyzes the reduction of nitrate to nitrite, and the development of a red color after zinc is added indicates the presence of residual nitrates and confirms a negative reaction. If no color develops, nitrate was reduced beyond nitrite (positive test).

II. SPECIMEN

The specimen is any isolated colony on primary or subculture plates.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

Perform and record QC as required. Include expiration date on label.

1. Nitrate disks

a. Combine the following in a flask.

potassium nitrate ……. 30 g
sodium molybdate dihydrate …… 0.1 g
distilled water ……100 ml

b. After dissolving these, sterilize by filtration (0.22-µm-pore-size filter).

c. Dispense 20-µl quantities of the solution onto sterile 1/4-in. filter paper disks that are spread inside empty, sterile petri dishes. Allow the disks to dry at room temperature for 72 h before collecting them in storage containers (e.g., glass vials).

d. Store at room temperature. Label with expiration date of 1 year.

2. Nitrate reagents

a. Nitrate A

sulfanilic acid …… 0.5 g
glacial acetic acid …… 30 ml
distilled water ……120 ml

b. Nitrate B

1,6-Cleve's acid (5-amino-2-naphthalene sulfonic acid) ……. 0.2 g
glacial acetic acid …….. 30 ml
distilled water ……120 ml

(1) Dissolve the ingredients of each solution in distilled water in separate containers.

(2) Store refrigerated (4 to 6°C) in dark bottles, and label "Nitrate A" and "Nitrate B," with date of preparation and expiration date of 3 months.

3. Disks and reagents are also commercially available (disks and reagents from Anaerobe Systems, Becton Dickinson, Hardy, PML, and Remel) (*see* Appendix 4.6–2).

4. Zinc

B. Supplies

1. Clean empty petri dish or glass slide

2. Dropper bottles or pipettes

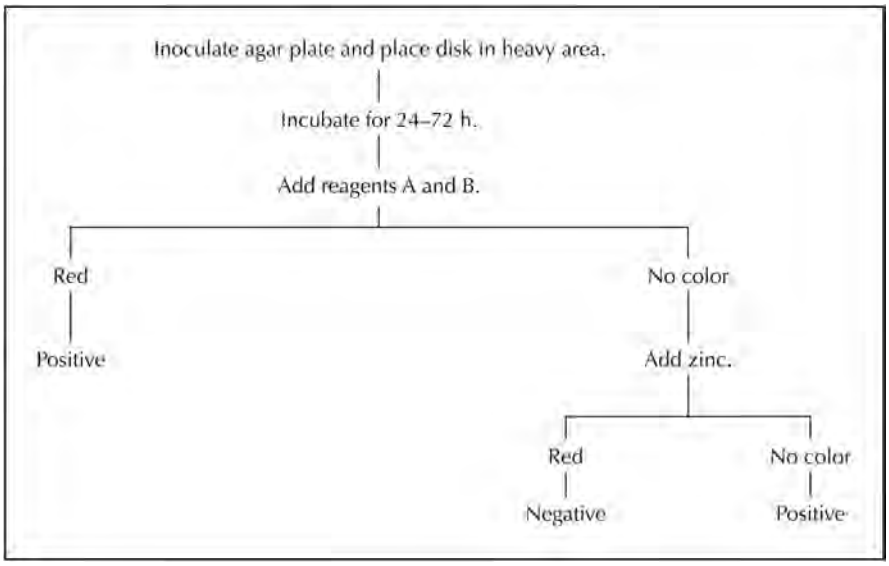


Figure 4.6.3–1 Flowchart for nitrate disk reduction test.

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Check the disks and reagents when they are prepared and monthly thereafter.

B. Inoculate *Escherichia coli* ATCC 25922 and *Acinetobacter lwoffii* ATCC 43498 on BAP, and test as described below under item V. The results should show the following.

1. *E. coli:* nitrate positive, development of red color

2. *A. lwoffii:* nitrate negative, no color change; after addition of zinc, development of red color

C. Record the results on a QC log (*see* Appendix 4.6–3 for a sample QC sheet).

V. PROCEDURE

See Fig. 4.6.3–1 for a flowchart of these procedures.

A. Inoculate organism on brucella or other nonselective anaerobic BAP.

B. Place the nitrate disk on the most heavily inoculated area.

C. Incubate anaerobically for 24 to 72 h at 35 to 37°C until heavy growth occurs around the disk.

D. Remove the disk from the surface of the plate, and place it in a clean petri dish or on a slide.

E. Add 1 drop each of nitrates A and B. If no color develops within 5 min, add a pinch of zinc dust or zinc granules to the disk and wait 5 min.

◩ **NOTE:** See procedure 4.6.2 for limitation of a positive nitrate disk reaction causing a false-negative spot indole test.

VI. RESULTS

A. Positive reaction: development of red or pink color after the reagents are added *or* no color development after zinc is added

B. Negative reaction: no color development after the reagents are added *and* development of red color after zinc is added

C. *Hint:* The red color of a positive test may be difficult to distinguish from the red color of the anaerobic BAP. Tilting the plate to allow the red liquid to flow to the edge improves detection.

SUPPLEMENTAL READING

Engelkirk, P. G., J. Duben-Engelkirk, and V. R. Dowell, Jr. 1992. *Principles and Practice of Clinical Anaerobic Bacteriology*. Star Publishing Co., Belmont, CA.

Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold. 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual*, 6th ed. Star Publishing Co., Belmont, CA.

MacFaddin, J. F. 2000. *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Lippincott Williams & Wilkins, Philadelphia, PA.

4.6.4 Catalase Test

I. PRINCIPLE

Some anaerobic bacteria possess catalase, an enzyme that decomposes hydrogen peroxide (H_2O_2) into oxygen and water. When a catalase-positive organism is emulsified with H_2O_2, it releases oxygen, which is detected by the formation of bubbles. *A 15% solution of H_2O_2 instead of the conventional 3% is preferred for anaerobic bacteria.*

II. SPECIMEN

The specimen should show adequate growth on primary plates or subculture plates. Since RBCs contain some catalase, it is recommended that a medium without blood, such as egg yolk agar, be used.

III. MATERIALS

A. Reagent

H_2O_2, 15% (Anaerobe Systems; *see* Appendix 4.6–1)
Store in a dark bottle, and keep refrigerated (4 to 6°C) when not in use.

B. Supplies

1. Clean empty petri dish or glass slide
2. Wooden sticks or loops

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Test hydrogen peroxide before using it and every day of use thereafter.

B. Test *Staphylococcus aureus* ATCC 25923 and *Streptococcus pyogenes* ATCC 19615 as described below under item V. The results should be as follows.

1. *S. aureus:* strong positive reaction
2. *S. pyogenes:* negative reaction

C. Record the results on a QC log (*see* Appendix 4.6–3 for a sample QC sheet).

V. PROCEDURE

A. Touch the center of a pure colony with a loop or sterile wooden stick, and transfer the sample onto the surface of a clean, dry glass slide or petri dish. If you are testing growth from blood-containing medium, avoid carryover of the agar.

B. Add 1 drop of 15% hydrogen peroxide to the smear. Do not introduce a platinum loop into the drop, because this may cause a false-positive reaction.

C. Observe for immediate bubbling.

D. An alternative is to add 1 drop of 15% hydrogen peroxide directly to the growth on a medium that does not contain blood and then observe for bubble formation.

VI. RESULTS

A. Positive reaction: immediate bubbling of the hydrogen peroxide

B. Negative reaction: no bubbling

Formation of rare bubbles after 20 to 30 s is considered a negative catalase test.

Precaution: Some bacteria may possess enzymes other than catalase that can decompose hydrogen peroxide.

SUPPLEMENTAL READING

Engelkirk, P. G., J. Duben-Engelkirk, and V. R. Dowell, Jr. 1992. *Principles and Practice of Clinical Anaerobic Bacteriology*. Star Publishing Co., Belmont, CA.

Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold. 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual*, 6th ed. Star Publishing Co., Belmont, CA.

MacFaddin, J. F. 2000. *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Lippincott Williams & Wilkins, Philadelphia, PA.

4.6.5 Identification by Using Special-Potency Disks

I. PRINCIPLE

Special-potency disks of vancomycin (5 µg), kanamycin (1,000 µg), and colistin (10 µg) are used as an aid in determining the Gram stain reaction of anaerobes as well as in preliminary categorization of some anaerobic genera and species (Table 4.6.5–1). In general, gram-positive organisms are resistant to colistin and susceptible to vancomycin, while most gram-negative organisms are resistant to vancomycin. This difference is especially useful with some clostridia that consistently stain gram negative.

II. SPECIMEN

The specimen is any isolated colony on primary or subculture plates.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

Perform and record QC as required. Include expiration date on label.

1. Special-potency antimicrobial agent disks are commercially available (Anaerobe Systems, Becton Dickinson, Hardy, PML, Remel; *See* Appendix 4.6–1).

vancomycin ……5 µg
kanamycin …… 1,000 µg
colistin …… 10 µg

a. Store a small supply of disks (one carton each) in a tight container with desiccants in a refrigerator.

b. Store the stock supply at −20°C.

2. Brucella or other anaerobic BAP

B. Supplies

1. Single-disk dispenser or forceps

2. Ruler (divided into millimeters)

Table 4.6.5–1 Identification by means of special-potency antimicrobial agent disks[a]

Organisms	Response[b] to:		
	Kanamycin, 1,000 µg	Vancomycin, 5 µg	Colistin, 10 µg
Gram positive	V	S[c]	R
Gram negative	V	R	V
Bacteroides fragilis group	R	R	R
Bacteroides ureolyticus group	S	R	S
Fusobacterium spp.	S	R	R
Porphyromonas spp.	R	S[d]	R
Prevotella spp.	R	R	V
Veillonella spp.	S	R	S

[a] Adapted with permission from H. R. Jousimies-Somer, P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold, *Wadsworth-KTL Anaerobic Bacteriology Manual*, 6th ed., 2002, Star Publishing Co., Belmont, CA.

[b] R, resistant; S, susceptible; V, variable.

[c] Exceptions: rare strains of *Lactobacillus* spp. and *Clostridium* spp. may be vancomycin resistant.

[d] *Porphyromonas* spp. are vancomycin susceptible but fluoresce or are pigmented.

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Test special-potency antimicrobial agent disks by lot when initially received and weekly thereafter.

B. Test *Bacteroides fragilis* ATCC 25285, *Clostridium perfringens* ATCC 13124, and *Fusobacterium necrophorum* ATCC 25286 as described below under item V. The results should show the following.

1. *B. fragilis:* resistant to all three antimicrobial agents

2. *F. necrophorum:* resistant to vancomycin and susceptible to kanamycin and colistin

3. *C. perfringens:* susceptible to vancomycin and kanamycin and resistant to colistin

C. Record the results on a QC log (*see* Appendix 4.6–3 for a sample QC sheet).

V. PROCEDURE

A. Allow the container with disks to reach room temperature before opening it.

B. Subculture the isolate on a BAP. To ensure an even, heavy lawn of growth, streak the first quadrant back and forth several times. Streak the other quadrants to yield isolated colonies.

C. Place the three antimicrobial agent disks on the first quadrant well apart from each other.

D. If you have several organisms to test, first streak all the plates and then add the disks to them at the same time.

E. Incubate the plate(s) anaerobically for 48 to 72 h at 35 to 37°C.

F. Examine for zones of inhibition of growth around the disks.

VI. RESULTS

A. Susceptible: zone of inhibition of ≥10 mm

B. Resistant: zone of inhibition of <10 mm

SUPPLEMENTAL READING

Engelkirk, P. G., J. Duben-Engelkirk, and V. R. Dowell, Jr. 1992. *Principles and Practice of Clinical Anaerobic Bacteriology*. Star Publishing Co., Belmont, CA.

Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold. 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual*, 6th ed. Star Publishing Co., Belmont, CA.

MacFaddin, J. F. 2000. *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Lippincott Williams & Wilkins, Philadelphia, PA.

4.6.6

Sodium Polyanethol Sulfonate Disk for Differentiation of Anaerobic Cocci

I. PRINCIPLE

Sodium polyanethol sulfonate (SPS), a commonly used anticoagulant, inhibits certain bacteria, such as *Peptostreptococcus anaerobius* and the aerobe *Gardnerella vaginalis.* Paper disks impregnated with 5% SPS can be used as a tool for differentiating *P. anaerobius* from other anaerobic cocci.

II. SPECIMEN

The specimen is any isolated colony of anaerobic gram-positive cocci on primary or subculture plates.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

Perform and record QC as required. Include expiration date on label.

1. SPS disks

a. Combine the following in a flask.

SPS .....5 g
distilled water .....100 ml

b. After dissolving SPS, sterilize the mixture by filtration (0.22-µm-pore-size filter).

c. Dispense 20 µl onto sterile 1/4-in.-diameter filter paper disks that are spread inside empty, sterile petri dishes. Allow these to dry for 72 h at room temperature.

d. Store the disks at room temperature, and label with an expiration date of 6 months.

2. SPS disks are also commercially available (Anaerobe Systems, Becton Dickinson, Hardy, PML, Remel; *see* Appendix 4.6–1). Store as indicated by the manufacturer.

3. Brucella or other anaerobic BAP

B. Supplies

1. Single-disk dispenser or forceps

2. Ruler (divided into millimeters)

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Test each lot upon receipt and monthly thereafter.

B. Test *P. anaerobius* ATCC 27337 and *Peptostreptococcus asaccharolyticus* ATCC 29745 as described below under item V. The results should show the following.

1. *P. anaerobius:* susceptible to SPS

2. *P. asaccharolyticus:* resistant to SPS

C. Record the results on a QC log (*see* Appendix 4.6–3 for a sample QC sheet).

V. PROCEDURE

A. Allow the container with disks to reach room temperature before use.

B. Subculture the isolate on a BAP. To ensure an even, heavy lawn of growth, streak the first quadrant back and forth several times. Streak the other quadrants to yield isolated colonies.

C. Place the SPS disk on the first quadrant.

D. If you have several organisms to test, first streak all the plates and then add the disks to them at the same time. You can use one plate for up to four tests.

E. Incubate the plate(s) anaerobically for 48 to 72 h at 35 to 37°C.

F. Examine for a zone of inhibition of growth around the disk.

VI. RESULTS

A. Susceptible: zone of inhibition of ≥12 mm

P. anaerobius usually gives a very large zone of inhibition (≥16 mm), whereas other anaerobic cocci that appear susceptible to SPS give smaller zones. To presumptively identify *P. anaerobius*, you must also consider the Gram stain, typical colonial morphology, and odor. Some strains of *Peptostreptococcus micros* may be susceptible or partially susceptible to SPS. Examine the Gram stain for the small cell size of *P. micros* and chaining characteristics of *P. anaerobius.*

B. Resistant: zone of inhibition of <12 mm

SUPPLEMENTAL READING

Engelkirk, P. G., J. Duben-Engelkirk, and V. R. Dowell, Jr. 1992. *Principles and Practice of Clinical Anaerobic Bacteriology*. Star Publishing Co., Belmont, CA.

Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold. 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual*, 6th ed. Star Publishing Co., Belmont, CA.

MacFaddin, J. F. 2000. *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Lippincott Williams & Wilkins, Philadelphia, PA.

Wideman, P. A., V. L. Vargo, D. Citrobaum, and S. M. Finegold. 1976. Evaluation of the sodium polyanethol sulfonate disk test for the identification of *Peptostreptococcus anaerobius. J. Clin. Microbiol.* **4:**330–333.

4.6.7

Bile Test/*Bacteroides* Bile Esculin Agar for Differentiation of Anaerobic Gram-Negative Rods

I. PRINCIPLE

Bacteroides fragilis group organisms, *Fusobacterium mortiferum,* and *Fusobacterium varium* are clinically significant anaerobic gram-negative bacilli that are capable of growing in the presence of bile. Thus, the ability to grow in the presence of 20% bile (equal to 2% oxgall) is a key reaction in separating *B. fragilis* group from other anaerobic gram-negative bacilli as well as in differentiating bile-resistant fusobacteria. Besides inoculating a bile-containing medium, such as *Bacteroides* bile esculin agar (BBE), the test can also be performed with disks impregnated with 20% bile.

II. SPECIMEN

The specimen is any isolated colony on primary or subculture plates.

III. MATERIALS

Reagents

A. Bile disks (Anaerobe Systems, Becton Dickinson, Hardy, PML, Remel; *see* Appendix 4.6–1)

B. Brucella or other anaerobic BAP

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Test each lot upon receipt and monthly thereafter.

B. Test *B. fragilis* ATCC 25285 and *Prevotella melaninogenica* ATCC 25845 as described below under item V. The results should show the following.

1. *B. fragilis:* resistant to the bile disk

2. *P. melaninogenica:* susceptible to the bile disk

C. Record the results on a QC log (*see* Appendix 4.6–3 for a sample QC sheet).

V. PROCEDURE

A. Allow the container with disks to reach room temperature before use.

B. Subculture the isolate on a BAP. To ensure an even, heavy lawn of growth, streak the first quadrant back and forth several times. Streak the other quadrants to yield isolated colonies.

C. Place the bile disk on the second quadrant.

D. If you have several organisms to test, first streak all the plates and then add the disks to them at the same time. You can use one plate for up to four tests.

E. Incubate the plate(s) anaerobically for 48 to 72 h at 35 to 37°C.

F. Examine for any zone of inhibition of growth.

VI. RESULTS

A. Susceptible: zone of inhibition present

B. Resistant: no zone of inhibition present

SUPPLEMENTAL READING

Engelkirk, P. G., J. Duben-Engelkirk, and V. R. Dowell, Jr. 1992. *Principles and Practice of Clinical Anaerobic Bacteriology.* Star Publishing Co., Belmont, CA.

Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold. 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual,* 6th ed. Star Publishing Co., Belmont, CA.

MacFaddin, J. F. 2000. *Biochemical Tests for Identification of Medical Bacteria,* 3rd ed. Lippincott Williams & Wilkins, Philadelphia, PA.

4.6.8 Fluorescence

I. PRINCIPLE

Fluorescence is manifested because specific molecules exposed to UV light (366 nm) absorb energy and become excited. As electrons subsequently return to their original lower-energy states, they emit photons at a different wavelength. The presence and color of fluorescing colonies can aid in the rapid detection and presumptive identification of certain anaerobic bacteria (*see* Table 4.6.8–1).

II. SPECIMEN

A. The specimen is any isolated colony on primary plates or any colony on subculture plates containing blood. Prolonged incubation (>72 h) is often required to produce fluorescing pigment for the black- or brown-pigmented *Porphyromonas* spp. and *Prevotella* spp. The use of laked blood or rabbit blood in medium has been found to enhance pigment production.

B. Use the sample itself (tissue, fluid, etc.).

C. The test can be done at the infected body site of a patient.

III. EQUIPMENT

A. Long-wave (366-nm) UV light source (UVP, Inc.; *see* Appendix 4.6–2)

B. Viewing cabinet (UVP, Inc.)

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Test pigmented *Prevotella* spp. on anaerobic BAP used in your laboratory for fluorescence.

B. Subculture *Prevotella melaninogenica* ATCC 25845 and *Bacteroides fragilis* ATCC 25285 onto BAP, incubate them anaerobically for 48 h at 35 to 37°C, and test them as described below under item V. The results should show the following.

1. *P. melaninogenica:* (brick) red fluorescence

2. *B. fragilis:* no fluorescence

C. Record the results on a QC log (*see* Appendix 4.6–3 for a sample QC sheet).

Table 4.6.8–1 Fluorescence of anaerobic bacteria

Organism(s)	Color of fluorescence
Porphyromonas asaccharolytica, *Porphyromonas endodontalis*	Red[a]
Porphyromonas gingivalis	None
Pigmented *Prevotella* spp.	Red[a]
Nonpigmented gram-negative bacilli	No fluorescence or pink, orange, or yellow
Fusobacterium spp.	Chartreuse
Veillonella spp.	Red
Eubacterium lentum	Red
Clostridium difficile	Chartreuse
Clostridium innocuum	Chartreuse

[a] Fluorescence disappears when black pigment has developed.

V. PROCEDURE

A. Expose the culture plate, patient sample, or infected body site to the UV light source in the dark by using a UV viewing cabinet or by darkening the room.
   1. When examining colonies on a plate, take the cover off and hold the plate close to the light source. Keep moving the plate until you find the optimal angle for best fluorescence.
   2. When examining slow-growing organisms such as *Eubacterium lentum* and *Veillonella* spp., look at the heaviest growth area for fluorescence. The fluorescence of *Veillonella* spp. fades rapidly in air, so examine the specimen within 15 min after it is exposed to air.

B. Wait 10 to 15 s for the fluorescence to occur.

C. Note the presence and color of fluorescence.

VI. RESULTS

A. Positive: distinct color detected with UV light
Brick red fluorescence is the only reliable color for presumptive identification of *Porphyromonas* spp. and pigmented *Prevotella* spp.

B. Negative: no fluorescence

SUPPLEMENTAL READING

Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold. 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual*, 6th ed. Star Publishing Co., Belmont, CA.

Shah, H. N., R. Bonnett, B. Mateen, and R. A. D. Williams. 1979. The porphyrin pigmentation of subspecies of *Bacteroides melaninogenicus. Biochem. J.* **180:**45–50.

Slots, J., and H. S. Reynolds. 1982. Long-wave UV light fluorescence for identification of black-pigmented *Bacteroides* spp. *J. Clin. Microbiol.* **16:**1148–1151.

4.6.9 Lipase Test

I. PRINCIPLE

Fats in egg yolk agar (EYA) are broken down by the enzyme lipase to produce glycerol and fatty acids. The fatty acids appear as a surface iridescent layer that covers the colony and may extend beyond the edge of the colony.

II. SPECIMEN

A. The specimen for the lipase tests consists of a 24- to 48-h pure culture from a primary enriched medium such as brucella agar.
B. Perform aerotolerance testing on the isolate to ensure that the organism is an anaerobe.

III. MATERIALS

A. Use commercially prepared EYA plates (Anaerobe Systems, Becton Dickinson [BD], Hardy, PML, Remel).
B. Alternatively, prepare media by using EYA emulsion (BD, Hardy, PML, Remel, Oxoid). Follow the manufacturer's instructions.

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Check the egg yolk medium for lipase activity when it is prepared or when each shipment or lot is received.
B. *Fusobacterium necrophorum* ATCC 25286 is positive.
C. *Bacteroides fragilis* ATCC 25285 is negative.
D. Record the results on a QC log (*see* Appendix 4.6–3 for a sample QC sheet).

V. PROCEDURE

A. Inoculate a portion of egg yolk medium with the organism to be tested.
B. It is best to inoculate the portion of egg yolk medium heavily and then streak to obtain isolated colonies.

◩ **NOTE:** Do not inoculate the whole plate; leave a portion to act as a negative control.

C. Incubate the plate under anaerobic conditions for 24 to 48 h.

VI. RESULTS

A. Examine egg yolk medium plates for an iridescent and multicolored layer on top of the colonies.
B. It may be necessary to hold plates at an angle to adequately view the iridescent multicolored layer. This is a positive test.
C. Hold plates for 48 h. Some rare organisms may require 72 h of incubation to exhibit results. After 72 h of incubation, however, egg yolk medium plates may produce irregular test results.

SUPPLEMENTAL READING

Engelkirk, P. G., J. Duben-Engelkirk, and V. R. Dowell, Jr. 1992. *Principles and Practice of Clinical Anaerobic Bacteriology*. Star Publishing Co., Belmont, CA.

Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold. 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual*, 6th ed. Star Publishing Co., Belmont, CA.

MacFaddin, J. F. 2000. *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Lippincott Williams & Wilkins, Philadelphia, PA.

4.6.10 Lecithinase Test

I. PRINCIPLE

Bacterial lecithinase splits lecithin into water-insoluble diglycerides, resulting in an opaque halo surrounding a colony.

II. SPECIMEN

A. The specimen for the lecithinase tests consists of a 24- to 48-h pure culture from a primary enriched medium such as brucella agar.

B. Perform aerotolerance testing on the isolate to ensure that the organism is an anaerobe.

III. MATERIALS

A. Use commercially prepared egg yolk agar (EYA) plates (Anaerobe Systems, Becton Dickinson [BD], Hardy, PML, Remel).

B. Alternatively, prepare media by using EYA emulsion (BD, Hardy, PML, Remel, Oxoid). Follow the manufacturer's instructions.

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Check the egg yolk medium for lecithinase activity when it is prepared or when each shipment or lot is received.

B. *Clostridium perfringens* ATCC 13124 is positive.

C. *Bacteroides fragilis* ATCC 25285 is negative.

D. Record the results on a QC log (*see* Appendix 4.6–3 for a sample QC sheet).

V. PROCEDURE

A. Inoculate a portion of egg yolk medium with the organism to be tested.

B. It is best to inoculate *only a portion* of egg yolk medium heavily and then streak to obtain isolated colonies.

◩ **NOTE:** Do not inoculate the whole plate; leave a portion to act as a negative control.

C. Incubate the plate under anaerobic conditions for 24 to 48 h.

VI. RESULTS

A. Examine egg yolk medium plates for a white opacity in the medium that surrounds the colony and extends beyond the edge of growth. The white opaque edge is very sharp and very even. This is a positive test.

B. A positive test may take 48 h for some organisms.

SUPPLEMENTAL READING

Engelkirk, P. G., J. Duben-Engelkirk, and V. R. Dowell, Jr. 1992. *Principles and Practice of Clinical Anaerobic Bacteriology.* Star Publishing Co., Belmont, CA.

Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold. 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual,* 6th ed. Star Publishing Co., Belmont, CA.

MacFaddin, J. F. 2000. *Biochemical Tests for Identification of Medical Bacteria,* 3rd ed. Lippincott Williams & Wilkins, Philadelphia, PA.

4.6.11 Pigment Production

I. PRINCIPLE

Some species of *Prevotella* and *Porphyromonas* produce a dark pigment that causes their colonies to become brown to black. *Prevotella* spp. produce black-brown protoporphyrin, but pigmentation of incubated colonies may be delayed for 4 days or even more, whereas the pigment of *Porphyromonas* spp. is protoheme that may appear within 4 days of incubation but may also be delayed for as long as 2 weeks.

II. SPECIMEN

A. The specimen for pigment production tests consists of a pure culture from a primary enriched medium such as brucella agar.
B. Plated media containing laked blood or rabbit blood are frequently used to enhance pigmentation.
C. Perform aerotolerance testing on the isolate to ensure that the organism is an anaerobe.

III. MATERIALS

Use commercially prepared anaerobic BAP, or use laked BAP or rabbit blood agar plates (BBL, Hardy, PML, Remel, Oxoid).

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Check the BAP or other medium for pigment production when it is prepared or when each shipment or lot is received.
B. *Prevotella melaninogenica* ATCC 25845 is positive.
C. *Bacteroides fragilis* ATCC 25285 is negative.
D. Record the results on a QC log (*see* Appendix 4.6–3 for a sample QC sheet).

V. PROCEDURE

A. Examine colonies from anaerobic plates for brown to black pigment.
B. Some strains may produce pigment in 4 to 6 days; others may take up to 2 weeks.
C. Colonies initially appear tan and then darken.
D. Pigmented *Prevotella* spp. and *Porphyromonas* spp. (except for *Porphyromonas gingivalis*) produce brick red fluorescence under a Wood's lamp (366 nm) at 2 to 3 days before they become pigmented. Brick red fluorescence results can substitute for pigment production.
E. In most clinical laboratory situations it is not necessary to hold primary plates longer than 7 days to detect pigment production, since other methods (*see* procedure 4.6.5, Identification by Using Special-Potency Disks, or procedure 4.6.8, Fluorescence) can be used to help identify pigmented *Prevotella* spp. and *Porphyromonas* spp.

VI. RESULTS

A. Black pigment is positive.

B. The absence of black pigment is negative.

SUPPLEMENTAL READING

Engelkirk, P. G., J. Duben-Engelkirk, and V. R. Dowell, Jr. 1992. *Principles and Practice of Clinical Anaerobic Bacteriology.* Star Publishing Co., Belmont, CA.

Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold. 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual,* 6th ed. Star Publishing Co., Belmont, CA.

MacFaddin, J. F. 2000. *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Lippincott Williams & Wilkins, Philadelphia, PA.

Shah, H. N., R. Bonnett, B. Mateen, and R. A. D. Williams. 1979. The porphyrin pigmentation of subspecies of *Bacteroides melaninogenicus. Biochem. J.* **180:**45–50.

4.6.12 Urease Test

I. PRINCIPLE

Some organisms have the ability to split urea into two molecules of ammonia by the enzymatic action of urease, resulting in alkalinity, which causes the indicator phenol to change from yellow to red.

II. SPECIMEN

A. The specimen for the urease test consists of a 24- to 48-h pure culture from a primary enriched medium such as brucella agar.

B. Perform aerotolerance testing on the isolate to ensure that the organism is an anaerobe.

III. MATERIALS

A. Use commercially available urea broth (BBL, Hardy, PML, Remel).

B. Alternatively, use commercially available rapid urea disks (Becton Dickinson, Hardy, PML, Key Scientific Products, Remel).

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Intense pink throughout the disk or broth is positive.

B. *Bacteroides ureolyticus* ATCC 33387 is positive.

C. *Bacteroides fragilis* ATCC 25285 is negative.

D. Record the results on a QC log (*see* Appendix 4.6–3 for a sample QC sheet).

V. PROCEDURE

A. Inoculate urea broth or urea disk with organism to be tested.

B. Incubate urea broth under anaerobic conditions for 1 h.

C. If you use a urea disk, heavily inoculate the disk. Observe any reaction for 1 h. Most positive reactions will become pink in 15 min.

D. Follow the manufacturer's instructions.

VI. RESULTS

A. A positive test is the rapid formation of a pink color.

B. A negative test is no color change in the expected time frame.

SUPPLEMENTAL READING

Engelkirk, P. G., J. Duben-Engelkirk, and V. R. Dowell, Jr. 1992. *Principles and Practice of Clinical Anaerobic Bacteriology*. Star Publishing Co., Belmont, CA.

Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold. 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual*, 6th ed. Star Publishing Co., Belmont, CA.

MacFaddin, J. F. 2000. *Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. Lippincott Williams & Wilkins, Philadelphia, PA.

Appendixes to Procedure 4.6

APPENDIX 4.6–1

Summary of tests used for the rapid identification of anaerobes[a]

Test	Principle	Reagent(s)	Results	Control organisms	Expected result(s)
Special-potency disks	Special-potency disks are used as an aid in determining the Gram stain reaction of anaerobes as well as in preliminary categorization of some genera and species. Susceptible zones, ≥ 10 mm; resistant zones, ≤10 mm	Vancomycin, 5 μg Kanamycin, 1,000 μg Colistin, 10 μg	See Table 4.6.5–1 for details. In general, gram-positive organisms are resistant to colistin and susceptible to vancomycin, while most gram-negative organisms are resistant to vancomycin, except for *Porphyromonas* spp.	*Bacteroides fragilis* ATCC 25285 *Fusobacterium necrophorum* ATCC 25286 *Clostridium perfringens* ATCC 13124	Resistant to all three antibiotics Resistant to vancomycin; susceptible to kanamycin and colistin Resistant to colistin; susceptible to vancomycin and kanamycin
Spot indole test	Indole is split from tryptophan by certain organisms. It is used in grouping and identifying many anaerobic bacteria.	*p*-Dimethyl-aminocinnamaldehyde. To perform test, ensure that media contain tryptophan, such as blood agar or egg yolk medium. Use a small piece of filter paper.	Positive indole, development of a blue or green color on filter paper within 30 s. A negative test is no color change or pinkish color.	*Escherichia coli* ATCC 25922 *Pseudomonas aeruginosa* ATCC 27853	Indole positive Indole negative
Nitrate disk reduction test	Nitrate can be reduced to nitrite and other reduction products by organisms possessing the enzyme nitrate reductase. The nitrate test is useful for separating *Bacteroides ureolyticus* grp organisms from *Fusobacterium* grp organisms, which have similar special-potency disk results. *B. ureolyticus* grp organisms are nitrate positive and *Fusobacterium* grp organisms are nitrate negative.	Nitrate disks are commercially available. Nitrate A and B reagents and zinc	See Fig. 4.6.3–1 for details. A positive reaction is indicated by the development of a red or pink color after the reagents are added. A negative reaction is indicated by no color development after the reagents are added and development of a red color after zinc is added.	*E. coli* ATCC 25922 *Acinetobacter lwoffii* ATCC 43498	Nitrate positive, red color Nitrate negative, no color change

(continued)

APPENDIX 4.6–1 *(continued)*

Summary of tests used for the rapid identification of anaerobes[a] *(continued)*

Test	Principle	Reagent(s)	Results	Control organisms	Expected result(s)
Catalase test	Some anaerobic bacteria possess catalase, an enzyme that decomposes hydrogen peroxide into oxygen and water.	A 15% solution of hydrogen peroxide is preferred.	A positive reaction is indicated by immediate bubbling; a negative reaction is indicated by no bubbling. Formation of bubbles after 20 s is considered a negative test.	*Staphylococcus aureus* ATCC 25923 *Streptococcus pyogenes* ATCC 19615	Catalase positive Catalase negative
SPS disk test	SPS is used for the differentiation of anaerobic cocci.	SPS disks are commercially available.	*Peptostreptococcus anaerobius* produces a large zone (≥12 mm) around the SPS disk. *Peptostreptococcus micros* may produce small zones around the SPS disk (≤10 mm).	*P. anaerobius* ATCC 27337 *Peptostreptococcus asaccharolyticus* ATCC 29745	Susceptible to SPS Resistant to SPS
Bile test	*B. fragilis, Fusobacterium mortiferum, Fusobacterium varium,* and *Bilophila wadsworthia* are capable of growing in the presence of bile (bile resistant). This is a key reaction in separating *B. fragilis* grp organisms from many other anaerobic gram-negative rods.	Bile disks are commercially available, or use BBE agar plates, which are also commercially available.	A positive test (bile resistance) is indicated by growth around bile disks or growth on BBE agar plates.	*B. fragilis* ATCC 25285 *Prevotella melaninogenica* ATCC 25845	Resistant to bile Susceptible to bile
Fluorescence	Some anaerobic organisms are capable of fluorescing when exposed to UV light. The presence and color of fluorescing colonies can aid in the rapid detection and identification of certain anaerobic bacteria.	Use any isolated colony from plates containing blood. Prolonged incubation, of >72 h, is necessary. Use a long-wave UV light source (366 nm).	A positive test is a distinct color detected with UV light from blood agar plates. See Table 4.6.8–1 for use as an aid in the presumptive identification of certain anaerobic bacteria.	*P. melaninogenica* ATCC 25845 *B. fragilis* ATCC 25285	Brick red fluorescence No fluorescence
Esculin	To determine the ability of an organism to hydrolyze the glycoside esculin to esculetin. Esculetin reacts with an iron salt to form a dark-brown or black complex.	Use commercially prepared BBE agar plates, or use esculin broth.	A positive esculin test is the presence of black to brown color. A positive test on BBE agar is a black colony.	*B. fragilis* ATCC 25285 *Bacteroides vulgatus* ATCC 29327	Black, positive esculin No black, esculin negative

(continued)

APPENDIX 4.6–1 *(continued)*

Summary of tests used for the rapid identification of anaerobes[a] *(continued)*

Test	Principle	Reagent(s)	Results	Control organisms	Expected result(s)
Lipase	Free fats in EYA are broken down by the enzyme lipase to produce glycerol and fatty acids. The fatty acids appear as a surface iridescent layer that covers the colony and may extend beyond the edge of colony.	Use commercially prepared EYA plates, or prepare media by using EYA media.	Examine egg yolk medium plates for an iridescent and multicolored layer on top of the colonies. This is a positive test; it may take 48 h.	*F. necrophorum* ATCC 25286 *B. fragilis* ATCC 25285	Lipase positive, multicolored layer Lipase negative, no color on top
Lecithinase	Bacterial lecithinase splits lecithin to insoluble diglycerides, resulting in an opaque halo surrounding a colony on a medium containing egg yolk.	Use commercially prepared EYA plates, or prepare media by using EYA media.	Examine egg yolk medium plates for a white opacity in the medium that surrounds the colony and extends beyond the edge of growth. This is a positive test.	*C. perfringens* ATCC 13124 *B. fragilis* ATCC 25285	Lecithinase positive Lecithinase negative
Pigment production	Some anaerobic gram-negative rods, namely, *Porphyromonas* spp. and some *Prevotella* spp., produce a dark pigment that causes their colonies to become brown to black.	Use commercially prepared anaerobic BAP, or use laked BAP plates or rabbit blood agar plates.	Examine colonies from anaerobic plates for brown to black pigment. Some strains may produce pigment in 4 to 6 days; others may take up to 2 weeks. A brown to black pigment is a positive test.	*P. melaninogenica* ATCC 25845 *B. fragilis* ATCC 25285	Black pigment No black pigment
Urease	To determine the ability of an organism to split urea. Hydrolysis of urea by the enzyme urease releases ammonia, the alkalinity of which causes the indicator phenol to change from yellow to red.	Use commercially available urea broth, or use commercially available rapid urea disks.	A color change from pale yellow to dark bright pink represents a positive test for urea hydrolysis.	*B. ureolyticus* ATCC 33387 *B. fragilis* ATCC 25285	Urease positive, pink color Urease negative, no color change

[a] Abbreviation: grp, group.

APPENDIX 4.6–2

Addresses of Suppliers

Anaerobe Systems
15906 Concord Circle
Morgan Hill, CA 95037
http://www.anaerobesystems.com

BD Biosciences
7 Loveton Circle
Sparks, MD 21152
http://www.bd.com/microbiology

Hardy Diagnostics
1430 W. McCoy Ln.
Santa Maria, CA 93455
http://www.hardydiagnostics.com

Key Scientific Products
1402 D Chisolm Trail
Round Rock, TX 78681
http://www.keyscientific.com

PML Microbiologicals, Inc.
27120 SW 95th Ave.
Wilsonville, OR 97070
http://www.pmlmicro.com

Remel, Inc.
P.O. Box 14428
12076 Santa Fe Dr.
Lenexa, KS 66215
http://www.remelinc.com

Sigma-Aldrich, Inc.
Iron Run Corporate Center
6950 Ambassador Dr.
Allentown, PA 18106
http://www.sigma-aldrich.com

UVP, Inc.
2066 W. 11th St.
Upland, CA 91786
http://www.uvp.com

APPENDIX 4.6–3 Sample QC Sheet

Test/Reagent: _____

Control strains #1: _____ Predicted result (PR) #1: _____

#2: _____ #2: _____

#3: _____ #3: _____

Date	Lot# or date prepared	Exp. date	Result #1	Result #2	Result #3	PR	Init.

4.7 Commercial Kit Overnight Biochemical Systems for the Identification of Anaerobes

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Biochemical systems for identification of an anaerobe rely on the metabolic breakdown of substrates and the production of end products during the growth of the isolated organism. There are at least two commercially packaged kit systems that fit this method for the identification of commonly isolated anaerobic bacteria in the clinical microbiology laboratory when species identification is needed: the API 20A (bioMérieux, Inc.) and the AN Microplate (Biolog) (1–6) (Table 4.7–1). Both rely on overnight incubation of the test card before reading. The API 20A is manually filled and read; the AN Microplate is automatically filled but manually (visually) read. The API 20A uses 16 carbohydrates; its indicator system is bromcresol purple that turns yellow at a pH of 6.8 when a positive reaction occurs. The AN Microplate has a database of 361 anaerobes that are exposed to 95 preselected carbon sources for specific identification. The Minitek (BD Biosciences) kit was described in the last two editions of this handbook, but there were no references on the BD website that indicated that these systems were being manufactured any longer. Procedure 4.8 describes the BD Crystal ANR ID, which had largely taken over the role of the older Minitek panels.

II. SPECIMEN

The ideal specimens for analysis are colonies grown for 24 to 48 h on brucella-BAP or another medium suitably enriched for growing anaerobes (*see* procedure 4.3).

III. MATERIALS

A. API 20A or AN Microplate (*see* Appendix 4.7–1 for addresses)
B. Manufacturer-recommended inoculator fluid
C. Pasteur pipettes or other transfer devices
D. Anaerobic jar, anaerobic pouch, or anaerobic chamber (*see* procedure 4.5)
E. Accompanying reagents
F. Mineral oil
G. Incubator, 35 to 37°C

ANALYTICAL CONSIDERATIONS

IV. PROCEDURE

The instructions below serve only as a general guide; it is imperative that you follow the manufacturer's packaged directions for each of the microbiochemical systems. The microtube systems require growth of the organism for substrate degradation.

A. Colonies are suspended from a 24- to 48-h culture from nonselective medium to make a turbid suspension equivalent to an appropriate McFarland standard per the manufacturer's instructions.
B. For the API 20A, use a Pasteur pipette to dispense the suspension into each cupule of the strip provided. The urea and indole require an oil overlay. The API 20A strip contains 16 carbohydrates and tests for indole, gelatin, urea, esculin, and catalase.

Table 4.7–1 Characteristics of manual biochemical systems

Parameter	API 20A[a]	AN Microplate[b]
No. of tests	21	95
Inoculum source	Plate	Plate
Equipment needed	No	Yes, for filling plates
Incubation time (h)	24	20–24
Database		361 anaerobes
Storage temp (°C)	2–8	2–8

[a] bioMérieux, Inc.

[b] Biolog.

IV. PROCEDURE *(continued)*

C. For the AN Microplate, the manufacturer's instructions need to be followed; the wells are automatically filled.

D. Incubate the API 20A strip for 24 h and the AN Microplate for 20 to 24 h in an anaerobic jar, pouch, or chamber at 35°C.

E. After incubation, add the required reagents according to the manufacturer's instructions. As with any manual identification system, there is some subjectivity in reading. Always read the package insert and, if possible, obtain color charts for comparison to determine positive and negative reactions.

F. Use the numerical identification systems provided by the manufacturer or computerized coding system to interpret the identification. A phone number is usually provided for calling the company if the number obtained is not available in the codebook.

V. QUALITY CONTROL

Include QC information on reagent container and in QC records.

Each manufacturer recommends specific QC procedures (Table 4.7–2). Record the results on a QC log (*see* Appendix 4.6–3 for a sample QC sheet).

POSTANALYTICAL CONSIDERATIONS

VI. LIMITATIONS OF THE PROCEDURE

A. Laboratories that use either of these biochemical systems must use only the identification tables or numerical systems provided by the manufacturer.

B. Most biochemical systems work well with common, hardy, saccharolytic organisms; slower-growing anaerobes and/or fastidious anaerobes like *Porphyromonas* and *Fusobacterium* spp. may not work as effectively as *Bacteroides* and *Prevotella* spp.

Table 4.7–2 Recommended QC strains

System	Organism	ATCC no.
API 20A	*Clostridium histolyticum*	19401
	Bacteroides ovatus	8483
	Propionibacterium acnes	11827
	Clostridium sordellii	9714
	Clostridium perfringens	13124

VI. LIMITATIONS OF THE PROCEDURE *(continued)*

C. Typically, most identification systems perform less effectively for the gram-positive anaerobic non-spore-forming rods, usually because the database for these is often smaller and taxonomy is changing rapidly and is being driven by molecular considerations.

D. Other tests should be used to supplement the biochemical kit systems, including bile tolerance, catalase, lipase and lecithinase action as demonstrated on an egg yolk agar plate, motility, and the use of special-potency antimicrobial agent disk susceptibility.

E. Some saccharolytic organisms may reduce the indicators or produce a color reaction that is difficult to interpret.

REFERENCES

1. **Carroll, K. C., and M. P. Weinstein.** 2007. Manual and automated systems for detection and identification of microorganisms, p. 192–217. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
2. **Citron, D. M., I. R. Poxton, and E. J. Baron.** 2007. *Bacteroides, Porphyromonas, Prevotella, Fusobacterium,* and other anaerobic gram-negative rods, p. 911–932. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
3. **Forbes, B. A., D. F. Sahm, and A. S. Weissfeld (ed.).** 2007. *Bailey and Scott's Diagnostic Bacteriology,* 12th ed. Mosby Elsevier, St. Louis, MO.
4. **Head, C. B., and S. Ratnam.** 1988. Comparison of API ZYM system with API-Ident, API 20A, Minitek Anaerobe II, and RapID-ANA systems for identification of *Clostridium difficile. J. Clin. Microbiol.* **26:**144–146.
5. **Karachewski, N. O., E. L. Busch, and C. L. Wells.** 1985. Comparison of PRAS II, RapID ANA, and API 20A systems for identification of anaerobic bacteria. *J. Clin. Microbiol.* **21:**122–126.
6. **Moore, H. B., V. L. Sutter, and S. M. Finegold.** 1975. Comparison of three procedures for biochemical testing of anaerobic bacteria. *J. Clin. Microbiol.* **1:**15–22.

APPENDIX 4.7–1

Manufacturers of Biochemical Systems

BD
1 Becton Drive
Franklin Lakes, NJ 07417
http://www.bd.com

Biolog
21124 Cabot Boulevard
Hayward, CA 94545
800-284-4949
E-mail: info@biolog.com
http://www.biolog.com/contactUs.htm

4.8 Rapid Enzymatic Systems for the Identification of Anaerobes

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Rapid identification of anaerobes can be accomplished with commercially available microsystems that detect preformed enzymes within a few hours, eliminating the need for growth of the isolates (1–4, 6–9, 12–14, 16). The systems that are available are listed in Table 4.8–1. In a study that recently surveyed what methods laboratories were using to identify isolated anaerobes, 66% reported using a system that detected preformed enzymes (5). The systems listed in Table 4.8–1 require only 4 h of aerobic incubation after inoculation with a turbid suspension of the organism equivalent to a no. 3 or 4 McFarland standard. There are differences in the numbers and types of organisms included in each database as well as in the substrates that are used for differentiation. Some use fluorogenic and some use chromogenic substrates. Most require some reagent addition after incubation, before reading. Database and numerical identification profiles are provided for each system.

There are data in the literature about evaluation of these systems. A few examples of these comparisons are given here. A study comparing the RapID ANA panel with conventional prereduced anaerobically sterilized biochemicals demonstrated a correct genus identification with 94% of fresh clinical isolates and 87% agreement to the species level (3). The BBL Crystal ANR ID system for anaerobes was evaluated with 322 clinically significant anaerobes and found to have an 89% agreement with conventional identification. It was reported as easy to use; no reagents had to be added, and it provided a quicker turnaround time than conventional biochemical systems (2). The Vitek ANI Card was evaluated when it was initially marketed for the identification of anaerobic bacteria and was found to perform well with common isolates: it had 100% correlation to conventional methods for *Bacteroides fragilis* and *Clostridium perfringens*. The performance with other groups was not as good; however, the authors suggested that supplemental testing could improve the accuracy of the card (14). A new card (ANC) is available with the Vitek 2 that combines identification of selected anaerobes and *Corynebacterium* spp. Its database has 63 taxa of anaerobes and corynebacteria and uses a colorimetric technology containing chromogenic substrates. There are 64 wells of substrates; it requires a no. 3 McFarland, and results are available in about 6 h. A study using the ANC at three tertiary care hospitals in Canada and the United States concluded that the anaerobic portion of the card performed well for the isolates contained in its database, identifying the clinical isolates tested correctly 95% of the time. Some isolates, especially gram-positive anaerobic *Clostridium* spp. and non-spore-formers, were among the incorrect calls compared to a molecular 16S sequencing identification (12).

Four of the preformed enzyme systems (Rapid ID 32A, RapID ANA II, Rapid ID CB Plus, and Crystal ANR ID) were evaluated for the specific identification of a variety of *Actinomyces* spp. by Santala et al. (13). The Remel Rapid ID CB Plus has a limited database, including only gram-positive bacilli. In that study, *Actinomyces israelii* was identified by all, but other *Actinomyces* spp. did not fare so well. The Crystal ANR ID system performed best of the four, and the authors recommended that more work be done before these were acceptable for identification of *Actinomyces* spp. (13).

16S rRNA is also being used with increasing frequency for the molecular identification of anaerobes. A study that compared the identification by this method versus biochemical methods of significant bloodstream isolates found PCR to be a more accurate means of identification. Of 316 isolates, 16S rRNA identified 100% to the genus level and 91% to the species level, compared to 88% by conventional methods for the genus level and 66% for species level identification (15). There are many other laboratories that have begun to look at molecular identification of anaerobes on a more regular basis, and this may become the more specific method of identification in the future (10, 17, 18). For now, the molecular methods are limited to laboratories that have the ability to utilize sequencing for routine identification. For molecular methods to become the norm for anaerobic identification, in addition to better availability, better guidelines are needed for interpretation of sequencing results, and there may still remain isolates that cannot be identified confidently by 16S rRNA gene sequencing alone (18).

II. SPECIMEN

The ideal specimen consists of isolated colonies grown for 24 to 48 h on brucella blood agar or another suitably enriched nonselective medium for growing anaer-

Table 4.8–1 Characteristics of rapid identification systems

Parameter	Rapid ID 32A[a]	RapID ANA II[b]	Anaerobe panel[c]	Anaerobe ANI Card[d]	Crystal ANR ID[e]
No. of tests	29	18	24	28	29
Inoculum source	Plate (use only Columbia blood agar)	Plate	Plate	Plate	Plate
Inoculum age	24–48 h	24–72 h	24–48 h	24–48 h	24–72 h
McFarland turbidity	No. 4 in 3.0 ml	No. 3 in 1.0 ml	No. 3 in 3.0 ml	No. 3 in 1.5 ml	No. 4 in 2.3 ml
Diluent	Suspension medium (purchased)	Provided	Sterile deionized H_2O	Sterile saline	Provided
Size of inoculum	55 µl/cupule	0.1 ml/well	50 µl/well	Semiautomatic filling	Semiautomatic filling
Database	Codebook, computer assisted	Codebook, computer assisted EPIC-ID System; a CD program is available	Codebook, computer assisted	Computer program	Electronic codebook. The database used depends upon the type of primary media used to prepare the inoculum.
Incubation time	4 h	4 h	4 h	4 h	4 h
Additional required reagents and apparatus	James reagent, Fast Blue reagent, nitrate reagents, mineral oil, 6% H_2O_2 Pasteur pipettes or electronic pipette Codebook or identification software	Spot indole *p*-dimethylaminocinnamaldehyde ANA II reagent (cinnamaldehyde) RapID inoculation fluid Pipette Codebook	Mineral oil Peptidase reagent 0.8% sulfanilic acid 3% H_2O_2 Xylene 0.5% *N,N*-dimethyl-α-naphthylamine Inoculation fluid Ehrlich's reagent Cover panel 50-µl pipette Codebook	Spot indole *p*-dimethylaminocinnamaldehyde Vitek System computer Off-line or handheld viewer Printer Sterile saline Filling stand Sealer module	Spot indole *p*-dimethylaminocinnamaldehyde BBL Crystal panel viewer BBL Crystal electronic computer codebook 15% H_2O_2 Pipette Sterile cotton swabs
QC organisms	*Clostridium sordellii* ATCC 9714 *Clostridium baratii* ATCC 27638 *Clostridium sporogenes* ATCC 19404 *Actinomyces viscosus* ATCC 15987 *Capnocytophaga sputigena* ATCC 33612 *Bacteroides fragilis* ATCC 23745 *Bacteroides vulgatus* ATCC 8482	*Clostridium sordellii* ATCC 9714 *Bacteroides distasonis* ATCC 8503 *Bacteroides uniformis* ATCC 8492 *Peptostreptococcus magnus* ATCC 29328	*Clostridium perfringens* ATCC 13124 *Clostridium sordellii* ATCC 9714 *Bacteroides fragilis* ATCC 25285	*Bacteroides ureolyticus* ATCC 33387 *Bacteroides vulgatus* ATCC 8482 *Propionibacterium acnes* ATCC 11827 *Porphyromonas gingivalis* ATCC 33277 *Bacteroides distasonis* ATCC 8503	*Bacteroides fragilis* ATCC 25285 *Bacteroides distasonis* ATCC 8503 *Peptostreptococcus asaccharolyticus* ATCC 29743 *Lactobacillus acidophilus* ATCC 314 *Fusobacterium varium* ATCC 27725

[a] bioMérieux, Inc., Hazelwood, MO.
[b] Remel, Inc., Lenexa, KS.
[c] Siemens MicroScan, Inc., West Sacramento, CA.
[d] bioMérieux, Inc., Hazelwood, MO.
[e] BD Biosciences, Sparks, MD.

II. SPECIMEN *(continued)*

obes. Schaedler agar is not recommended, because the excess glucose in the medium may interfere with enzyme production. However, the Crystal ANR ID plate does have a separate database that allows the use of Schaedler agar (2). Also consult the package insert or call the manufacturer if you choose to deviate from the recommendations.

III. MATERIALS

A. Rapid ID 32A and Anaerobe ANI Card (bioMérieux, Inc.), Rapid Anaerobe panel (Siemens MicroScan, Inc.), Crystal ANR ID (BD Biosciences), or RapID ANA II (Remel, Inc.) (1–4, 6, 7, 13, 15) (*see* Appendix 4.8–1 for addresses)

B. Recommended inoculation fluid

C. Pasteur pipette, if required by system

D. 35°C incubator (non-CO_2)

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

Each manufacturer recommends specific QC procedures (Table 4.8–1).

ANALYTICAL CONSIDERATIONS

V. PROCEDURE

A. Suspend enough colonies from a 24- to 48-h culture from a nonselective medium to make a turbid suspension equivalent to a McFarland no. 3 to no. 4 standard. Each test kit has its own medium and McFarland requirements (Table 4.8–1).

B. Once the suspension is prepared, inoculate the panel within 15 min. For some organisms, growth from two plates is necessary to achieve a McFarland no. 4 standard.

C. Incubate in a standard aerobic 35°C incubator for 4 h. *Precaution: Do not incubate in CO_2.*

D. After incubation, add the required reagents (Table 4.8–1). The development of specific colors in the chromogenic tests indicates a positive reaction. Read the reactions, and record as either positive or negative.

E. To interpret the results of the test, use the numerical identification system from the package to obtain a code number.

F. Compare the code number obtained with the code numbers in the manufacturer's database book to identify the organism. Careful adherence to interpretation of colors is important for correct identification of the isolate.

POSTANALYTICAL CONSIDERATIONS

VI. LIMITATIONS OF THE PROCEDURE

A. Specific medium requirements differ from manufacturer to manufacturer; however, systems generally do not use media containing glucose because the sugar suppresses glycolytic activity.

B. Rapid enzymatic test kits should be used in conjunction with other conventional information such as Gram stain, colonial morphology, and organism growth requirements. Special-potency antimicrobial agent disks and other presumptive tests can be very useful in verifying identification and Gram stain reaction. All aggregate reactions must be considered.

C. *Interpretation of colors produced can be difficult but is critical for obtaining accurate reproducible results.*

D. Organisms that have been sequentially transferred for long periods may demonstrate aberrant reactions and incorrect identification. Organisms that have not been recently subcultured may demonstrate aberrant results.

◩ **NOTE:** The inoculum size is critical to obtain correct results; refer to the manufacturer's instructions.

E. Advantages of rapid enzymatic systems

1. Systems do not require growth to obtain an identification.

2. Incubation is in a standard non-carbon dioxide incubator.

VI. LIMITATIONS OF THE PROCEDURE *(continued)*

3. Identification in 4 h is possible.
4. Systems are good for fastidious anaerobic organisms and are not dependent on saccharolytic activity.
5. Many isolates can be identified without the expense of PRAS biochemicals.

F. Disadvantages of rapid enzymatic systems

1. Identification systems are limited by their databases and do not also include recent taxonomic changes in a timely or automatic fashion.
2. Databases that are predominantly made using frozen isolates from one geographical region suffer for lack of ability to pick up diverse phenotypes.
3. Some reactions are difficult to interpret.
4. Relatively inert organisms may be misidentified.

REFERENCES

1. **Burlage, R. S., and P. D. Ellner.** 1985. Comparison of the PRAS II, An-Ident, and RapID-ANA systems for identification of anaerobic bacteria. *J. Clin. Microbiol.* **22:**32–35.
2. **Cavallaro, J. J., L. S. Wiggs, and J. M. Miller.** 1997. Evaluation of the BBL Crystal anaerobe identification system. *J. Clin. Microbiol.* **35:**3186–3191.
3. **Celig, D. M., and P. C. Shreckenberger.** 1991. Clinical evaluation of the RapID-ANA panel for identification of anaerobic bacteria. *J. Clin. Microbiol.* **29:**457–462.
4. **Dellinger, C. A., and L. V. H. Moore.** 1986. Use of the RapID-ANA system to screen for enzyme activities that differ among species of bile-inhibited *Bacteroides. J. Clin. Microbiol.* **23:**289–293.
5. **Goldstein, E. J., D. M. Citron, P. J. Goldman, and R. J. Goldman.** 2008. National hospital survey of anaerobic culture and susceptibility methods: III. *Anaerobe* **14:**68–72.
6. **Jenkins, S. A., D. B. Drucker, M. G. L. Keanly, and L. A. Langull.** 1991. Evaluation of the Rapid ID 32A system for the identification of *Bacteroides fragilis* and related organisms. *J. Appl. Bacteriol.* **71:**360–365.
7. **Karachewski, N. O., E. L. Busch, and C. L. Wells.** 1985. Comparison of PRAS II, RapID ANA, and API 20A systems for identification of anaerobic bacteria. *J. Clin. Microbiol.* **21:**122–126.
8. **Moll, W. M., J. Ungerechts, G. Marklein, and K. P. Schaal.** 1996. Comparison of BBL Crystal ANR ID kit and API Rapid ID 32 A for identification of anaerobic bacteria. *Zentralbl. Bakteriol.* **284:**329–347.
9. **Moncla, B. J., P. Braham, L. K. Rabe, and S. L. Hillier.** 1991. Rapid presumptive identification of black-pigmented gram-negative anaerobic bacteria by using 4-methylumbelliferone derivatives. *J. Clin. Microbiol.* **29:**1955–1958.
10. **Nagy, E., E. Urbán, J. Sóki, G. Terhes, and K. Nagy.** 2006. The place of molecular genetic methods in the diagnostics of human pathogenic anaerobic bacteria. A minireview. *Acta Microbiol. Immunol. Hung.* **53:**183–194.
11. **Porschen, R. K., and E. H. Spaulding.** 1974. Phosphatase activity of anaerobic organisms. *Appl. Microbiol.* **27:**744.
12. **Rennie, R. P., C. Brosnikoff, L. Turnbull, L. B. Reller, S. Mirrett, W. Janda, K. Ristow, and A. Krilcich.** 2008. Multicenter evaluation of the Vitek 2 anaerobe and *Corynebacterium* identification card. *J. Clin. Microbiol.* **46:**2646–2651.
13. **Santala, A.-M., N. Sarkonen, V. Hall, P. Carlson, H. Jousimies-Somer, and E. Könönen.** 2004. Evaluation of four commercial test systems for identification of *Actinomyces* and some closely related species. *J. Clin. Microbiol.* **42:**418–420.
14. **Schreckenberger, P. C., D. M. Celig, and W. M. Janda.** 1988. Clinical evaluation of the Vitek ANI card for identification of anaerobic bacteria. *J. Clin. Microbiol.* **26:**225–230.
15. **Simmon, K. E., S. Mirrett, L. B. Reller, and C. A. Petti.** 2008. Genotypic diversity of anaerobic isolates from bloodstream infections. *J. Clin. Microbiol.* **46:**1596–1601.
16. **Stoakes, L., K. M. Kelly, K. Manarin, B. Schieven, R. Lannigan, D. Groves, and Z. Hussain.** 1990. Accuracy and reproducibility of the MicroScan rapid anaerobe identification system with an automated reader. *J. Clin. Microbiol.* **28:**1135–1138.
17. **Song, Y.** 2005. Anaerobiosis: molecular biology, genetics and other aspects—minireview. PCR-based diagnostics for anaerobic infections. *Anaerobe* **11:**79–91.
18. **Woo, P. C., S. K. Lau, J. L. Teng, H. Tse, and K. Y. Yuen.** 2008. Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical laboratories. *Clin. Microbiol. Infect.* **14:**908–934.

APPENDIX 4.8–1

List and Addresses of Manufacturers of Commercial Enzymatic Test Systems

Crystal ANR ID
BD
1 Becton Drive
Franklin Lakes, NJ 07417
http://www.bd.com/microbiology

Rapid ID 32A and Anaerobe Vitek ANI Card and Vitek 2 Anaerobe and Corynebacterium Card
bioMérieux, Inc.
595 Anglum Rd.
Hazelwood, MO 63042
http://www.biomerieux.com

RapID ANA II
Remel, Inc.
12076 Santa Fe Dr.
Lenexa, KS 66215
http://www.remelinc.com

Rapid Anaerobe ID
Siemens Healthcare Diagnostics
1717 Deerfield Rd.
Deerfield, IL 60015-0778
http://diagnostics.siemens.com

4.9 Rapid Biochemical Tests (4 Hours or Less) for the Identification of Anaerobes

4.9.1 Introduction

Many anaerobic isolates may be identified to the genus and species levels using a variety of preformed-enzyme tests or other rapid biochemical tests. In some instances, tests for identification beyond the level that can be attained with the various spot tests described in procedure 4.6 are needed. Presumptive or definitive identification of anaerobes is possible by using individual biochemical tests or a combination of preformed enzymatic manifestations.

Rapid biochemical tests are individual tests that may need to be performed when system approaches such as those described in procedure 4.6 are used. See Appendix 4.9–1 for a summary of rapid biochemical tests for the identification of anaerobes. See Appendix 4.9–2 for a list and addresses of manufacturers of rapid biochemical tests.

4.9.2 Alkaline Phosphatase

I. PRINCIPLE

Hydrolysis of 4-nitrophenyl phosphate by alkaline phosphatase releases free 4-nitrophenol, which is yellow. This test may be used to distinguish between the indole-positive species *Peptostreptococcus hydrogenalis* and *Peptostreptococcus asaccharolyticus.*

II. MATERIALS

A. Reagents
1. Wee-Tabs (Key Scientific Products)
2. Alkaline phosphatase is available in commercial identification kits (*see* procedure 4.8).
3. Enzymatic tablets (Pro-Lab Diagnostics)

B. Supplies
1. Loop or wooden applicator stick
2. Disposable petri dish
3. Glass slide

III. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Organisms
1. Positive control: *P. hydrogenalis*
2. Negative control: *P. asaccharolyticus*

B. Performance frequency
1. Perform QC test with each new lot of substrate or reagent.
2. Perform QC test each day the test is performed.
3. Record the results on a QC log (*see* Appendix 4.6–3 for a sample QC sheet).

IV. PROCEDURE

A. Use an actively growing (48- to 72-h) culture on an agar plate medium that does not contain carbohydrates.

B. Prepare a heavy suspension of organisms (>2.0 McFarland turbidity) in 0.25 ml of saline.

C. Add tablet, vortex the suspension, and incubate at 37°C for a maximum of 4 h.

V. RESULTS

A. Positive: yellow color

B. Negative: no color or very pale yellow

VI. LIMITATIONS AND PRECAUTIONS

A. This test is only part of the overall scheme for the identification of some anaerobes.

B. Further biochemical characterization may be necessary for specific identification.

C. Insufficient inoculum may give false-negative results.

D. Allowing the test to react less or significantly longer than 4 h may lead to uninterpretable results.

SUPPLEMENTAL READING

Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold. 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual,* 6th ed. Star Publishing Co., Belmont, CA.

Key Scientific Products. 2001. Technical insert. Key Scientific Products, Round Rock, TX.

4.9.3 Glutamic Acid Decarboxylase

I. PRINCIPLE

The glutamic acid decarboxylase test is used to determine the enzymatic ability of an organism to decarboxylate glutamic acid to form an amine, with resulting alkalinity. Glutamic acid decarboxylase is a rapid test for presumptive identification of most of the *Bacteroides fragilis* group, *Clostridium perfringens, Clostridium sordellii, Clostridium baratii, Eubacterium limosum,* and 53% of *Peptostreptococcus micros* strains.

II. MATERIALS

A. Glutamic acid tube (GDC tube [Remel, Inc.] or conventional tube)
B. Microbiological loop
C. Standard 35°C aerobic incubator

III. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Organisms
- **1.** Positive control: *B. fragilis*
- **2.** Negative control: *Fusobacterium nucleatum*

B. Performance frequency
- **1.** Perform QC test with each new lot of substrate or reagent.
- **2.** Perform QC test each day the test is performed.
- **3.** Record the results on a QC log (*see* Appendix 4.6–3 for a sample QC sheet).

IV. PROCEDURE

A. Pick up a heavy inoculum (heavy visible paste) from a fresh pure culture of the anaerobe to be tested.
B. Stab the inoculum in several spots at the very top of the medium, and then macerate it well throughout the upper one-fourth of the medium by moving the loop in and out of the medium several times.
C. Do not inoculate the agar in the butt of the tube.
D. Incubate aerobically at 35°C.
E. Examine at 30 min and 1 h if desired. Incubate tests for 4 h before a final negative determination.

V. RESULTS

A. A positive test for glutamic acid decarboxylate is indicated by an alkaline shift of the indicator from green to deep blue.
B. No change or a light to medium blue is considered negative.

VI. LIMITATIONS AND PRECAUTIONS

A. This test is only part of the overall scheme for identification and provides a high probability for presumptive identification of the *B. fragilis* group, *C. perfringens,* and *C. sordellii.*
B. Further biochemical characterization may be necessary for specific identification.

VI. LIMITATIONS AND PRECAUTIONS *(continued)*

C. When testing the *B. fragilis* group, negative tests cannot be expressed as "not *B. fragilis* group," since approximately 40% of *Bacteroides distasonis* and 55% of *Bacteroides vulgatus* strains are negative.

D. Many *Fusobacterium* strains give intermediate reactions.

E. Only a distinct deep blue color should be considered positive.

F. Too light an inoculum may yield false-negative results.

G. After prolonged standing, the test may be difficult to read owing to the presence of an indistinct yellowish-purple color. Compare with an uninoculated tube.

SUPPLEMENTAL READING

Jilly, B. J. 1984. Rapid glutamic acid decarboxylase test for identification of *Bacteroides* and *Clostridium* spp. *J. Clin. Microbiol.* **19:**592–593.

Johnson, K. S. 1987. Rapid glutamic acid decarboxylase reactions as a screening test for *Bacteroides fragilis* group species and for *Clostridium perfringens*, abstr. C15, p. 326. *Abstr. 87th Annu. Meet. Am. Soc. Microbiol.* 1987. American Society for Microbiology, Washington, DC.

Remel, Inc. 1999. Technical insert. Remel, Inc., Lenexa, KS.

4.9.4 L-Alanyl-Alanylaminopeptidase

I. PRINCIPLE

Hydrolysis of L-alanyl-alanylaminopeptide by alanylaminopeptidase releases beta-naphthylamine, which complexes with paradimethylaminocinnamaldehyde in the presence of acetic acid to produce a pink to purple color. This procedure may be used as a rapid test to separate *Fusobacterium* species from *Bacteroides* species.

II. MATERIALS

A. Reagents

1. L-Alanyl-L-alanylaminopeptidase disk (ALN Disk; Remel, Inc.)
2. Paradimethylaminocinnamaldehyde reagent
3. Enzymatic tablets (Pro-Lab Diagnostics)

B. Supplies

1. Deionized water
2. Loop or wooden applicator stick
3. Disposable petri dish
4. Glass slide

III. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Organisms

1. Positive control: *Bacteroides fragilis*
2. Negative control: *Fusobacterium mortiferum*

B. Performance frequency

1. Perform QC test with each new lot of substrate or reagent.
2. Perform QC test each day the test is performed.
3. Record the results on a QC log (*see* Appendix 4.6–3 for a sample QC sheet).

IV. PROCEDURE

A. Place disk on slide or petri dish lid, and moisten slightly with water.

B. Rub visible inoculum onto ALN Disk with loop or wooden applicator stick, and allow to react for 2 min.

C. Add 1 drop of cinnamaldehyde reagent, and look for color change in 30 s.

V. RESULTS

A. Red to pink is positive for possible *Bacteroides* species.

B. The absence of color change is negative for *Fusobacterium* species.

VI. LIMITATIONS AND PRECAUTIONS

A. This test is only part of the overall scheme for the identification of some anaerobes.

B. The organism tested must be an anaerobic gram-negative rod.

C. Cultures older than 3 days should be subcultured before the test is performed.

D. Further biochemical characterization may be necessary for specific identification.

E. Insufficient inoculum may give false-negative results.

F. Allowing the test to react for less or significantly longer than 30 s may lead to uninterpretable results.

SUPPLEMENTAL READING

Thomson, R. B., Jr. 2007. Specimen collection, transport, and processing, p. 377. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. American Society for Microbiology, Washington, DC.

Remel, Inc. 2001. Technical insert. Remel, Inc., Lenexa, KS.

4.9.5 L-Proline-Aminopeptidase

I. PRINCIPLE

Hydrolysis of L-proline-beta-naphthylamide by proline aminopeptidase releases beta-naphthylamine, which complexes with paradimethylaminocinnamaldehyde in the presence of acetic acid to produce a pink to purple color.

This test is used to screen for the presumptive identification of suspected colonies of *Clostridium difficile* on selective media. Proline-aminopeptidase may also be used as a rapid test in the differentiation of other anaerobic organisms.

II. MATERIALS

A. Reagents

1. L-Proline-beta-naphthylamide disk (PRO Disk, Remel, Inc.; Key Scientific, Pro-Lab Diagnostics)
2. Paradimethylaminocinnamaldehyde reagent

B. Supplies

1. Deionized water
2. Loop or wooden applicator stick
3. Disposable petri dish
4. Glass slide

III. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Organisms

1. Positive control: *C. difficile*
2. Negative control: *Clostridium perfringens*

B. Performance frequency

1. Perform QC test with each new lot of substrate or reagent.
2. Perform QC test each day the test is performed.
3. Record the results on a QC log (*see* Appendix 4.6–3 for a sample QC sheet).

IV. PROCEDURE

A. Place disk on slide or petri dish lid, and moisten slightly with water.
B. Rub visible inoculum (heavy) onto disk with loop or wooden applicator stick.
C. Allow to react for 2 min or up to 5 min.
D. Add 1 drop of cinnamaldehyde reagent, and look for color change in 30 s.

V. RESULTS

A. Color change (red to pink) within 30 s means that the organism may be *C. difficile.*
B. The absence of color change means that the organism is not *C. difficile.*

VI. LIMITATIONS AND PRECAUTIONS

A. This test is only part of the overall scheme for the identification of some anaerobes.
B. The test organism must be an anaerobic gram-positive rod.
C. Further biochemical characterization may be necessary for specific identification.
D. An insufficient inoculum may give false-negative results.
E. Allowing the test to react for less or significantly longer than 30 s may lead to uninterpretable results.

SUPPLEMENTAL READING

Remel, Inc. 2001. Technical insert. Remel, Inc., Lenexa, KS.

4.9.6 4-Methylumbelliferone Derivative Substrates

I. PRINCIPLE

Preformed enzyme (glucosidase) links to a fluorescent substrate (4-methylumbelliferone) to yield rapid determination of enzymatic activity. In the presence of the glucosidase enzyme, the derivative substrate 4-methylumbelliferyl releases 4-methylumbelliferone, a fluorescent compound easily detected by long-wave (360-nm) UV light.

II. MATERIALS

A. 4-Methylumbelliferone glucoside substrate (Sigma-Aldrich, Inc.)
B. Glucosidase substrates suspended in distilled water to a concentration of 4 mM
C. Substrates frozen in snap-cap tubes (12 by 75 mm)

III. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Use known negative and positive controls for each substrate.
B. Perform QC test with each new lot of substrate or reagent.
C. Perform QC test each day the test is performed.
D. Record the results on a QC log (*see* Appendix 4.6–3 for a sample QC sheet).

IV. PROCEDURE

A. If derivative substrates are frozen, thaw and suspend in 20 μl of 1 M sodium phosphate buffer (pH 5.0).
B. Add enough solution to soak a Whatman no. 2 filter paper strip.
C. Allow solution spot to dry for 10 min.
D. Smear a heavy loopful of bacteria (24 to 48 h of incubation) directly on the substrate, and incubate the filter paper strip at 37°C for 15 min.
E. Examine the filter paper strip under a long-wavelength handheld lamp.

V. RESULTS

A. The hydrolytic product of enzymatic action on the glucoside derivative produces an immediate light blue fluorescence under a Wood's lamp.
B. Light blue fluorescence under a Wood's lamp (365 nm) is positive; no color is negative.

VI. LIMITATIONS AND PRECAUTIONS

A. A light inoculum will yield false-negative results.
B. The buffer must be acidic. An alkaline buffer will yield false-positive results.
C. Observe in a dark room to avoid false-negative results.

SUPPLEMENTAL READING

Key Scientific Products. Wee-Tabs technical insert. Key Scientific Products, Round Rock, TX.

Mangels, J. I., I. Edvalson, and M. Cox. 1993. Rapid presumptive identification of *Bacteroides fragilis* group organisms with use of 4-methylumbelliferone-derivative substrates. *Clin. Infect. Dis.* **16**(Suppl. 4)**:**S319–S321.

Moncla, B. J., P. Braham, L. K. Rabe, and S. L. Hillier. 1991. Rapid presumptive identification of black-pigmented gram-negative anaerobic bacteria by using 4-methylumbelliferone derivatives. *J. Clin. Microbiol.* **29:**1955–1958.

4.9.7

Combination Enzymatic Tablets for Nitrophenol, Aminopeptidase, and Methylumbelliferyl Substrates

I. PRINCIPLE

Some bacteria may produce enzymes which hydrolyze various chromogenic substrates, producing a positive test. Combination tablets allowing two or more enzymatic tests to be performed in a single tube can be used to detect enzymatic activity based upon a color change or to detect other substrates producing 4-methylumbelliferone enzymatically from methylumbelliferyl substrates, leading to fluorescent end products when exposed to a Wood's lamp. These test systems are a convenient and economical way to achieve identification of some anaerobes.

II. MATERIALS

A. Reagents

1. Tablets can be purchased containing two or more test substrates in various predetermined combinations (see manufacturer for choice of combination tablets and substrates available; Wee-Tabs, Key Scientific Products, Round Rock, Tex.).
2. Individual tablets may be purchased from Pro-Lab Diagnostics.
3. Peptidase reagent (cinnamaldehyde)

B. Supplies

1. Microbiological loop or sterile swab
2. Sterile distilled water
3. Long-wave UV light (Wood's lamp, 366 nm)

III. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Each lot of disks should be tested with organisms of known reactivity prior to use.

B. See manufacturer's insert for QC organism recommendations.

C. Test each new lot of peptidase reagent.

D. Perform QC test each day the test is performed.

E. Record the results on a QC log (*see* Appendix 4.6–3 for a sample QC sheet).

IV. PROCEDURE

A. Add at least 0.25 ml but not more than 0.5 ml (about 5 drops) of distilled water to tablet.

B. Inoculate heavily from fresh 24-h growth from anaerobic BAP. The heavier the inoculum, the better (>2.0 McFarland turbidity).

C. Incubate for at least 2 h at 37°C.

V. RESULTS

Read and interpret in the exact sequence listed below.

A. Nitrophenol tests (glucosidase): observe for an immediate bright yellow color indicating a positive test.

B. 4-Methylumbelliferone: observe the tube for fluorescence by holding under a long-wave UV lamp. An immediate blue-green fluorescence indicates a positive test.

C. Aminopeptidase: perform the aminopeptidase test last by adding 2 drops of peptidase reagent. A dark red within 15 min indicates a positive test.

VI. LIMITATIONS AND PRECAUTIONS

A. These tests may be only part of an overall identification scheme for some anaerobes.

B. Further biochemical characterization may be necessary for specific identification of some anaerobic bacteria.

C. Insufficient inoculum and organisms older than 24 h may give false-negative results.

D. Allowing the test to react for less or significantly more time than manufacturer's recommendations may lead to uninterpretable results.

E. The glucosidase test must be read first, followed by the 4-methylumbelliferone test and then the aminopeptidase test. This sequence must be followed for reliable results.

SUPPLEMENTAL READING

Hudspeth, M. K., S. H. Gerardo, D. M. Citron, and E. J. C. Goldstein. 1997. Growth characteristics and a novel method for identification (the Wee-Tab System) of *Porphyromonas* species isolated from infected dog and cat bite wounds in humans. *J. Clin. Microbiol.* **35:**2450–2453.

Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold. 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual*, 6th ed. Star Publishing Co., Belmont, CA.

Key Scientific Products. Wee-Tabs package insert. Key Scientific Products, Round Rock, TX.

Mangels, J. I., I. Edvalson, and M. Cox. 1993. Rapid presumptive identification of *Bacteroides fragilis* group organisms with use of 4-methylumbelliferone-derivative substrates. *Clin. Infect. Dis.* **16**(Suppl. 4)**:**S319–S321.

Moncla, B. J., P. Braham, L. K. Rabe, and S. L. Hillier. 1991. Rapid presumptive identification of black-pigmented gram-negative anaerobic bacteria by using 4-methylumbelliferone derivatives. *J. Clin. Microbiol.* **29:**1955–1958.

4.9.8 Appendixes to Procedure 4.9

APPENDIX 4.9–1

Summary of single or combination rapid enzymatic tests for anaerobes

Test	Principle	Reagents	Results
Alkaline phosphatase	Hydrolysis of 4-nitrophenyl phosphate by alkaline phosphatase releases free 4-nitrophenol, which is yellow. Used to help identify anaerobic gram-positive cocci.	Alkaline phosphatase in kits or in individual tablets	A yellow color within 4 h is positive; no color is negative.
Glutamic acid decarboxylase	Enzymatic action on glutamic acid by a specific decarboxylase releases an amine indicated by a dark blue color. Used for the presumptive identification of *Bacteroides fragilis* group and some *Clostridium* spp.	Glutamic acid tube, commercially available	A positive test for glutamic acid decarboxylase is indicated by a color shift from green to dark blue; no change is negative.
L-Alanyl-alanylaminopeptidase	Hydrolysis of L-alanyl-alanylaminopeptide releases β-naphthylamine, which complexes with cinnamaldehyde to produce a pink to purple color. Used to separate *Fusobacterium* spp. from *Bacteroides* spp.	ALN Disk, commercially available	A positive test is indicated by a pink to purple color in 30 s. A negative test is indicated by no color change.
L-Proline-aminopeptidase	Hydrolysis of L-proline-β-naphthylamine releases β-naphthylamine, which complexes with cinnamaldehyde to produce a pink to purple color. Used to presumptively identify *Clostridium difficile.*	PRO Disk, commercially available	A positive test is indicated by a pink to purple color in 30 s. A negative test is indicated by no color change.
4-Methylumbelliferone derivative substrates	A glucosidase is linked to a fluorescent substrate, 4-methylumbelliferone, to yield rapid determination of enzymatic activity as indicated by fluorescence when viewed with a Wood's lamp.	4-Methylumbelliferone glucoside substrate, commercially available, and 360-nm UV light	An immediate light blue fluorescence when viewed with a Wood's lamp is positive. A negative test is no color.
Combination glucosidase naphthylamide tablets	Combination tablets in which two or more enzymatic tests can be performed in a single tube. Some substrates may also be linked to 4-methylumbelliferone to permit detection of fluorescent end products.	Glucosidase/naphthylamine/methylumbelliferyl commercially available tablets	A positive glucosidase is indicated by bright yellow within 2 h; a positive peptidase is indicated by red within 15 min; 4-methylumbelliferone shows a blue-green fluorescence immediately.

APPENDIX 4.9–2

List of Suppliers for Rapid Biochemical Tests

Key Scientific Products
1402 D Chisolm Trail
Round Rock, TX 78681
http://www.keyscientific.com

Pro-Lab Diagnostics
9701 Dessau Rd.
Unit 802
Austin, TX 78754
http://www.pro-lab.com

Remel, Inc.
P.O. Box 14428
12076 Santa Fe Dr.
Lenexa, KS 66215
http://www.remelinc.com

Sigma-Aldrich, Inc.
Iron Run Corporate Center
6950 Ambassador Dr.
Allentown, PA 18106
http://www.sigma-aldrich.com

4.10 Anaerobic Gram-Negative Bacilli

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The anaerobic gram-negative bacteria are part of the microbiota of the oral cavity, gastrointestinal tract, and urogenital tract (3, 6, 12). Many taxonomic changes have been instituted based on newer biochemical and molecular sequencing considerations. There are new genera and species for "old names" and newly described organisms, and these will only increase in numbers as better culture techniques and more advanced methods for molecular identification are utilized (3–13, 15, 17, 20, 21). These changes have made understanding current taxonomy and correlating old and new names in the clinical literature more difficult for laboratorians and clinicians alike. Clinical decisions still require accurate, timely identification of clinically relevant anaerobic pathogens by the clinical microbiology laboratory. We, as laboratorians, need to keep abreast of the changes and relay those changes as efficiently as possible to our colleagues in a manner that maintains the old and new; i.e., always consider sharing with the clinician the old and new genera, for example, so that they can associate the two. Table 4.10–1 gives information about some of the changes in taxonomy of the anaerobic gram-negative bacilli.

The *Bacteroides fragilis* group, composed of the bile-resistant *Bacteroides* spp., includes the following 11 species: *B. fragilis, B. thetaiotaomicron, B. distasonis, B. vulgatus, B. ovatus, B. stercoris, B. uniformis, B. caccae, B. merdae, B. eggerthii,* and *B. goldsteinii*. As a result of molecular sequencing and reanalysis of relatedness of species, there has been a proposal to move *B. distasonis, B. merdae,* and *B. goldsteinii* to a new genus, *Parabacteroides* (16). We use the *Bacteroides* genus for these three isolates in this edition. The pigmented gram-negative bacilli, once included in the *Bacteroides* genus, are now referred to as members of the genera *Prevotella, Porphyromonas,* and *Alistipes* (15). Members of the *Prevotella* genus also include nonpigmented species. All of these bile-sensitive strains can be further categorized as saccharolytic or asaccharolytic. Many of the bile-resistant strains of *Bacteroides* are normal members of the microbiota of the gastrointestinal tract, predominantly in the colon. Once outside of the colon, they can cause abscesses in the abdominal cavity, bacteremia, peritonitis, and other significant clinical diseases. Many of the bile-sensitive species of gram-negative bacilli are normal members of the oral microbiota but can be responsible for dental diseases as well as pulmonary and other systemic diseases; most occur in mixed infections with other oral aerobes and anaerobes.

Table 4.10–1 Changes in taxonomy among the anaerobic gram-negative bacilli

New designation	Old designation
Alistipes putredinis	*Bacteroides putredinis*
Alistipes finegoldii	None (new)
Dialister micraerophilus	None (new)
Dialister propionicifaciens	None (new)
Faecalibacterium prausnitzii	*Fusobacterium prausnitzii*
Porphyromonas uenonis	*Porphyromonas endodontalis* (nonoral)
Sneathia sanguinegens	*Leptotrichia sanguinegens*
Tannerella forsythia	*Bacteroides forsythus*
Sutterella wadsworthensis	*Campylobacter (Bacteroides) gracilis* (some strains)
Campylobacter gracilis	*Bacteroides gracilis* (some strains)
Campylobacter rectus	*Wolinella recta*
Campylobacter curvus	*Wolinella curva*

I. PRINCIPLE *(continued)*

This procedure describes characteristics of and identification methods for the clinically important members of the anaerobic gram-negative bacilli, including *Bacteroides* spp., *Prevotella* spp., *Porphyromonas* spp., *Fusobacterium* spp., *Sutterella* spp., *Bilophila wadsworthia*, and some *Campylobacter* spp. Anaerobic gram-negative bacilli are the most frequently encountered of all anaerobes in clinical specimens, with the *Bacteroides fragilis* group isolated more frequently than any other anaerobe (3, 6, 12).

Initial differentiation of anaerobic gram-negative bacilli is based on Gram stain and colonial morphology, motility, pigment production, fluorescence under long-wave UV light, susceptibility to special-potency antimicrobial agent disks, other disk or spot tests, and often the use of commercial kit systems for complete genus and species identification. On occasion, gas-liquid chromatography (GLC) or fatty acid analysis may also be required (3, 10, 12). Communication with the patient's physician is crucial to determine the extent of workup required, especially when specimens are found to be positive for mixed anaerobic and aerobic organisms without any predominating organisms. Correlation with the direct Gram stain is essential to further facilitate appropriate identification procedures.

II. ISOLATES

Identification of anaerobic gram-negative bacilli requires isolation of the organism in pure culture. Follow the directions in procedure 4.4 for details on how to obtain a pure culture of the isolate to be identified and how to confirm that the isolate is an anaerobe. Establish that the isolate is a gram-negative bacillus by Gram stain reaction; see other sections in this handbook for identification of other types of isolates. The following steps will aid in identification.

A. Place special-potency antimicrobial agent disks of kanamycin (1,000 μg), vancomycin (5 μg), and colistin (10 μg) on a brucella BAP or another enriched medium for isolates that are gram-negative bacilli or coccobacilli (3, 10, 12). See procedures 4.3, 4.4, and 4.6 for other media and procedures.

B. Add a nitrate disk (Anaerobe Systems, Becton Dickinson [BD], Hardy, PML, Remel) to a heavily inoculated section (*see* procedure 4.6).

C. Using the results obtained with special-potency antimicrobial agent disks, refer to Fig. 4.10–1 for directions to the appropriate follow-up chart and to determine the tests needed for identification. Use the pure-culture growth on the brucella BAP to perform additional tests.

ANALYTICAL CONSIDERATIONS

III. GRAM STAIN AND COLONY MORPHOLOGY

A. Gram stain morphology

1. The steps taken to identify anaerobic isolates depend on the Gram stain reaction, yet anaerobic organisms often have a variable stain. Any unclear reaction may be resolved by use of one or all of the following.

a. Use a young culture (log-phase growth) grown on medium with small or no amounts of carbohydrates.

b. Alter Gram stain procedure by use of methanol instead of heat to fix smear (*see* procedure 4.4); use basic fuchsin as a counterstain (3, 12).

c. Use buffered Gram stain reagents (*see* procedure 4.4).

d. Reduce the acetone concentration in the decolorizer solution (300 ml of acetone, 700 ml of 95% ethanol) (3, 10, 12).

2. Most *Bacteroides* spp. do not display unique morphology on Gram stain. They appear as pale, filamentous, pleomorphic, gram-negative rods with or without vacuoles and irregular staining.

3. The pigmented *Prevotella* spp. and *Porphyromonas* spp. tend to stain as pale coccobacilli.

4. Cell morphology may be a significant differentiating factor for some *Fusobacterium* spp.

a. *Fusobacterium nucleatum* is a thin, pale, gram-negative rod with tapering ends (fusiform). Other *Fusobacterium* spp. may be differentiated from *F. nucleatum* on the basis of indole reaction, lipase, and bile (Fig. 4.10–2

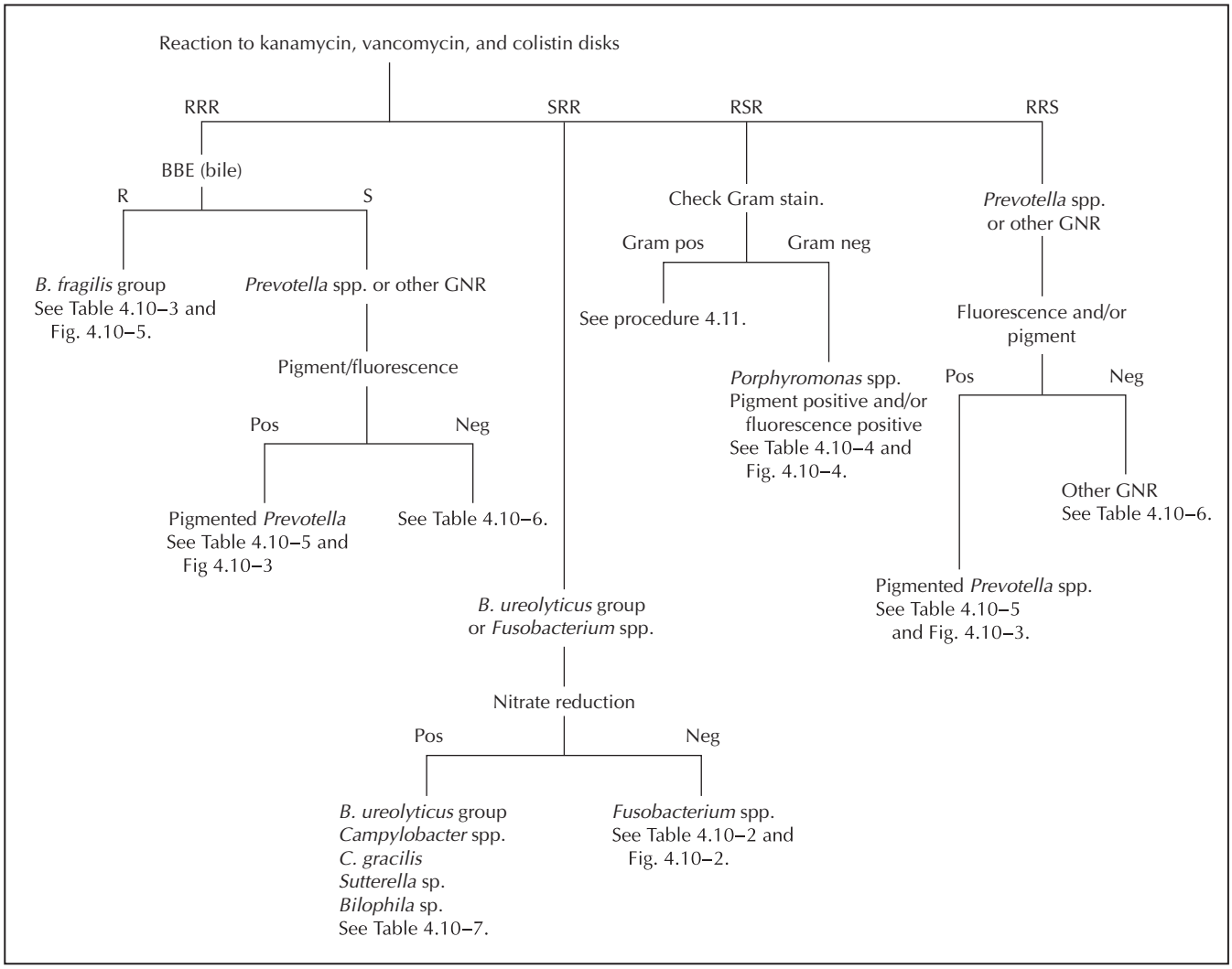


Figure 4.10–1 Reaction to kanamycin, vancomycin, and colistin disks. Pos, positive; Neg, negative; GNR, gram-negative rods; R, resistant; S, susceptible.

III. GRAM STAIN AND COLONY MORPHOLOGY *(continued)*

and Table 4.10–2). Caveat: The needle-shaped morphology is shared with the microaerophilic, indole-negative *Capnocytophaga* spp.

b. Other *Fusobacterium* spp. (*see* Table 4.10–2) tend to be pleomorphic on Gram stain. *Fusobacterium mortiferum* (and sometimes *Fusobacterium necrophorum*) displays more bizarre and extremely pleomorphic forms, with filaments, round bodies, and swollen areas.

5. *Bacteroides ureolyticus, Campylobacter gracilis,* other anaerobic *Campylobacter* spp., *Bilophila,* and *Sutterella* are thin gram-negative rods with rounded ends (3, 12).

B. Colony morphology

1. Anaerobic gram-negative bacilli display a variety of colony morphologies, some of which are characteristic of certain species. Presumptive identification can sometimes be made on the basis of colony morphology and Gram stain results.

a. Record the colony description, such as size, shape, and unusual characteristics (pitting, spreading, hemolysis, "breadcrumblike" or "fried-egg-like" appearance, iridescence, pigments, UV reactions) (*see* procedure 4.4).

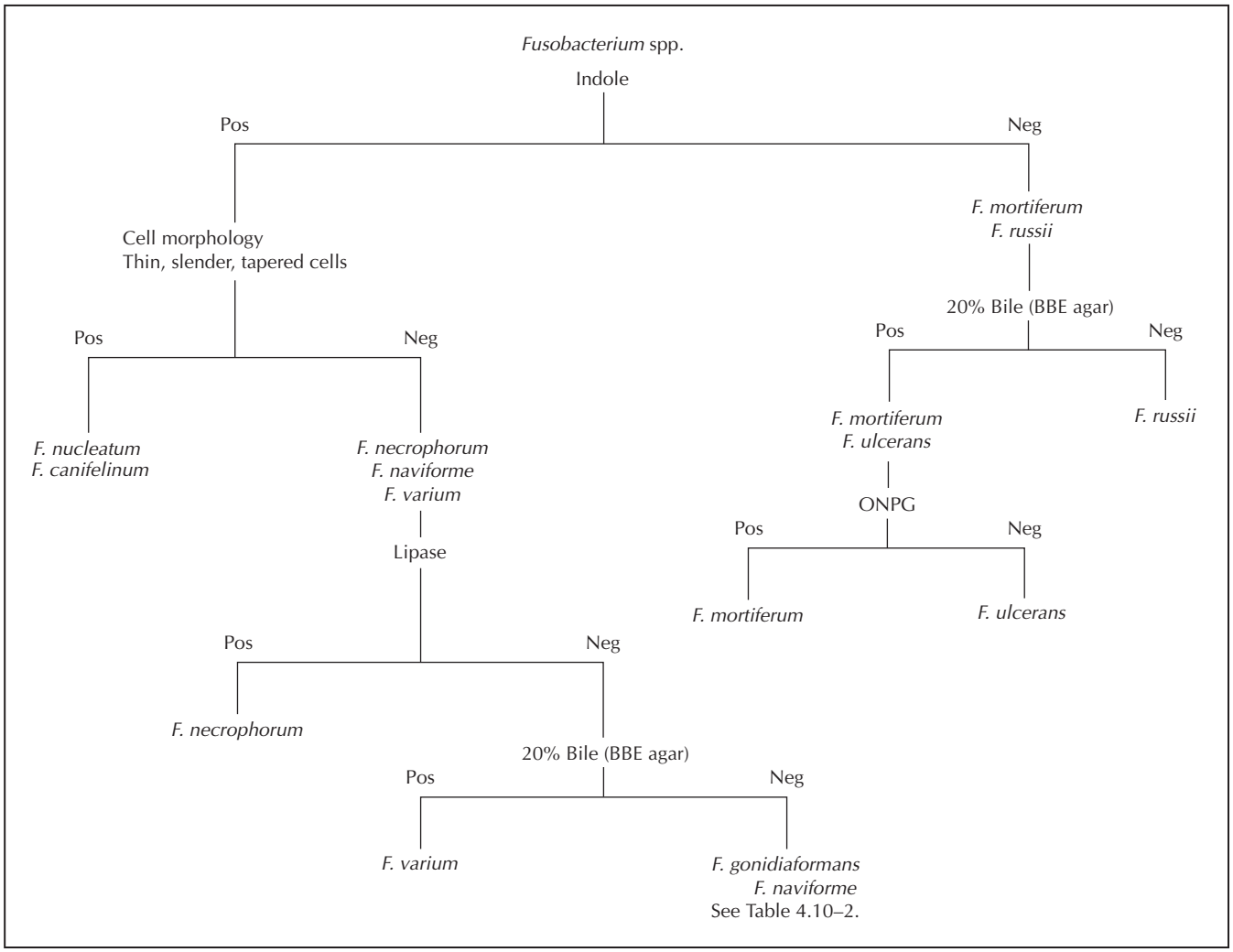


Figure 4.10–2 Identification of *Fusobacterium* spp. Pos, Positive; Neg, negative. ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

Table 4.10–2 Characteristics of *Fusobacterium* spp.[a]

Species	Cell morphology	Colony morphology	Indole	Growth on BBE or in 20% bile	Esculin	Lipase	ONPG[b]
F. nucleatum	Thin, tapered ends	Breadcrumblike, speckled	+	−	−	−	−
F. necrophorum	Round, not tapered ends; bizarre forms common	Umbonate, greening of agar	+	$-^{+}$	−	$+^{-}$	−
F. mortiferum	Bizarre; round "bodies"	Fried-egg appearance	−	+	+	−	+
F. varium	Round, not tapered ends	Smooth (fried egg)	$+^{-}$	+	−	−	−
F. naviforme	Boat-shaped	Mottled	+	−	−	−	−
F. russii[c]	Round ends	Smooth	−	−	−	−	−
F. canifelinum[c]	Slender; pointed ends		+	−		−	−
F. ulcerans[d]	Large, rounded ends	Smooth	−	+	−	−	−

[a] +, positive; −, negative; $+^{-}$, most cells are positive and some are negative; $-^{+}$, most cells are negative and some (even subspecies) may be positive. Adapted from reference 3.

[b] ONPG, *o*-nitrophenyl-β-D-galactopyranoside; test with API ZYM or Roscoe tablets.

[c] Has been associated with animal bites.

[d] Can cause "tropical ulcers" (3, 4, 12); also nitrate positive.

III. GRAM STAIN AND COLONY MORPHOLOGY *(continued)*

b. Perform aerotoleance testing on all colony types prior to proceeding with other tests (*see* procedure 4.4).

c. Note growth characteristics on enriched and differential primary isolation media (*see* procedure 4.4).

2. *B. fragilis* group

In general, on enriched primary media, *B. fragilis* group organisms form colonies that are 2 to 3 mm in diameter, circular, convex, and gray to gray-white (shiny, silver-like). A *Bacteroides* bile esculin (BBE) plate may be included for primary isolation and will select for those species able to grow in the presence of 20% bile and to hydrolyze esculin (Table 4.10–3).

a. Black colonies on BBE that are >1 mm in diameter and Gram stain as pale, pleomorphic, gram-negative rods can be presumptively identified as belonging to the *B. fragilis* group.

b. *Bacteroides vulgatus* produces >1-mm-diameter colonies on BBE but usually does not hydrolyze esculin (Table 4.10–3).

c. *B. goldsteinii* closely resembles *B. merdae* but may be variably catalase positive, in contrast to the catalase-negative *B. merdae*. If Roscoe tables are used, *B. goldsteinii* will be positive for β-glucuronidase, α-glucosidase, and β-glucosidase; *B. merdae* will be negative for these enzymes (19).

d. Some non-*B. fragilis* group organisms are resistant to bile. *Bilophila wadsworthia*, *F. mortiferum*, and *F. varium* as well as aerobes like *Enterococcus* spp. and members of the *Enterobacteriaceae* will grow on BBE medium. These organisms, however, do not usually produce >1-mm colonies, so be certain to evaluate any BBE growth carefully.

3. *B. ureolyticus* group organisms (*B. ureolyticus*, *C. gracilis*, *Campylobacter* spp., and *Sutterella*) produce colonies that are small and translucent or trans-

Table 4.10–3 Characteristics of the *Bacteroides fragilis* group[a]

Species	Growth in 20% bile	Indole	Catalase	Esculin hydrolysis	Arabinose	Cellobiose	Rhamnose	Salicin	Sucrose	Trehalose	Xylan	α-Fucosidase
Bacteroides caccae	+	−	−	+	+	+⁻	+⁻	−⁺	+	+	−	+
Bacteroides distasonis	+	−	+⁻	+	−⁺	+	V	+	+	+	−⁺	−
Bacteroides eggerthii	+	+	−	+	+	−⁺	+⁻	−	−	−	+	−
Bacteroides fragilis	+	−	+	+	−	+	−⁺	−	+	−	−	+
Bacteroides goldsteinii	+	−	V	+	−	+	+	+	+	+	−	−
Bacteroides merdae	+	−	−⁺	+	−⁺	V	+	+	+	+	−	−
Bacteroides ovatus	+	+	+⁻	+	+	+	+	+	+	+	+	+
Bacteroides stercoris	+	+	−	+⁻	−⁺	+	+	−⁺	+	−	V	V
Bacteroides thetaiotaomicron	+	+	+	+	+	+	+	−⁺	+	−	+	+
Bacteroides uniformis	+ʷ	+	−⁺	+	+	+	−⁺	+	+	−	V	+⁻
Bacteroides vulgatus	+	−	−⁺	−⁺	+	−	+	−	+	−	−⁺	+

[a] +, positive; −, negative; +⁻, most strains are positive, but occasional strains are negative; −⁺, most strains are negative, but occasional strains are positive; w, weak; V, variable. Adapted from reference 12.

III. GRAM STAIN AND COLONY MORPHOLOGY *(continued)*

parent and may produce greening of the agar. Three colony morphologies exist: smooth and convex, pitting, and spreading. All three colony types may appear in the same culture (3, 20).

- **a.** It may be necessary to subculture colonies of each morphology to resolve questions of pleomorphism.
- **b.** Pitting (corroding) is usually best detected if the surface of the agar is inspected at a 30 to 45° angle.
- **c.** Ability to corrode agar and spread can be medium dependent and may be enhanced by using moist, fresh media or prereduced anaerobically sterilized (PRAS) media.
- **d.** Colony variants may produce multiple forms (pitting, spreading, convex). Improper methods of maintaining stock cultures may cause pure isolates to revert to producing multiple colony types.
- **e.** *B. ureolyticus* group colonies are usually much smaller and more translucent than those of fusobacteria.

4. *Fusobacterium* species (Fig. 4.10–2 and Table 4.10–2)

- **a.** Along with its distinctive cell morphology, *F. nucleatum* possesses three different characteristic colony forms: breadcrumb, speckled, and smooth. The colony sizes vary from <0.5 to 2 mm, and the colors vary from white (breadcrumb) to gray and gray-white (7, 12). The agar surrounding *F. nucleatum* colonies becomes greenish upon exposure to air.
- **b.** *F. necrophorum* produces umbonate colonies with greening of the agar. Strains that are lipase positive can also be beta-hemolytic.
- **c.** *F. mortiferum/varium* produces fried-egg colonies (translucent with opaque centers and irregular borders) on the surface of the agar.
- **d.** *Capnocytophaga* spp. (not obligate anaerobes) produce iridescent or speckled, flat, spreading colonies with fingerlike projections and yellow centers.

IV. PIGMENTATION AND FLUORESCENCE

Include QC information on reagent container and in QC records.

A. Pigmentation (*see* procedure 4.6)

1. Use of primary medium containing 5% laked sheep blood (also laked rabbit blood) enhances production of pigment by pigmented *Prevotella* spp. and *Porphyromonas* spp. Organisms that fluoresce brick red or produce brown to black colonies on blood-containing medium are placed into pigmented *Prevotella* spp. or *Porphyromonas* spp.

2. Pigmentation usually occurs within 4 days but may be delayed, taking as long as 21 days for some isolates (11–13). Pigmentation begins as light tan to brown and then darkens upon incubation.

3. Note the degree of pigmentation; it may be useful in differentiating this group from other groups. *Porphyromonas* spp. tend to produce dark brown to black colonies (Table 4.10–4), while some pigmenting *Prevotella* spp. do not develop pigmentation as dark (Table 4.10–5). *Prevotella bivia*, although not usually considered a part of the pigmented group, may produce pigment upon prolonged incubation (*see* Table 4.10–6).

4. It is generally not practical in a clinical laboratory to hold primary plates beyond 7 days to detect delayed pigment production, particularly when brick red fluorescence is usually detected in 48 to 72 h.

B. Long-wave UV fluorescence (*see* procedure 4.6)

1. Test all primary plates with growth or any suspicious colonies for fluorescence by exposure to long-wave UV light (Wood's lamp, 366 nm) in order to recognize black-pigmented *Prevotella* spp. and *Porphyromonas* spp. (except for *Porphyromonas gingivalis*) before a distinct pigment develops (Fig. 4.10–3 and 4.10–4 and Tables 4.10–4 and 4.10–5) (18). Usually brick red

Table 4.10–4 Characteristics of *Porphyromonas* spp. of human origin[a]

Species	Pigment	Fluorescence	Indole	Catalase	α-Fucosidase[b]	α-Galactosidase[b]	β-Galactosidase[b]	NAG[b]	Trypsin	Chymotrypsin
P. asaccharolytica[c]	+	+	+	−	+	−	−	−	−	−
P. catoniae	−	+	−	−	+	$-^{+}$	+	+	$-^{+}$	$+^{-}$
P. uenonis[d]	+	+	+	−	−	−	−	−	−	−
P. endodontalis	+	+	+	−	−	−	−	−	−	−
P. gingivalis	+	−	+	−	−	−	−	+	+	−
P. somerae	+	W	−	−	−	−	+	+	−	+

[a] Adapted from reference 3. +, positive; −, negative; $-^{+}$, most strains are negative and some are positive; $+^{-}$, most strains are positive and some are negative; W, weak reactions usual; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; NAG, *N*-acetyl-β-glucosaminidase. All organisms in this table are esculin negative. *P. asaccharolytica* may be lipase positive, but all others in the table are lipase negative.

[b] These results are obtained with Roscoe tablets (WEE-Tabs), used according to the manufacturer's directions.

[c] *P. asaccharolytica* may be lipase positive; all others in this table are lipase negative.

[d] *P. uenonis* is very similar to *P. endodontalis* but has been associated with nonoral human infections.

Table 4.10–5 Characteristics of pigmented and saccharolytic *Prevotella* spp.[a]

Species	Indole	Lipase	Esculin	Cellobiose	Lactose	α-Fucosidase	α-Galactosidase	β-Galactosidase	NAG[b]
P. corporis	−	−	−	−	−	−	−	−	−
P. denticola	−	−	+	−	+	+	+	+	+
P. intermedia/nigrescens	+	+	−	−	−	+	−	−	−
P. loescheii	−	V	+	+	+	+	+	+	+
P. melaninogenica	−	−	−	−	+	+	+	+	+
P. pallens	+	−	−	−	−	+	−	−	−
P. tannerae	−	−	−	−	+	+	−	+	+

[a] +, positive; −, negative; V, variable.

[b] NAG, *N*-acetyl-β-glucosaminidase.

IV. PIGMENTATION AND FLUORESCENCE *(continued)*

fluorescence can be observed in 48 to 72 h, whereas pigment production may often take 4 to 5 days.

2. Some nonpigmented anaerobic gram-negative organisms can fluoresce a variety of shades of pink, orange, and chartreuse. Therefore, only those isolates that fluoresce brick red can be presumptively identified as pigmented *Prevotella* spp. or *Porphyromonas* spp. (*see* Tables 4.6–2 and 4.10–4) (*see* also procedure 4.6).
   - **a.** *Fusobacterium* spp. can fluoresce chartreuse (yellow-green).
   - **b.** *Veillonella* spp. can fluoresce red (but not brick red). Differentiation is easily achieved by Gram stain.
   - **c.** *P. gingivalis* may lack the ability to fluoresce but will produce black colonies, so note both pigmentation and fluorescence (Table 4.10–4).
   - **d.** If colonies are too old (>5 days), they lose their ability to fluoresce by direct exposure to UV light (7, 9, 10, 18). A preparation of 5 to 10 pigmented colonies emulsified in absolute methanol (1 ml) in a glass test tube can be exposed to UV light to test for fluorescence and may increase the likelihood of a positive result (18).

V. MOTILITY

Most significant clinical isolates fall into the "gram-negative nonmotile" classification. However, if there is any difficulty in identifying an isolate or if the Gram stain shows helical or curved rods, perform either a flagellum stain or a motility test (3, 12). Determine motility as follows.

Table 4.10–6 Characteristics of 20% bile-sensitive, nonpigmented, and saccharolytic *Prevotella* spp.[a]

Organism	Gelatin	Esculin hydrolysis	Indole	α-Fucosidase[b]	ONPG[b]	NAG[b]	β-Xylosidase[b]	Fermentation of:						
								Glucose	Lactose	Salicin	Sucrose	Xylose	Arabinose	Cellobiose
P. baroniae	+	+	−	+	+	+	−[c]	+	+	+	+	−	−	+
P. bivia	+	−	−	+	+	+	−	+	+	−	−	−	−	−
P. buccae	+	+	−	−	+	−	+	+	+	+	+	+	+	+
P. buccalis	−	+	−	+	+	+	−	+	+	−	+	−	−	+
P. dentalis	−	V	−	−	+	+	V	+	+	−	W	V	+	+
P. disiens	+	−	−	−	−	−	−	+	−	−	−	−	−	−
P. enoeca	+	V	−	+	+	+	−	+	+	−	−	−	−	−
P. heparinolytica	−	+	+	+	+	+	+	+	+	+	+	+	+	+
P. marshii	+	−	−	+	−	−	−[c]	+	−	−	−	−	−	−
P. multiformis	+	−	−	V	+		−	+	+	−	+	−	−	+
P. multisaccharivorax	+	+	−	−	V		+	+	+	V	+	+	V	+
P. oralis	+	+	−	+	+	+	−[c]	+	+	+	+	−	−	+
P. oris	V	+	−	+	+	+	+	+	+	+	+	+	+	+
P. oulorum[d]	−	+	−	−	+	+	−	+	+	−	+	−	−	−
P. shahii	+	−	−	+	W	+	−[c]	+	+	−	+	−	−	−
P. veroralis	V	+	−	+	+	+	−	+	+	−	+	−	−	+
P. zoogleoformans	V	+	−	+	+	+	+	+	+	V	+	V	V	+

[a] Adapted from reference 3. +, positive; −, negative; W, weak positive reactions; V, variable reactions; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; NAG, *N*-acetyl-β-glucosaminidase.

[b] These results are obtained with Roscoe tablets (WEE-Tabs), used according to the manufacturer's directions.

[c] These results were obtained using 4-methylumbelliferyl substrates.

[d] *P. oulorum* is also catalase and lipase positive.

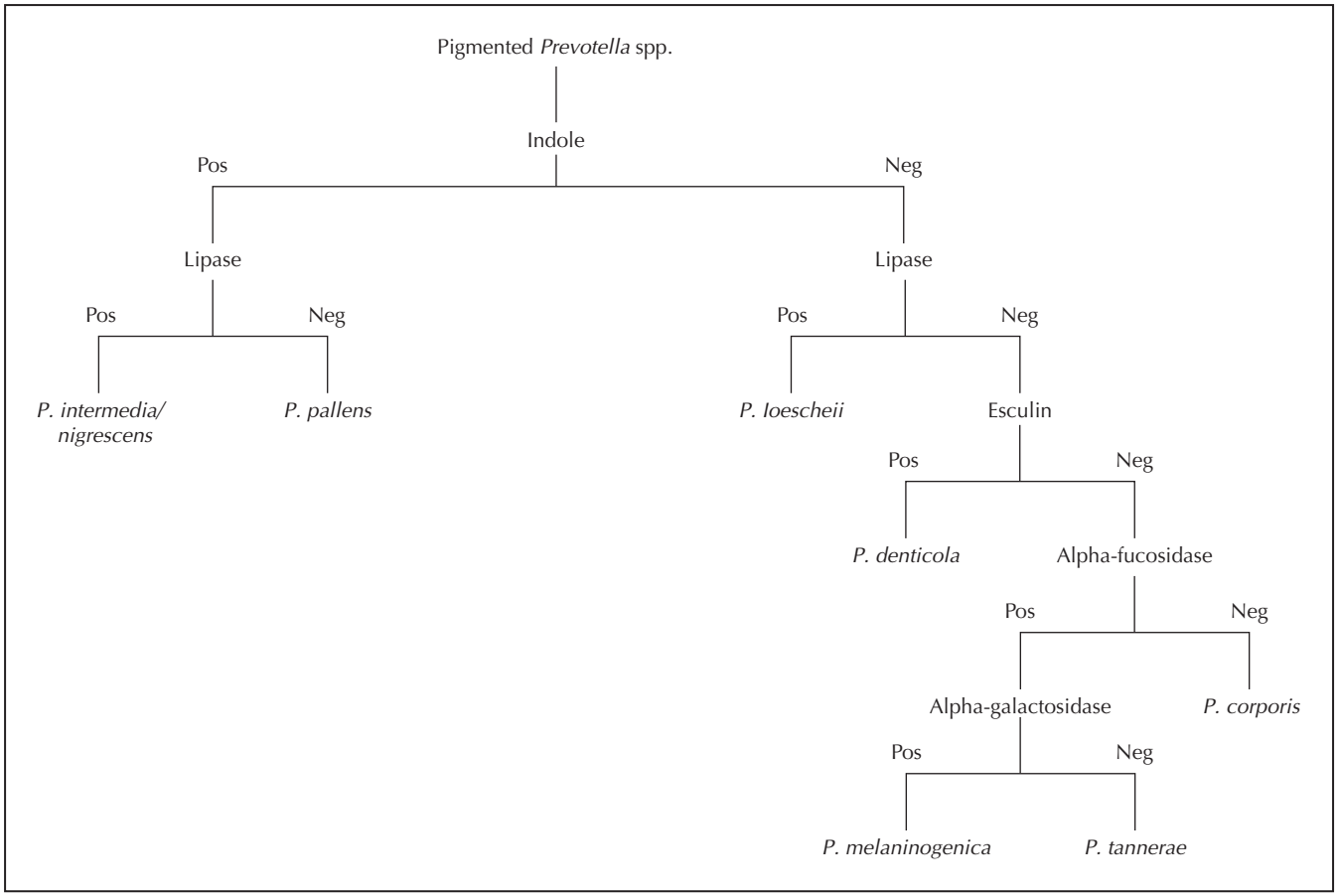


Figure 4.10–3 Identification of pigmented *Prevotella* spp. Pos, positive; Neg, negative.

V. MOTILITY *(continued)*

A. Use wet-mount examination and dark-field or phase-contrast microscopy of young broth cultures (3 h old). The addition of formate and fumarate may be necessary.

B. Use Todd-Hewitt broth partially solidified with 0.3% agar. Incubate 3 to 6 days; observe for diffuse growth away from stab.

C. Use Columbia broth supplemented with 1% soluble starch (BD), 2% rabbit serum, and 0.24% agar (3). Incubate for 3 to 6 days.

D. Dark-field or phase-contrast microscopy is preferred, since these semisolid-medium motility tests are time-consuming and difficult to interpret. However, many clinical laboratories may not have a dark-field or phase-contrast microscope.

VI. RAPID PRESUMPTIVE IDENTIFICATION TESTS

Include QC information on reagent container and in QC records.

Use rapid tests to rule out or confirm presumptive identifications based on Gram stain and colony morphology (*see* procedure 4.4 for additional information). When typical morphology (cell and colony) is apparent and is combined with rapid tests, the resulting preliminary identification may be useful until more exhaustive tests are completed or are needed by clinician (*see* procedures 4.6 to 4.9 for an explanation).

A. Special-potency antimicrobial agent disks

Determine susceptibility to special-potency antimicrobial agent disks (vancomycin, 5 μg; kanamycin, 1,000 μg; and colistin, 10 μg) (*see* procedure 4.6).

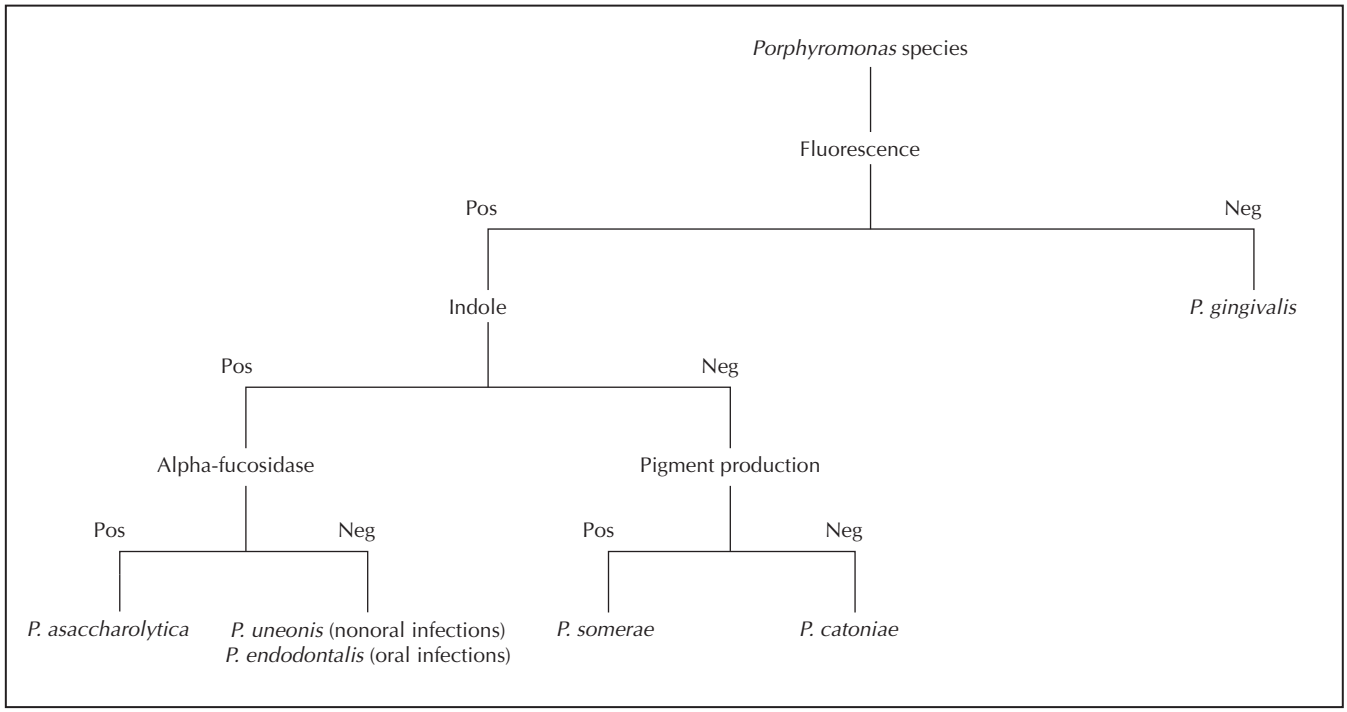


Figure 4.10–4 Identification of *Porphyromonas* spp. Some *Porphyromonas* spp. of animal origin that may be recovered from bite infections are not listed. Pos, positive; Neg, negative.

VI. RAPID PRESUMPTIVE IDENTIFICATION TESTS *(continued)*

These disks are used as an aid in determining the Gram stain reaction and in separating different gram-negative species and genera (12).

1. *B. fragilis* group organisms can be identified by the special-potency antimicrobial agent disk pattern showing resistance to all three disks and resistance to 20% bile or on BBE agar (Fig. 4.10–1 and Table 4.10–3).
2. *B. ureolyticus* group organisms are susceptible to kanamycin and colistin and resistant to vancomycin special-potency disks. These organisms reduce nitrate and are nitrate reductase positive (Fig. 4.10–1).
3. *Fusobacterium* spp. are susceptible to kanamycin and colistin and resistant to vancomycin special-potency disks. These organisms are nitrate reductase negative (Fig. 4.10–1).
4. *Porphyromonas* spp. are resistant to kanamycin and colistin and susceptible to vancomycin special-potency disks (Fig. 4.10–1).
5. *Prevotella* spp. are resistant to kanamycin and vancomycin special-potency disks and vary in their susceptibility to colistin. *Prevotella* spp. may have a disk pattern typical of the *B. fragilis* group (resistance to the three antimicrobial agent disks), but these organisms do not grow in 20% bile (Fig. 4.10–1).

B. Spot indole (*see* procedure 4.6 for use of spot indole)

1. Some species of the *B. fragilis* group (*see* Table 4.10–3 and Fig. 4.10–5) can be differentiated by spot indole (12).
2. *F. nucleatum* is differentiated from other *Fusobacterium* spp. and from *Capnocytophaga* spp. (microaerophiles) by typical cell morphology, its ability to produce indole, and other tests (*see* Fig. 4.10–2 and Table 4.10–2).
3. An indole- and lipase-positive coccobacillus that forms black-pigmented colonies or fluoresces red under UV light is identified as belonging to the *Prevotella intermedia/nigrescens* group (Table 4.10–5).

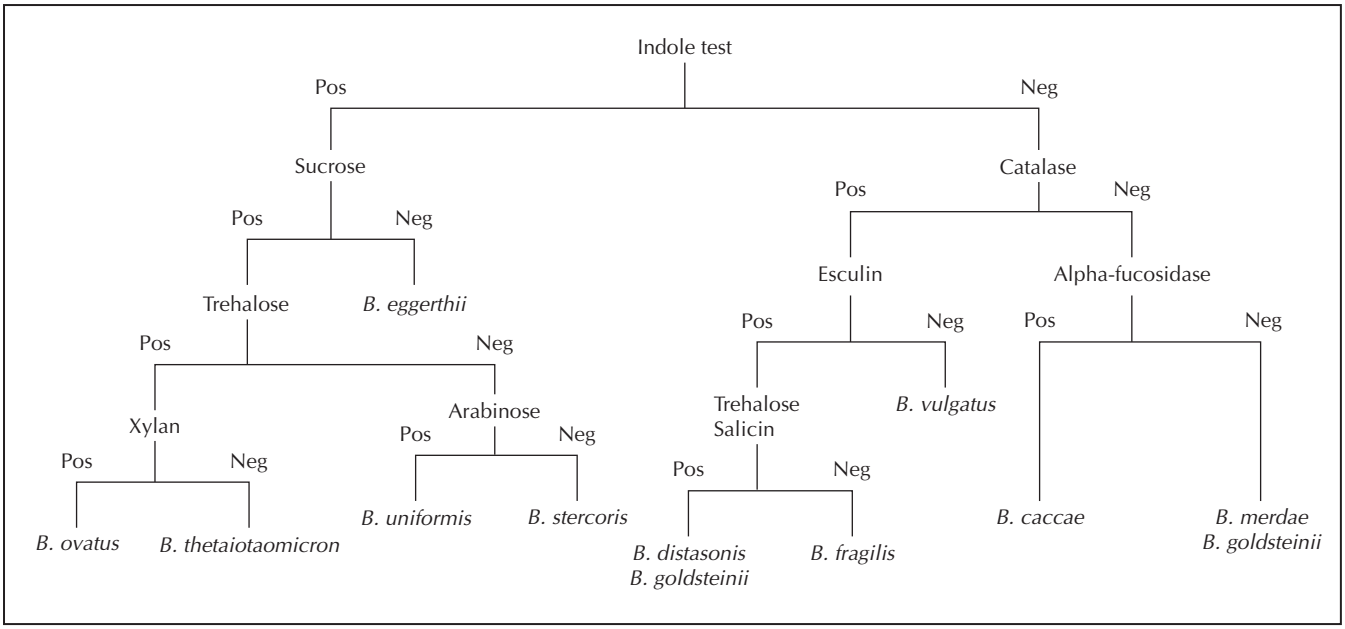


Figure 4.10–5 Identification of the *Bacteroides fragilis* group. Pos, positive; Neg, negative.

VI. RAPID PRESUMPTIVE IDENTIFICATION TESTS *(continued)*

4. An indole-positive, lipase-positive, pleomorphic gram-negative rod that produces beta-hemolytic colonies on blood agar is *F. necrophorum* (*see* Table 4.10–2 and Fig. 4.10–2).
5. *Precaution: Indole is diffusible, so the spot indole test should be performed only on plates with pure cultures* (see *procedure 4.6*).

C. Nitrate and urease (*see* procedure 4.6 for use of nitrate and urea disk tests)

1. A thin, gram-negative bacillus with rounded ends that is resistant to vancomycin and susceptible to both kanamycin and colistin should be tested for nitrate reduction. Place a nitrate disk (Anaerobe Systems, BD, Hardy, Remel) on the anaerobic BAP along with special-potency antimicrobial agent disks (*see* procedure 4.6).
2. The agar-pitting *B. ureolyticus* group organisms resemble some of the less frequently isolated *Fusobacterium* spp. in cell morphology and special-potency disk pattern but are easily differentiated by the ability to reduce nitrates (Fig. 4.10–1 and Table 4.10–7).
3. Nitrate-positive, corroding isolates that are urease positive and have the proper patterns of susceptibility to special-potency disks are identified as *B. ureolyticus* (Fig. 4.10–1 and Table 4.10–7). A rapid urea test using sterile urea broth or a urea disk can give a positive result in 15 to 30 min (*see* procedure 4.6).
4. *C. gracilis* is nonmotile and urease negative and thus can be differentiated from *B. ureolyticus* (Table 4.10–7).
5. *B. wadsworthia*, which phenotypically resembles *B. ureolyticus*, is nitrate and urease positive but grows in 20% bile (or on BBE agar) and has a strong catalase reaction (2) (Table 4.10–7).
6. *Sutterella wadsworthensis* is nitrate positive but is urea negative and catalase negative, allowing for differentiation from *B. wadsworthia* (*see* Table 4.10–7).
7. Small, gram-negative cocci that have colonies fluorescing red (not brick red) under UV light and are nitrate positive are *Veillonella* spp.

Table 4.10–7 Characteristics of *Bacteroides* spp. and other anaerobic gram-negative bacilli that are weakly saccharolytic or nonsaccharolytic[a]

Species	Growth in 20% bile	Indole	Esculin	Gelatin	Catalase	Nitrate	α-Fucosidase[b]	β-Xylosidase[b]	Pitting of agar	Motility	Stimulated with F/F[c]	Urease
Alistipes putredinis	+⁻	+	−	+	+⁻	−	NT	NT	−	−	−	+
Bacteroides capillosus	−⁺	−	+	W	−	−	NT	NT	−	−	−	−
Bacteroides coagulans	+	V	−	+	−	−	NT	NT	−	−	−	−
Bacteroides splanchnicus	+	+	+	NT	−	NT	+	−	−		−	
Bacteroides ureolyticus	−	−	−	−	−⁺	+	NT	NT	+	−	+	+
Bilophila wadsworthia	+	−	−	−	+	+	NT	NT	−	−	−	+⁻
Campylobacter spp.	−	−	−	−	−	+	NT	NT	V	+⁻	+	−
Campylobacter gracilis	−	−	−	−	−	+	NT	NT	V	−	+	−
Sutterella wadsworthensis	+[d]	−	−	−	−	+	NT	NT	V	−	+	−
Tannerella forsythia[e]	−	−	+	+	−	−	NT	NT	−	−	−	−
Tissierella praeacuta[f]	+	−	−	+	−	V	NT	NT	−	+	−	−

[a] Adapted from reference 3. +, positive; −, negative; −⁺, most strains are negative and some are positive; +⁻, most strains are positive and some are negative; W, weak reactions usual; NT, not tested; V, variable reactions.

[b] These results are obtained with Roscoe tablets (WEE-Tabs), used according to the manufacturer's directions.

[c] F/F, formate and fumarate; +, stimulated by addition of formate and/or fumarate to the medium.

[d] *S. wadsworthensis* will not grow on a BBE agar plate, even though it will be resistant to a 20% bile disk.

[e] *Tannerella forsythia* is the new name for *Bacteroides forsythus* (12a, 13a, 16a).

[f] *Tissierella praeacuta* is the new name for *Bacteroides praeacutus* (5).

VI. RAPID PRESUMPTIVE IDENTIFICATION TESTS *(continued)*

D. Bile (*see* procedure 4.6 for use)

1. The ability to grow in the presence of bile is an important characteristic in the primary identification of many anaerobic gram-negative organisms. Use of a primary-isolation BBE plate, bile broth, or bile disk plate provides a quick means of separating organisms that grow on bile.
2. *B. fragilis* group organisms are resistant to bile; other *Bacteroides* spp. may be resistant (*see* Fig. 4.10–1 and 4.10–5 and Tables 4.10–3 and 4.10–6).
3. Some *Fusobacterium* spp. grow on bile (Table 4.10–2 and Fig. 4.10–2). A kanamycin-sensitive, bile-tolerant organism should be identified as a *Fusobacterium* sp. (either as *F. mortiferum* or *F. varium*). Some bile-resistant strains of *F. necrophorum* may also be lipase positive and require additional testing (*see* procedures 4.8 and 4.9).
4. *B. wadsworthia* grows in 20% bile and forms small colonies on BBE in 3 to 4 days that are clear with black centers, resembling "fish eyes" (2) (Table 4.10–7).
5. *S. wadsworthensis* is tolerant to the bile disk but is urease and catalase negative (Table 4.10–7).

   ◩ **NOTE:** An unusual characteristic of *S. wadsworthensis* is that it does not grow in 20% bile broth or on BBE medium.

E. Lipase

1. Lipase (egg yolk agar plate) (*see* procedure 4.6) can be combined with rapid tests to aid identification of pigmented *Prevotella* spp., *Porphyromonas* spp., and *Fusobacterium* spp.
2. An indole- and lipase-positive coccobacillus that produces black-pigmented or brick red fluorescent colonies is identified as a member of the *P. intermedia/nigrescens* group (Table 4.10–5).
3. A lipase-positive, indole-negative pigmented gram-negative rod may be identified as *Prevotella loescheii* (Table 4.10–5).
4. An indole- and lipase-positive isolate with a fusobacterium special-potency antimicrobial agent disk pattern is identified as *F. necrophorum* (Table 4.10–2). Lipase-positive strains of *F. necrophorum* will usually be beta-hemolytic (12).

F. Other rapid tests, spot tests, or enzymatic tests

Other rapid tests, spot tests, or enzymatic tests may be helpful in the identification of anaerobic gram-negative rods (*see* procedure 4.6 for more information) (3, 11, 12).

POSTANALYTICAL CONSIDERATIONS

VII. DEFINITIVE IDENTIFICATION

Use the appropriate figure or table to determine additional tests required for identification of an isolate (*see* also procedures 4.7 to 4.9). The definite identification of some species requires certain additional biochemical tests beyond the scope of this procedure, such as PRAS biochemicals, metabolic end product analysis, genetic analysis, and/or whole-cell fatty acid profiling by GLC (*see* CMPH 2.6 and 2.7 and references 6, 11, and 12). Even in some research or reference laboratories, a number of anaerobic gram-negative strains will not be identified completely. Clinicians usually want to know if anaerobes are present. The choice of antimicrobial agents is usually empirical. Persistent resistance to therapy may lead to requests for antimicrobial susceptibility testing. Periodic antimicrobial profiles of the most common anaerobic isolates should be conducted and shared with the medical community.

REFERENCES

1. **Baron, E. J., P. Summanen, J. Downes, M. C. Roberts, H. Wexler, and S. M. Finegold.** 1989. *Bilophila wadsworthia,* gen. nov. and sp. nov., a unique gram-negative anaerobic rod recovered from appendicitis specimens and human faeces. *J. Gen. Microbiol.* **135:**3405–3411.
2. **Baron, E. J., M. Curren, G. Henderson, H. Jousimies-Somer, K. Lee, K. Lechowitz, C. A. Strong, P. Summanen, K. Tunér, and S. M. Finegold.** 1992. *Bilophila wadsworthia* isolates from clinical specimens. *J. Clin. Microbiol.* **30:**1882–1884.
3. **Citron, D. M., I. R. Poxton, and E. J. Baron.** 2007. *Bacteroides, Porphyromonas, Prevotella, Fusobacterium,* and other anaerobic gram-negative rods, p. 911–932. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
4. **Claros, M. C., Y. Papke, N. Kleinkauf, D. Adler, D. M. Citron, S. Hunt-Gerardo, T. Montag, E. J. C. Goldstein, and A. C. Rodloff.** 1999. Characteristics of *Fusobacterium ulcerans,* a new and unusual species compared with *Fusobacterium varium* and *Fusobacterium mortiferum. Anaerobe* **5:**137–140.
5. **Collins, M. D., and H. N. Shah.** 1986. Reclassification of *Bacteroides praeacutus* Tissier (Holdeman and Moore) in a new genus, *Tissierella,* as *Tissierella praeacuta* comb. nov. *Int. J. Syst. Bacteriol*. **36:**461–463.
6. **Finegold, S. M., and W. L. George.** 1989. *Anaerobic Infections in Humans.* Academic Press, Inc., San Diego, CA.
7. **Finegold, S. M., and H. Jousimies-Somer.** 1997. Recently described anaerobic bacteria: medical aspects. *Clin. Infect. Dis.* **25:**S88–S93.
8. **Finegold, S. M., M.-L. Vaisanen, M. Rautio, E. Eerola, P. Summanen, D. Molitoris, Y. Song, C. Liu, and H. Jousimies-Somer.** 2004. *Porphyromonas uenonis* sp. nov., a pathogen for humans distinct from *P. asaccharolytica* and *P. endodontalis. J. Clin. Microbiol*. **42:**5298–5301.
9. **Han, Y. H., R. M. Smibert, and N. H. Krieg.** 1991. *Wolinella recta, Wolinella curva, Bacteroides ureolyticus,* and *Bacteroides gracilis* are microaerophiles, not anaerobes. *Int. J. Syst. Bacteriol*. **41:**218–222.
10. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore.** 1977. *Anaerobe Laboratory Manual,* 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
11. **Jousimies-Somer, H., and P. Summanen.** 2002. Recent taxonomic changes and terminology update of clinically significant anaerobic gram negative bacteria (excluding spirochetes). *Clin. Infect. Dis.* **35:**S17–S21.
12. **Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold.** 2002. *Wadsworth Anaerobic Bacteriology Manual,* 6th ed. Star Publishing Co., Belmont, CA.

12a. **Judicial Commission of the International Committee on Systematics of Prokaryotes.** 2008. The adjectival form of the epithet in *Tannerella forsythensis* Sakamoto *et al.* 2002 is to be retained and the name is to be corrected to *Tannerella forsythia* Sakamoto *et al.* 2002. Opinion 85. *Int. J. Syst. Evol. Microbiol.* **58:**1974.

13. **Kononen, E., J. Matto, M. L. Vaisanen-Tunkelrott, E. V. G. Frandsen, I. Helander, S. Asikainen, S. M. Finegold, and H. R. Jousimies-Somer.** 1998. Biochemical and genetic characterization of a *Prevotella intermedia/nigrescens*-like organism. *Int. J. Syst. Bacteriol.* **48:**39–46.

13a. **Maiden, M. F. J., P. Cohee, and A. C. R. Tanner.** 2003. Proposal to conserve the adjectival form of the specific epithet in the reclassification of *Bacteroides forsythus* Tanner *et al.* 1986 to the genus *Tannerella* Sakamoto *et al.* 2002 as *Tannerella forsythia* corrig., gen. nov., comb. nov. Request for an opinion. *Int. J. Syst. Evol. Microbiol.* **53:**2111–2112.

14. **Mangels, J. I., I. Edvalson, and M. Cox.** 1993. Rapid presumptive identification of *Bacteroides fragilis* group organisms with use of 4-methylumbelliferone-derivative substrates. *Clin. Infect. Dis.* **16:**S319–S321.
15. **Rautio, M., E. Eerola, M. L. Väisänen-Tunkelrott, D. Molitoris, M. D. Collins, and H. Jousimies-Somer.** 2003. Reclassification of *Bacteroides putredinis* (Weinberg et al., 1937) in a new genus *Alistipes* gen. nov., as *Alistipes putredinis* comb. nov., and description of *Alistipes finegoldii* sp. nov., from human sources. *Syst. Appl. Microbiol.* **26:**182–188.
16. **Sakamoto, M., and Y. Benno.** 2006. Reclassification of *Bacteroides distasonis, Bacteroides goldsteinii,* and *Bacteroides merdae* as *Parabacteroides distasonis* gen. nov., comb. nov., *Parabacteroides goldsteinii* comb. nov., and *Parabacteroides merdae* comb. nov. *Int. J. Syst. Evol. Microbiol.* **56:**1599–1605.

16a. **Sakamoto, M., M. Suzuki, M. Umeda, I. Ishikawa, and Y. Benno.** 2002. Reclassification of *Bacteroides forsythus* (Tanner et al. 1986) as *Tannerella forsythus* corrig., gen. nov., comb. nov. *Int. J. Syst. Evol. Microbiol.* **52:**841–849.

17. **Shah, H. N., and M. D. Collins.** 1988. Proposal for reclassification of *Bacteroides asaccharolyticus, Bacteroides gingivalis,* and *Bacteroides endodontalis* in a new genus, *Porphyromonas. Int. J. Syst. Bacteriol.* **38:**128–131.
18. **Slots, J., and H. S. Reynolds.** 1982. Long-wave UV light fluorescence for identification of black-pigmented *Bacteroides* spp. *J. Clin. Microbiol.* **16:**1148–1151.
19. **Song, Y., C. Liu, J. Lee, M. Bolanos, M. L. Vaisanen, and S. M. Finegold.** 2005. *Bacteroides goldsteinii* sp. nov. isolated from clinical specimens of human intestinal origin. *J. Clin. Microbiol.* **43:**4522–4527.
20. **Vandamme, P., M. I. Daneshvar, F. E. Dewhirst, B. J. Paster, K. Kersters, H. Goossens, and C. W. Moss.** 1995. Chemotaxonomic analyses of *Bacteroides gracilis* and *Bacteroides ureolyticus* and reclassification of *B. gracilis* as *Campylobacter gracilis* comb. nov. *Int. J. Syst. Bacteriol.* **45:**145–152.
21. **Wexler, H. M., D. Reeves, P. H. Summanen, E. Molitoris, M. McTeague, J. Duncan, K. Wilson, and S. M. Finegold.** 1996. *Sutterella wadsworthensis* gen.nov., sp. nov., bile-resistant microaerophilic *Campylobacter gracilis*-like clinical isolates. *Int. J. Syst. Bacteriol.* **46:**252–258.

4.11 Anaerobic Gram-Positive Bacilli

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Anaerobic gram-positive bacilli of clinical relevance in human infections are divided into two distinct groups: members of the genus *Clostridium,* which are spore-forming gram-positive anaerobic bacilli, and a group composed of more than 34 genera of non-spore-forming anaerobic gram-positive bacilli. Of the non-spore-formers, 6 genera are most commonly associated with clinical infections: *Actinomyces, Bifidobacterium, Eubacterium, Eggerthella, Lactobacillus,* and *Propionibacterium* (11–13). Two additional genera, *Mobiluncus* and *Atopobium,* have more recently been found in association with bacterial vaginosis and other infections; however, they are not easily recovered, and their pathogenicity is not as well understood (4, 12, 13). There have been many taxonomic changes among the anaerobic gram-positive non-spore-forming bacilli. Some of these species that have been found in clinical samples are listed in Table 4.11–1. Many of the anaerobic gram-positive bacilli are part of the normal microbiota of the oral cavity, gastrointestinal tract, genitourinary tract, and skin. They can, however, be associated with skin and soft tissue infections, periodontitis and other oral infections, pulmonary infections (usually in combination with other aerobes and anaerobes), genitourinary tract infections, and, in the case of *Propionibacterium acnes,* infected CSF shunts. *Many of the non-sporeformers can be resistant to metronidazole, an antimicrobial agent that is usually effective against most other anaerobes.*

More than 130 species of *Clostridium* have been identified. The percentage of clostridial isolates that are commonly recovered in properly collected specimens is small, however (5, 6, 11). *Clostridium perfringens* is one of the most commonly isolated species, followed by *C. clostridioforme, C. septicum, C. ramosum, C. innocuum,* and *C. tertium* (6, 11, 12). *Clostridium* spp. can cause acute, severe, or chronic infections. Some are always pathogenic, and some are toxigenic *(C. botulinum, C. tetani,* and *C. difficile);* others are rarely pathogenic. Some species are resistant to common antimicrobials used against aerobes (11).

Identification of the anaerobic gram-positive bacilli usually poses the most difficulty in the routine anaerobic laboratory (5, 6, 10–13). Determination of whether these bacilli produce spores is a key issue initially in sorting out their identity; however, some *Clostridium* spp. do not regularly produce spores in culture, making their identification confusing. Some *Clostridium* spp. are aerotolerant to small amounts of oxygen, especially with increased CO_2 in the environment, and they may be mistaken for the aerobic spore-forming *Bacillus* spp. (*C. tertium* and *C. histolyticum* in particular). Some *Clostridium* spp. and non-sporeformers may stain gram negative, confusing their identification even further. Use of prereduced anaerobically sterilized (PRAS) biochemicals, GLC and fatty acid analysis, and a commercial kit system will usually be necessary for complete identification; as sequencing methods become more available, these may replace the more conventional approach, but for now, commercial identification kits, in combination with GLC, fatty acid analysis, growth on a variety of specialized agar media, and utilization of special-potency antimicrobial disks, are used in most laboratories (11–13).

II. SPECIMEN

A. Pure isolates of anaerobic gram-positive bacilli on brucella agar with 5% sheep blood agar or on other enriched media are used for various tests, for inoculum preparation, and for biochemical identification systems (*see* procedures 4.6 to 4.9).

B. Perform aerotolerance testing (*see* procedure 4.4).

C. There are a few aerotolerant strains of *Clostridium (C. tertium, C. carnis,* and *C. histolyticum)* as well as a few aerotolerant strains of non-spore-forming bacilli (*Actinomyces, Lactobacillus,* and *Propionibacterium* spp.). Additional testing to determine their physiological requirements for oxygen may be necessary (10–13).

Table 4.11–1 Changes in taxonomy among the anaerobic gram-positive bacilli

New designation	Old designation
Old *Eubacterium* species	
Eggerthella lenta	*Eubacterium lentum*
Eggerthella hongkongensis	None (new)
Eggerthella sinensis	None (new)
Olsenella profusa	*Eubacterium* group D52
Collinsella aerofaciens	*Eubacterium aerofaciens*
Slackia exigua	*Eubacterium exiguum*
Pseudoramibacter alactolyticus	*Eubacterium alactolyticum*
Bulleidia extructa	*Eubacterium* sp.
Holdemania filiformis	*Eubacterium* sp.
Solobacterium moorei	*Eubacterium* sp.
Filifactor sp.	*Eubacterium* sp.
Mogibacterium spp.	*Eubacterium* spp.
Anaerotruncus spp.	*Eubacterium* spp.
Old *Bifidobacterium* species	
Parascardovia denticolens	*Bifidobacterium denticolens*
Scardovia inopinata	*Bifidobacterium inopinatum*
Old *Lactobacillus* species	
Catenibacterium mitsuokai	*Lactobacillus vitulinus* and *Lactobacillus catenaformis*
Olsenella uli	*Lactobacillus uli*
Atopobium spp.	Anaerobic *Lactobacillus* spp.
Other	
Propionimicrobium lymphophilum	*Propionibacterium lympholymphilum*
Propionibacterium propionicum	*Arachnia propionica*

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents, media, and supplies

1. PRAS brucella agar with 5% sheep blood supplemented with vitamin K and hemin or other suitable enriched agar media (*see* procedure 4.3)
2. CHOC plates
3. PRAS egg yolk agar plates
4. PRAS chopped meat-carbohydrate
5. Identification disks: nitrate
6. Special-potency antimicrobial agent disks: kanamycin, 1,000 μg; vancomycin, 5 μg; colistin, 10 μg
7. Gram stain reagents
8. Nitrate A and B reagents
9. Spot indole reagent (*p*-dimethylaminocinnamaldehyde)
10. 15% H_2O_2
11. Disposable inoculating loops
12. Commercial anaerobic identification system or individual enzymatic substrate (*see* procedure 4.9)

B. Equipment

1. Microscope
2. Anaerobic chamber, glove box, or anaerobic jars

C. Tests necessary for complete identification of anaerobic gram-positive bacilli

1. Gram stain
2. Motility
3. Indole
4. Nitrate
5. Catalase
6. Special-potency antimicrobial agent disks: kanamycin, 1,000 μg; vancomycin, 5 μg; colistin, 10 μg
7. Lecithinase
8. Lipase
9. Spore test
10. Esculin hydrolysis
11. Gelatin liquefaction
12. Oxygen tolerance
13. Urea
14. Carbohydrate fermentation and/or enzymatic reactions
15. Fluorescence

ANALYTICAL CONSIDERATIONS

IV. PROCEDURE

A. Day 1

1. Pick colony of anaerobic gram-positive rod from brucella sheep blood agar or other suitably enriched media and subculture to the following.
   - **a.** Chopped meat-carbohydrate for spore test and GLC (if necessary) and as a backup medium
   - **b.** Egg yolk agar for detection of lipase and lecithinase
   - **c.** Brucella sheep blood agar with nitrate, kanamycin, vancomycin, and colistin disks
   - **d.** CHOC in CO_2 for aerotolerance
2. Gram stain colony for specific characteristics and record results (*see* Appendixes 4.11–1 to 4.11–4 and Table 4.11–2).
   - **a.** If organism is pleomorphic and diphtheroidal, do indole and catalase tests. If both tests are positive, report *Propionibacterium acnes.*
   - **b.** If cells are large, gram-positive bacilli arranged in pairs as "boxcars" and if the colony produces a double zone of hemolysis on blood agar, report as *C. perfringens* (1 to 2 h of refrigeration will develop the zone of hemolysis).
   - **c.** If spores are seen and the cells are not large boxcar type, report *Clostridium* sp. but not *C. perfringens.*
3. Wet mount for motility
   Nonmotile anaerobic gram-positive rods are *C. perfringens, C. ramosum, C. innocuum,* and nonsporeformers (exceptions are *Lactobacillus* and rare *Eubacterium* spp.).
4. Catalase
   Propionibacterium and *Actinomyces viscosus* are positive.

Table 4.11–2 Cost-effective identification tips for the commonly recovered *Clostridium* spp.

Species	Spore location[a]	Swarming	Indole	Lecithinase	Fluorescence	Double zone of hemolysis	Urease	Yellow colonies on CCFA	Gram stain morphology
C. perfringens	Not seen	−	−	+	−	+	−	−	Boxcar; spores not seen
C. clostridioforme	ST	−	−	−	−	−	−	−	Usually gram negative; football-shaped cells
C. innocuum	T	−	−	−	+, chartreuse	−	−	−	Small cells, terminal spores
C. ramosum	T	−	−	−	+, red	−	−	−	Usually gram negative; thin, round, terminal spores
C. bifermentans	ST	−	+	+	−	−	−	−	Large cells, subterminal spores in chains
C. difficile	ST	−	−	−	+, chartreuse	−	−	+	Long, thin cells; oval spores
C. sordellii	ST	−[b]	+	+	−	−	+	−	Straight cells, central to subterminal spores
C. septicum	ST	+	−	−	−	−	−	−	Long, thin cells; pleomorphic; oval spores

[a] +, positive; −, negative; ST, subterminal; T, terminal.

[b] *C. sordellii* may form large, spreading colonies.

IV. PROCEDURE *(continued)*

B. Day 2 (or when adequate growth is achieved)

1. Examine brucella sheep blood agar plate.

a. Record results with kanamycin, vancomycin, colistin, and nitrate disks.

b. Record colonial morphology (*see* Appendix 4.11–1).

c. Record odor. *Clostridium difficile* smells of horse barn, and *P. acnes* has a strong odor of tryptophan.

2. Examine egg yolk agar plate.
Record lecithinase and/or lipase reaction (Fig. 4.11–1).

3. Stain chopped meat-carbohydrate.

a. Gram stain, and look for spores. If spores are present, record their presence and locations.

b. Spore stain can also be used (11).

4. Perform indole, fluorescence, and urea tests. Use cycloserine cefoxitin fructose agar (CCFA) as needed (*see* procedure 4.3).

5. Observe for swarming. *Hint:* This can be difficult at times to confirm. Drag loop or stick on medium to observe for presence of growth.

C. Days 3 to 5

Perform spore test if needed (6, 10, 11).

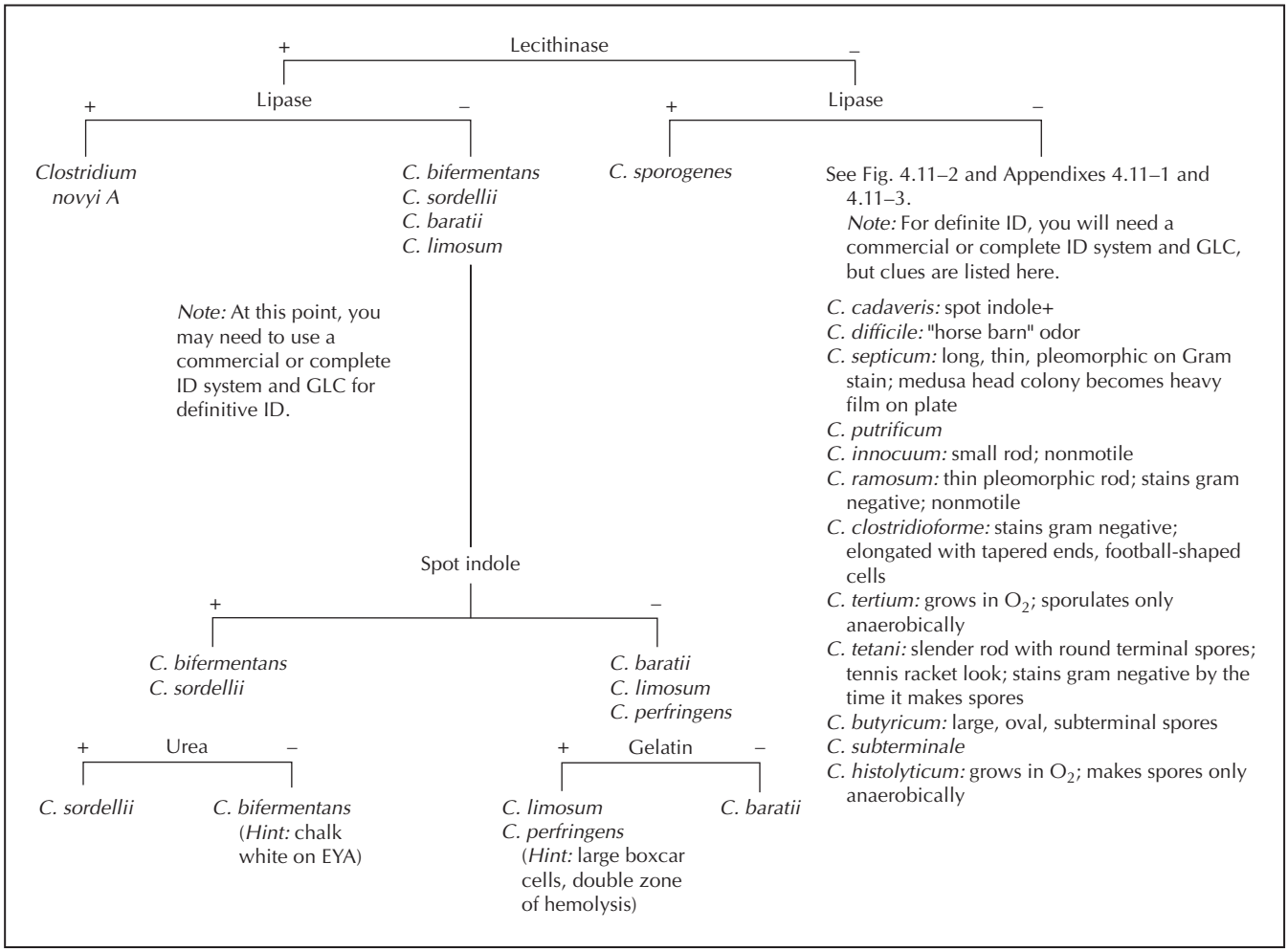

Lecithinase
+
–
Lipase
+
–
Lipase
+
–
Clostridium novyi A
C. bifermentans
C. sordellii
C. baratii
C. limosum
C. sporogenes
See Fig. 4.11–2 and Appendixes 4.11–1 and 4.11–3.
Note: For definite ID, you will need a commercial or complete ID system and GLC, but clues are listed here.
Note: At this point, you may need to use a commercial or complete ID system and GLC for definitive ID.
C. cadaveris: spot indole+
C. difficile: "horse barn" odor
C. septicum: long, thin, pleomorphic on Gram stain; medusa head colony becomes heavy film on plate
C. putrificum
C. innocuum: small rod; nonmotile
C. ramosum: thin pleomorphic rod; stains gram negative; nonmotile
C. clostridioforme: stains gram negative; elongated with tapered ends, football-shaped cells
C. tertium: grows in O_2; sporulates only anaerobically
C. tetani: slender rod with round terminal spores; tennis racket look; stains gram negative by the time it makes spores
C. butyricum: large, oval, subterminal spores
C. subterminale
C. histolyticum: grows in O_2; makes spores only anaerobically
Spot indole
+
–
C. bifermentans
C. sordellii
C. baratii
C. limosum
C. perfringens
+
Urea
–
+
Gelatin
–
C. sordellii
C. bifermentans (Hint: chalk white on EYA)
C. limosum
C. perfringens (Hint: large boxcar cells, double zone of hemolysis)
C. baratii

Figure 4.11–1 Identification tips for spore-forming rods using egg yolk agar (EYA). ID, identification.

V. IDENTIFICATION

A. *Clostridium* spp.

See Appendix 4.11–2 and Fig. 4.11–2 for Gram stain and colonial characteristics of species. See Table 4.11–2 for cost-effective identification tips for commonly isolated *Clostridium* spp.

1. Vegetative cells rod shaped, can vary from coccoid to filamentous
2. Obligate anaerobes (majority)
   Exceptions (will grow on CHOC in air) are *C. carnis*, *C. histolyticum*, and *C. tertium.*
3. Gram positive (most)
   Exceptions (sometimes appear as gram negative) are *Clostridium clostridioforme*, *C. ramosum*, and *Clostridium tetani* (by time of spore formation).
4. Motile
   Exceptions are *C. perfringens*, *C. ramosum*, and *C. innocuum.*
5. Catalase is generally not produced. If it is produced, it will be weak and in small amounts.
6. Since aerotolerant clostridia *(C. tertium*, *C. carnis*, and *C. histolyticum)* can show growth on solid media incubated in 5 to 10% CO_2, it is possible to confuse them with certain facultatively anaerobic *Bacillus* species. However, members of the genus *Clostridium* usually form spores only under anaerobic conditions and almost never produce catalase, whereas *Bacillus*

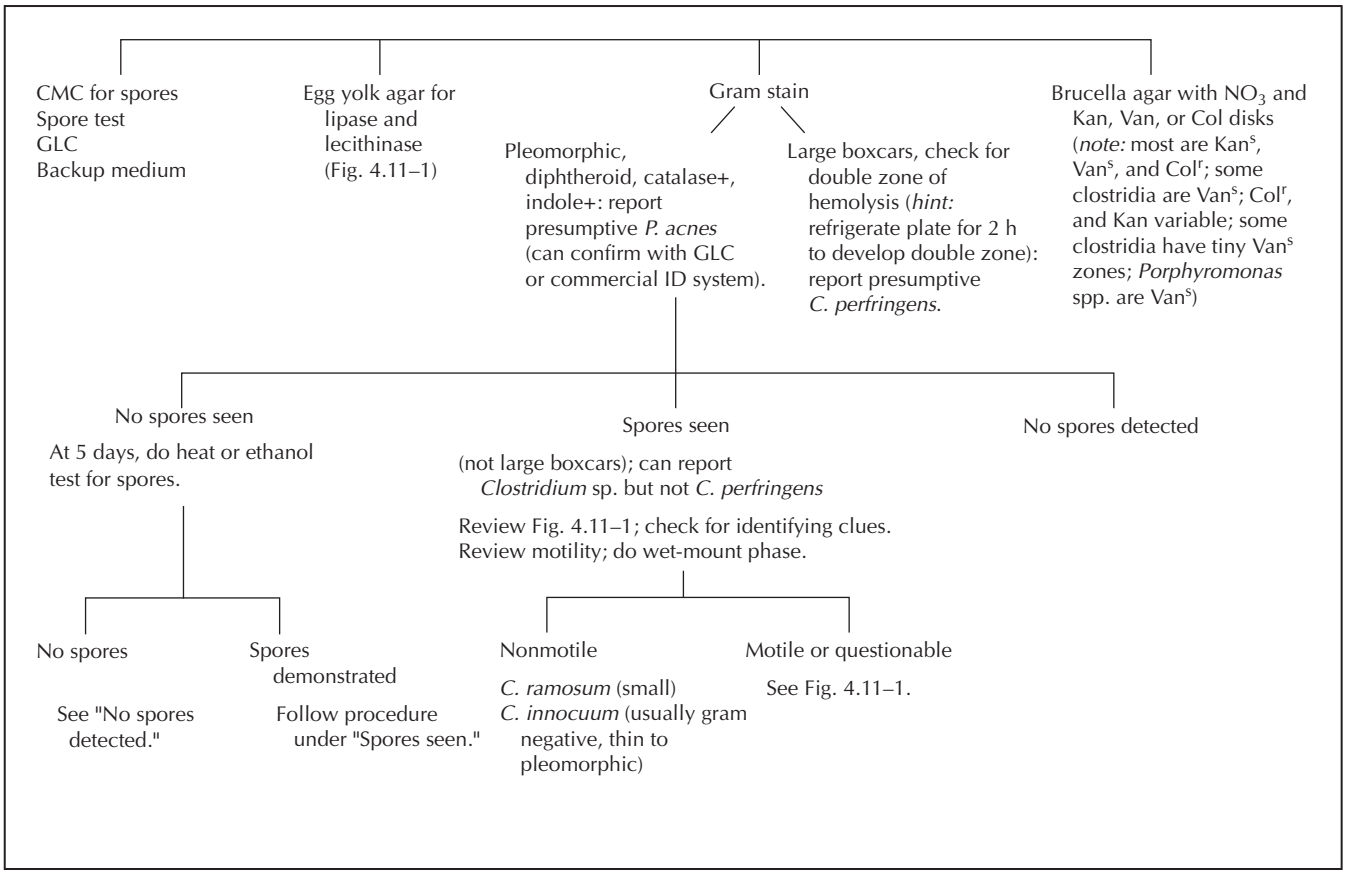

CMC for spores
Spore test
GLC
Backup medium
Egg yolk agar for lipase and lecithinase (Fig. 4.11–1)
Gram stain
Pleomorphic, diphtheroid, catalase+, indole+: report presumptive *P. acnes* (can confirm with GLC or commercial ID system).
Large boxcars, check for double zone of hemolysis (*hint:* refrigerate plate for 2 h to develop double zone): report presumptive *C. perfringens.*
Brucella agar with NO_3 and Kan, Van, or Col disks (*note:* most are Kan^s, Van^s, and Col^r; some clostridia are Van^s; Col^r, and Kan variable; some clostridia have tiny Van^s zones; *Porphyromonas* spp. are Van^s)
No spores seen
At 5 days, do heat or ethanol test for spores.
Spores seen
(not large boxcars); can report *Clostridium* sp. but not *C. perfringens*
No spores detected
Review Fig. 4.11–1; check for identifying clues.
Review motility; do wet-mount phase.
No spores
See "No spores detected."
Spores demonstrated
Follow procedure under "Spores seen."
Nonmotile
C. ramosum (small)
C. innocuum (usually gram negative, thin to pleomorphic)
Motile or questionable
See Fig. 4.11–1.

Figure 4.11–2 Procedure for identification of pure-colony anaerobic gram-positive bacillus from brucella or other agar. Refer to previous sections for details on collection, isolation, culture, and examination of plates and on obtaining pure colonies of anaerobic gram-positive rods. CMC, chopped meat-carbohydrate; ID, identification; Kan, kanamycin; Van, vancomycin; Col, colistin; r, resistant; s, susceptible.

V. IDENTIFICATION *(continued)*

species will form spores under aerobic or anaerobic conditions and are catalase positive.

7. Some species are obligate anaerobes, such as *Clostridium haemolyticum* and *Clostridium novyi* type B, and will not grow when exposed to even trace amounts of oxygen.
8. Swarming or spreading clostridia *(C. tetani, Clostridium septicum, and C. sordellii)* can be differentiated by spore location, indole, and urease production.
9. An organism that produces a horse barn smell, produces yellow "ground-glass" colonies on CCFA, and fluoresces chartreuse is *C. difficile.*
10. *C. ramosum* is a thin, gram-variable rod that has a small round or oval terminal spore. It can commonly be misidentified as a gram-negative rod. *Hint:* When in doubt, perform special-potency disk procedure and observe for an oval terminal spore often appearing as a blue dot.

B. Gram-positive non-spore-forming bacilli

See Appendixes 4.11–1, 4.11–3, and 4.11–4 for more details. These bacilli include the more commonly isolated *Actinomyces, Bifidobacterium, Eggerthella, Eubacterium, Lactobacillus,* and *Propionibacterium,* as well as many more newly named genera and species.

1. Distinctively variable morphology
   - **a.** Vary from branched, pleomorphic, diphtheroidal, or streptococcuslike to long and slender
   - **b.** Gram positive to gram variable
2. Considerable variation in relationship to oxygen
   - **a.** Most are obligate anaerobes.
   - **b.** Some facultative anaerobes or microaerophilic *Actinomyces* spp. (exception: *Actinomyces meyeri*) are microaerophilic.
   - **c.** *Propionibacterium* spp. may grow aerobically in the presence of CO_2.
3. Nonmotile
   Exceptions are rare *Lactobacillus* spp. and rare *Eubacterium* spp.
4. Catalase not produced
   Exceptions are *Propionibacterium* and *A. viscosus.*
5. Most commonly encountered in infections
   - **a.** *Propionibacterium acnes:* infections of implanted devices such as ventricular-arterial shunts or artificial joints
   - **b.** *Actinomyces* spp.: actinomycosis
   - **c.** *Eubacterium, Eggerthella,* and other newer genera of related organisms: blood cultures, genitourinary tract infections, and pulmonary infections
6. A clubbing, palisading, gram-positive bacillus that is indole positive, nitrate positive (usually), and catalase positive is *P. acnes.*
7. A small, straight-sided gram-positive bacillus that is nitrate positive and fluoresces red under UV light (366 nm) is *Eggerthella lenta.*

◩ **NOTE:** Other non-spore-forming, anaerobic gram-positive bacilli may be isolated from a variety of clinical specimens. Often they are part of a mixed aerobic and anaerobic microbiota, and it may be difficult to sort out their clinical relevance.

VI. REPORTING RESULTS

A. Rapid presumptive reporting to the physician can be extremely valuable. In some situations, the identification to the genus and species levels of an anaerobic gram-positive rod may take a number of days because of the time required to detect spores or because slow growers take a long time to form end products for GLC. Therefore, it is essential that preliminary results from rapid tests, Gram stains, etc., be reported as promptly as possible.

VI. REPORTING RESULTS
(continued)

B. Reports of results should include the following.
- **1.** Organized report form with a clear format
- **2.** Rapid reporting of Gram stain and other preliminary tests
- **3.** Legibility on workcards (especially important if more than one technologist is reporting results); clear entries if computer is used
- **4.** Telephone calls to the physician or ward about Gram stains and preliminary results with sterile body fluids or growth in blood cultures, CSF, or any sterile body fluid or tissue, per laboratory guidelines for reporting critical values and/or other reporting
- **5.** Telephone calls to the physician or ward of any abnormal results
- **6.** Documentation of information relayed by telephone
- **7.** Method of flagging abnormal results
- **8.** Interpretation of any results that might be confusing

POSTANALYTICAL CONSIDERATIONS

VII. PROCEDURE NOTES

A. Confirm aerotolerance testing of isolate. Some *Clostridium* spp. grow aerobically and may be confused with *Bacillus* spp. (*see* procedure 4.4; more details are within this procedure as well).

B. Confirm purity of all isolates (*see* procedure 4.4).

C. Confirm Gram stain reaction (*see* procedures 4.2 and 4.4).

D. Confirm presence of spores. Do not confuse spores with vacuoles. There will be one spore per cell in the same location in every cell. Vacuoles (one or more) appear in various locations within the cell.

E. Confirm motility. Do not confuse Brownian movement with motility.

F. Confirm that you have a bacillus. Look at a Gram-stained sample taken from around a penicillin disk on a plate where your organism is growing. A rod elongates and sometimes takes on long, bizarre shapes; cocci remain coccal.

REFERENCES

1. **Clarridge, J. E., and O. Zhang.** 2002. Genotypic diversity of clinical *Actinomyces* species: phenotype, source, and disease correlation among genospecies. *J. Clin. Microbiol.* **40:**3442–3448.
2. **Dewhirst, F. E., B. J. Paster, N. Tzellas, B. Coleman, J. Downes, D. A. Spratt, and W. G. Wade.** 2001. Characterization of novel human oral isolates and cloned 16S rDNA sequences that fall in the *Coriobacteriaceae:* description of *Olsenella* gen. nov., reclassification of *Lactobacillus uli* as *Olsenella uli* comb. nov. and description of *Olsenella profusa* sp. nov. *Int. J. Syst. Evol. Microbiol.* **51:**1797–1804.
3. **Downes, J., B. Olsvik, S. J. Hiom, D. A. Spratt, S. L. Cheeseman, I. Olsen, A. J. Weightman, and W. G. Wade.** 2000. *Bulleidia extructa* gen. nov., sp. nov., isolated from the oral cavity. *Int. J. Syst. Evol. Microbiol.* **50:**979–983.
4. **Ferris, M. J., A. Masztal, and D. H. Martin.** 2004. Use of species-directed 16S rRNA gene PCR primers for detection of *Atopobium vaginae* in patients with bacterial vaginosis. *J. Clin. Microbiol.* **42:**5892–5894.
5. **Finegold, S. M., and W. L. George.** 1989. *Anaerobic Infections in Humans.* Academic Press, Inc., San Diego, CA.
6. **Forbes, B. A., D. F. Sahm, and A. S. Weissfeld (ed.).** 2007. *Bailey and Scott's Diagnostic Bacteriology*, 12th ed. Mosby Elsevier, St. Louis, MO.
7. **Funke, G., N. Alvarez, C. Pascual, E. Falsen, E. Akervall, L. Sabbe, L. Schouls, N. Weiss, and M. D. Collins.** 1997. *Actinomyces europaeus* sp. nov., isolated from human clinical specimens. *Int. J. Syst. Bacteriol.* **47:**687–692.
8. **Hall, V., M. D. Collins, R. Hutson, E. Falsen, and B. I. Duerden.** 2002. *Actinomyces cardiffensis* sp. nov. from human clinical sources. *J. Clin. Microbiol.* **40:**3427–3431.
9. **Hall, V., P. R. Talbot, S. L. Stubbs, and B. I. Duerden.** 2001. Identification of clinical isolates of *Actinomyces* species by amplified 16S ribosomal DNA restriction analysis. *J. Clin. Microbiol.* **39:**3555–3562.
10. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore.** 1977. *Anaerobe Laboratory Manual*, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
11. **Johnson, E. A., P. Summanen, and S. M. Finegold.** 2007. *Clostridium*, p. 889–910. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.

REFERENCES *(continued)*

12. **Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold.** 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual,* 6th ed. Star Publishing Co., Belmont, CA.
13. **Könönen, E., and W. G. Wade.** 2007. *Propionibacterium, Lactobacillus, Actinomyces,* and other non-spore-forming anaerobic gram-positive rods, p. 872–888. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
14. **Nakazawa, F., S. E. Poco, M. Sato, T. Ikeda, S. Kalfas, G. Sundqvist, and E. Hoshino.** 2002. Taxonomic characterization of *Mogibacterium diversum* sp. nov. and *Mogibacterium neglectum* sp. nov., isolated from human oral cavities. *Int. J. Syst. Evol. Microbiol.* **52:**115–122.
15. **Ramos, C. P., E. Falsen, N. Alvarez, E. Akervall, B. Sjoden, and M. D. Collins.** 1997. *Actinomyces graevenitzii* sp. nov., isolated from human clinical specimens. *Int. J. Syst. Bacteriol.* **47:**885–888.
16. **Sabbe, L. J. M., D. Van de Merwe, L. Schouls, A. Bergmans, M. Vaneechoutte, and P. Vandamme.** 1999. Clinical spectrum of infections due to newly described *Actinomyces* species *A. turicensis, A. radingae,* and *A. europaeus. J. Clin. Microbiol.* **37:**8–13.
17. **Sarkonen, N., E. Könönen, P. Summanen, M. Könönen, and H. Jousimies-Somer.** 2001. Phenotypic identification of *Actinomyces* and related species isolated from human sources. *J. Clin. Microbiol.* **39:**3955–3961.
18. **Stackebrandt, E., P. Schumann, K. P. Schaal, and N. Weiss.** 2002. *Propionimicrobium* gen. nov., a new genus to accommodate *Propionibacterium lymphophilum* (Torrey 1916) Johnson and Cummins 1972, 1057^{AL} as *Propionimicrobium lymphophilum* comb. nov. *Int. J. Syst. Evol. Microbiol.* **52:**1925–1927.
19. **Tietz, A., K. E. Aldridge, and J. E. Figueroa.** 2005. Disseminated coinfection with *Actinomyces graevenitzii* and *Mycobacterium tuberculosis:* case report and review of the literature. *J. Clin. Microbiol.* **43:**3017–3022.
20. **Vandamme, P., E. Falsen, M. Vancanneyt, M. Van Esbroeck, D. Van de Merwe, A. Bergmans, L. Schouls, and L. Sabbe.** 1998. Characterization of *Actinomyces turicensis* and *Actinomyces radingae* strains from human clinical samples. *Int. J. Syst. Bacteriol.* **48:**503–510.

APPENDIX 4.11–1

Gram stain, colonial characteristics, and common biochemical clues to identify species of *Actinomyces* and *Propionibacterium*[a]

Organism	Gram stain morphology	Colonial morphology	Usual source(s)	Aerotolerance	Urease	Esculin	Catalase	Nitrate	α-GLU	Indole
Actinomyces spp.										
A. turicensis	Straight to curved rods; pseudobranching; gram variable	Glistening; opaque, butyrous	Genital sites	+	−	−	−	−	+	−
A. radingae[b]	Coccoid; pseudobranching	Small gray convex colony; butyrous; α-heme	Skin related	(+)	−	+	−	V	+	−
A. israelii	Short rods; branching; pleomorphic	White, opaque; "molar tooth" colonies	Genital; pulmonary; abdominal	(+)	−	+	−	+[−]	+	−
A. naeslundii	Short rods; branching or beading	Gray-white, translucent	Oral	+	+	V	−	+[−]	V	−
A. europaeus	Short rods; no filaments or branching	Smooth; translucent gray colonies; β-heme	UTI	+	−	V	−	V	+	ND
A. odontolyticus[c]	Short rods; branching or beading		Oral	+	−	V	−	+	−	−
A. meyeri	Short rods; branching or beading			−	−	V	−	V	+	−
A. graevenitzii	Short rods; some branching; may have swollen ends	Opaque colonies; may adhere to agar		+	−	−	−	−	−	−
A. viscosus	Short rods; branching or beading		Oral	+	+	V	+	+	V	−
Newer species										
A. cardiffensis				(+)	−	−	−	V	+	ND
A. dentalis				−	−	+	−	−	+	ND
A. hongkongensis				−	−	−	−	−	−	ND
A. nasicola				(+)	−	−	−	−	+	ND
A. oricola				+	−	+	−	−	+	ND
Propionibacterium spp.										
P. acnes				+	ND	−	+	+		+
P. avidum				+		+	+	−		−
P. granulosum				+		−	+	−		−
P. propionicum				−		−	−	+		−
Other										
Propionimicrobium lymphophilum	Coccoid; singly or in pairs or short chains; clumps	White, glistening small colonies	Initial isolate: lymph nodes	−		−	V	V		−

[a] α-GLU, alpha-glucosidase; +, positive; −, negative; V, variable; (+), better growth anaerobically; ND, not determined; UTI, urinary tract infections. Adapted from references 1, 8, 12, 13, and 15–20.

[b] May enhance growth in broth with 5% rabbit or horse blood.

[c] May produce pink to red colonies in ambient air.

APPENDIX 4.11–2

Gram stain and colonial characteristics of anaerobic gram-positive bacilli

Organism	Gram stain characteristics[a]	Colonial characteristics
Clostridium baratii	Large, with blunt ends; nonmotile; spores (ST) rarely seen	No hemolysis
C. bifermentans	Large, motile, oval (ST) spores in chains	Gray, irregular edge; narrow zone of hemolysis (note: chalk white on egg yolk agar)
C. botulinum	Large, motile, spores (ST)	Variable hemolysis
C. butyricum	Round or blunt ends, motile, large oval (ST) spores	Nondescript
C. cadaveris	Motile, oval (T) spores	
C. clostridioforme	Stains gram negative; elongated with tapered ends; football-shaped cells; spores rarely seen	Small, convex, translucent; mottled or mosaic surface
C. difficile	Relatively long, thin, motile, oval (T) spores readily seen; horse barn or stable odor	Slightly raised; umbonate with filamentous edge; translucent, with crystalline internal speckling; chartreuse fluorescence
C. histolyticum	Pleomorphic, motile, oval (ST) spores	Smooth and rough colonies; rough have flat edges with rhizoids; aerotolerant
C. innocuum	Small, nonmotile spores (T)	White, glossy, raised; chartreuse fluorescence
C. novyi	Medium, motile, oval (ST) spores	Gray, translucent; irregular surface; may swarm; double zone of hemolysis
C. perfringens	Large, blunt square ends; boxcar appearance; spores rarely seen, nonmotile	Gray, opaque; low, flat, somewhat rhizoid; tend to spread but not swarm; double zone of hemolysis
C. ramosum	Frequently gram negative; thin, pleomorphic, in chains with bulges; nonmotile; spores (T) round or oval, rarely seen	Translucent; circular or slightly irregular; entire; convex; red fluorescence
C. septicum	Long, thin, some oval; tend to be pleomorphic, sometimes producing long thin filaments; chain formation common; motile, oval (ST) spores	Medusa head-like growth becomes heavy film that covers plate; flat, gray, glistening, semitranslucent; markedly irregular to rhizoid margins
C. sporogenes	Oval (ST) spores, filamentous in older cultures, motile	Raised gray-yellow center, rhizoid edge; swarms; colonies adhere firmly to agar
C. sordellii	Straight, in singles and pairs; spores central to ST, cause slight swelling of cell; free spores often seen, motile	Translucent to opaque; flat or raised; can have mottled internal structure; swarms or spreads
C. tertium	Large oval (T) spores; sporulates only anaerobically; motile	Small, low, translucent, glossy; aerotolerant
C. tetani	Slender, motile, round (T) spores; tennis racket appearance	Translucent, gray; irregular edge; narrow zone of hemolysis

[a] Spore location: ST, subterminal; T, terminal.

APPENDIX 4.11–3

Phenotypic characteristics of *Clostridium* spp.[a,b]

Type and species	Gelatin hydrolysis	Glucose fermentation	Lecithinase	Lipase	Indole	Aerobic growth	Urea	Nitrate	Motility	Spore shape and location[c]	Esculin
Saccharolytic proteolytic											
C. bifermentans	+	+	+	−	+	−	−	−		OS	+
C. sordellii	+	+	+	−	+	−	+⁻	−		OS	−
C. perfringens	+	+	+	−	−	−	−	+⁻	−	RS	−
C. novyi A	+	+	+	+	−	−	−	−		OS	−
C. sporogenes	+	+	−	+	−	−	−	−		OS	+
C. cadaveris	+	+	−	−	+	−	−	−		OT	−
C. septicum	+	+	−	−	−	−	−	V		OS	+
C. difficile	+	+	−	−	−	−	−	−		OS	+
C. putrificum	+	+	−	−	−	−	−	−		T	−⁺
Saccharolytic nonproteolytic											
C. baratii	−	+	+	−	−	−	−	−		S, RS	+
C. tertium	−	+	−	−	−	+	−	−		OT	+
C. butyricum	−	+	−	−	−	−	−	−	−	OA	+
C. innocuum	−	+	−	−	−	−	−	−	−	OT	+
C. ramosum	−	+	−	−	−	−	−	−		R, OT, RS	+
C. clostridioforme	−	+	−	−	−⁺	−	−	+⁻		OS, RS	+
Asaccharytotic proteolytic											
C. tetani	+	−	−	−	V	−	−	−		RT	−
C. hastiforme	+	−	−	−	−	−	−	−⁺		S	−
C. subterminale	+	−	−⁺	−	−	−	−	−		OS, RS	−⁺
C. histolyticum	+	−	−	−	−	+⁻	−	−		OS	−
C. limosum	+	−	+	−	−	−	−	−		S	−

[a] Adapted from reference 11 with permission.

[b] +, positive reactions for 90 to 100% of strains; −, negative reactions for 90 to 100% of strains; +⁻, most strains positive, some strains negative; −⁺, most strains negative, some strains positive; V, variable (strains may be either + or −).

[c] OA, only anaerobic; RS, rarely seen; O, oval; R, round; S, subterminal; T, terminal.

APPENDIX 4.11–4

Characteristics of selected anaerobic gram-positive bacilli that are new species or were formerly identified as *Eubacterium*, *Bifidobacterium*, or other organisms[a]

Organism(s)	Bacillary form	Morphology	Metabolism	Glucose	Indole	Esculin	Catalase	Nitrate	ADH
New organisms									
Actinobaculum spp.	Straight or slightly curved rods	Singly; clusters	Saccharolytic						
Cryptobacterium spp.	Short rods; diphtheroidal								
Anaerofustis spp.	Thin rods		Saccharolytic						
Anaerostipes spp.	Thin rods	Short chains	Saccharolytic						
Bryantella spp.	Short rods	Pairs; short chains	Requires formate and fumarate						
Oribacterium spp.	Elongated to ovoid	Singly; pairs	Saccharolytic; motile						
Shuttleworthia	Short or slightly curved	Pairs; short chains	Saccharolytic	+	+	+	−	−	−
Turicibacter sanguinis	Irregular long rods	Long chains	Saccharolytic						
Faecalibacterium spp.	Pleomorphic rods		Saccharolytic						
Former *Eubacterium* spp.									
Eggerthella lenta	Straight rod with rounded ends		Saccharolytic; growth in 20% bile	−	−	V	+	+	+
Collinsella aerofaciens	Coccoid	Chains	Saccharolytic; stimulated by Tween 80	+	−	−	−	−	V
Slackia exigua			Stimulated by arginine	−	−	−	−	−	+
Bulleidia extructa	Short, slightly curved rods	Singly; pairs	Saccharolytic; stimulated by Tween 80	+	−	−	−	−	+
Holdemania filiformis	Short rods	Pairs; short chains	Saccharolytic; may grow weakly in 20% bile	+	−	+	−	−	−
Pseudoramibacter alactolyticus	Pleomorphic	Pairs	Saccharolytic	+	−	−	−	−	−
Solobacterium moorei	Straight or slightly curved rods	Pairs; short chains	Saccharolytic	+	−	+	−	−	+
Mogibacterium spp.	Short rods	Singly; clumps	Asaccharolytic	−	−	−	−	−	−
Anaerotruncus spp.	Thin rods		Saccharolytic	−	−	−	−	−	+
Atopobium vaginae				+	−	−	−	−	+
Olsenella spp.		Singly; pairs; short chains	Bile: variable growth	+					
Bifidobacterium spp.	Diphtheroidal; bifurcate or forked ends								

[a] Adapted from material in references 2–4 and 12–14. Where no information is included, not much is known about the biochemical characteristics of the organism at this time. +, positive; −, negative; V, variable; ADH, arginine dihydrolase.

4.12 Anaerobic Cocci

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The anaerobic cocci are a prominent part of the normal human microbiota of the skin, gastrointestinal tract, oral cavity, upper respiratory tract, and female genital tract. Anaerobic gram-positive cocci can be important human pathogens, and next to the anaerobic gram-negative bacilli, these anaerobes are the most commonly isolated anaerobes in clinically relevant infections (3–5, 9). In four surveys of human anaerobic pathogens in various clinical specimens, anaerobic cocci constituted 24 to 31% of all isolates (3, 9). The types of infections in which anaerobic gram-positive cocci predominate include infections in patients with a variety of head and neck infections, including periodontitis, chronic otitis media, chronic sinusitis, and brain abscesses; infectious processes in the female genital tract, including tubo-ovarian abscesses; and infections of the abdominal cavity, including peritonitis and perforated appendices. Many of these infections are polymicrobic, including other aerobes and anaerobes (3–5, 9). Anaerobic gram-negative cocci, although not infrequently encountered in the clinical laboratory from clinical specimens, are not often associated with a great number of infections (4, 5).

The use of DNA composition, hybridization data, and cellular fatty acid profiles in conjunction with analysis of 16S rRNA gene patterns continues to result in revisions of the taxonomy of the clinically relevant anaerobic gram-positive cocci. In the previous edition of this handbook, some of these changes were incorporated in Table 4.12–1; this table has been included again with a few further changes and additions to reclarify the present taxonomic status of the gram-positive anaerobic cocci and gram-negative anaerobic cocci. The only species that remains in the genus *Peptococcus* is *Peptococcus niger*. It is rarely isolated from clinical specimens and is not discussed further. Members of the genus *Peptostreptococcus* include *Peptostreptococcus anaerobius, P. stomatis,* and *P. micros*. The last was designated *Micromonas micros* in the last CMPH edition; however, more recent literature states that *Micromonas* is an illegitimate genus name (8, 9, 11). We conform to that here and refer to the organism again as *Peptostreptococcus micros*. A few additional species of *Anaerococcus* have been included in this table: *Anaerococcus vaginalis, A. octavius, A. hydrogenalis, A. lactolyticus, A. tetradius,* and *A. prevotii*. In a study by Song et al. in 2007, the last two species were rarely found; instead, many organisms previously identified as *A. tetradius* or *A. prevotii* were actually reidentified with aid of sequencing as *A. vaginalis* (2, 10–12). The *Peptoniphilus* genus includes *P. asaccharolyticus, P. ivorii, P. harei,* and *P. lacrimalis*. Although much of the literature and many of the commercial kits still identify *P. asaccharolyticus* commonly, the Song article suggests that many of these may actually be more appropriately identified as *P. harei* when molecular methods are utilized (10–12). *Atopobium parvulum* is the most recent designation for what was once called *Streptococcus parvulus* (1, 5, 7, 9). There are members of the *Atopobium* genus that are classified as gram-positive anaerobic non-spore-forming bacilli, and there are newer designations for some species of what was once in the genus *Eubacterium* (*see* procedure 4.11). *Staphylococcus saccharolyticus* is one of two anaerobic staphylococci, the other being *Staphylococcus aureus* subsp. *anaerobius* (9). Table 4.12–2 summarizes changes in taxonomy of the anaerobic gram-positive cocci.

In most cases, when an anaerobic gram-negative coccus is isolated, it is in a mixed culture with other anaerobes and aerobes. It is difficult to know what the significance of that coccus is, and identification as "anaerobic gram-negative coccus" may be adequate. If identification is pursued, one of three genera will most likely be the answer: *Veillonella, Acidaminococcus,* or *Megasphaera.* Members of the genus *Veillonella,* in particular, that are isolated most commonly from the human microbiota include *Veillonella atypica, V. dispar, V. parvula,* and a recently described species, *V. montpellierensis* (6).

Anaerobic cocci can be identified by Gram stain, colonial morphology, rapid (spot) tests, and various biochemical reactions, in tube or kit systems. In some instances, prereduced anaerobically sterilized (PRAS) biochemicals, gas-liquid chromatography (GLC), or fatty acid analysis may be necessary (3, 5, 9). Rarely recovered strains of anaerobic cocci or strains that have only been described for animals are not described here.

Table 4.12–1 Characteristics of commonly recovered *Peptostreptococcus*, *Peptoniphilus*, *Anaerococcus*, *Finegoldia*, *Atopobium*, and *Staphylococcus* spp.[a]

Species	Indole	PYR	Urease	Glucose fermentation	Alkaline phosphatase	α-Galactosidase	Gram stain	Colony morphology	Prior designation
Peptostreptococcus anaerobius[b]	−	−	−	+	−	−	Large cocci, often in chains	Gray with raised center; nonhemolytic; sweet odor	
P. micros	−	+	−	−	+	−	Clusters or short chains; <0.6 μm	Small, convex; dull white color; "halo" around colonies	*Micromonas (Peptostreptococcus) micros*
P. productus	−		−	+	−	+	Ovoid; in pairs or chains	Glistening and gray colonies	
Finegoldia magna	−	+	−	−[w]	d	−	Pairs, tetrads, and clusters; >0.6 μm	White; convex; small colonies	
Peptoniphilus asaccharolyticus	+[−]	−	−	−	−	−	Clumps, pairs, or tetrads	Small; slightly yellow pigment	*Peptostreptococcus asaccharolyticus*
P. ivorii	−	−	−	−	−	−	Clumps	Convex, slightly yellow colonies	
P. harei	V	−	−	−	−	−	Variable in shape and size	Flat, translucent colonies	
P. lacrimalis	−	−	−	−	−	−	Short chains or clumps	Pink-white colonies	
Anaerococcus vaginalis	V	−	−	+	d	−	Variable size; clumps and tetrads	Convex, gray to white colonies	
A. octavius	−	+[w]	−	+	−	−	Clumps	Yellow-white, glistening; raised colonies	
A. hydrogenalis	+	−	V	+	+[w]	−	Short chains or clumps	Small; nonhemolytic	
A. lactolyticus	−	−	+	+	+	−	Short chains or clumps	Pink-white colonies	
A. tetradius	−	+[w]	+	+	−	−	Clumps and tetrads	Nonglistening; gray; convex	*Peptostreptococcus tetradius*
A. prevotii	−	+	V	V	−	V	Clumps and tetrads	Nonglistening; gray; convex	*Peptostreptococcus prevotii*
Atopobium parvulum[c]	−			+		+	Short chains; elongated cocci	Resemble lactobacilli on agar	*Streptococcus parvulus*
Staphylococcus saccharolyticus[d]	ND		ND	+	ND	ND	Clusters and tetrads	Catalase and coagulase negative	
S. aureus subsp. *anaerobius*				+				Catalase and coagulase negative	

[a] PYR, pyrrolidonyl arylamino dihydrolase; +, positive; −, negative; d, often a delayed positive; w, weak; V, some strains are positive and some are negative. Adapted from references 5, 6, 9, and 10.

[b] Inhibited by SPS.

[c] Growth in broth is enhanced with addition of Tween 80. In addition, lactose is fermented, which separates out this species of *Atopobium* from other species.

[d] *S. saccharolyticus* is catalase positive if colonies are removed from hemin-containing media; cells are nitrate positive. Initial growth will be anaerobic only, but some growth may occur on BAP if subcultured and incubated in CO_2.

II. SPECIMEN

A. Appropriate specimens should be transported to the laboratory using appropriate anaerobic transport systems, because anaerobic cocci are very susceptible to the toxic effects of oxygen (*see* procedure 4.2).

B. Pure cultures of anaerobic cocci on brucella agar with 5% sheep blood or on other nonselective enriched media with 5% sheep blood are used for various rapid tests and for the inoculum that can be used for the commercially available rapid enzymatic biochemical identification kits (*see* procedures 4.6, 4.8, and 4.9).

III. MATERIALS

Include QC information on reagent container and in QC records.

Reagents, media, and supplies
Perform and record QC as required. Include expiration date on label.

A. 5% Sheep blood brucella agar supplemented with vitamin K (menadione) and hemin
B. CHOC plates for aerotolerance testing
C. Disposable loops and needles
D. Gram stain reagents (*see* procedure 4.4)
E. Special-potency antimicrobial agent disks: colistin, 10 µg; vancomycin, 5 µg; and kanamycin, 1,000 µg (*see* procedure 4.6)
F. Identification disks: sodium polyanethol sulfonate (SPS) and nitrate (*see* procedure 4.6)
G. Spot indole reagents (*see* procedure 4.6)
H. Rapid biochemical tests and commercial rapid enzymatic biochemical identification kits (*see* procedures 4.8 and 4.9)

ANALYTICAL CONSIDERATIONS

IV. PROCEDURE

A. Day 1

The first day that workable colonies are seen on the primary brucella agar is considered day 1.

1. Pick one colony from the primary brucella agar, and subculture it to PRAS brucella agar or other suitable media.
2. Streak the first quadrant of the subculture brucella agar to ensure heavy growth. Streak the other quadrants for isolation.
3. If the Gram stain reveals gram-positive cocci, place SPS and nitrate disks. Many anaerobic gram-positive cocci do not always stain as gram-positive cocci; therefore, the addition of the special-potency disks is useful for establishing the true Gram stain reaction of those microorganisms (*see* procedure 4.6).
4. Pick the same colony (*see* step IV.A.1) and subculture it to CHOC for aerotolerance testing. Incubate the plate at 35°C in 5% CO_2 for 24 h.
5. Pick the same colony and make a smear for Gram stain if not previously performed.
6. Document the description of colony morphology, Gram stain reaction, and all work performed on the anaerobe worksheet.
7. If the original colony is too small, the aerotolerance testing and Gram stain can be done on day 3 by using growth from the brucella agar subculture plate.
8. If using bags, pouches, or jars, leave primary isolation plates for 48 h.

B. Day 2

1. Examine the CHOC aerotolerance plate. Growth on CHOC indicates that the organism is not an anaerobe. There is no need to proceed with its anaerobic identification.
2. If there is no growth on the CHOC aerotolerance agar, incubate the plate for another 24 h.

IV. PROCEDURE *(continued)*

3. Examine the brucella agar if the anaerobic incubation system is a chamber. If there is good growth, proceed to read biochemical tests.

C. Day 3

1. Examine the CHOC again to confirm that the organism is an anaerobe.
2. Examine the brucella agar incubated on day 1. If there is good growth, proceed to read the potency disk results.
3. If the organism is a gram-negative coccus, perform the nitrate test and see Fig. 4.12–1 for presumptive identification (*see* procedure 4.10).
4. If the organism is a gram-positive coccus, read SPS result and nitrate test and proceed according to Fig. 4.12–1 and Table 4.12–1.
5. Perform spot indole and/or rapid urease test if necessary (*see* procedure 4.6).
6. If the organism cannot be identified by rapid tests, proceed to set up rapid biochemical spot tests or rapid enzymatic biochemical kits (*see* procedures 4.8 and 4.9). In some instances, the definitive identification of anaerobic gram-positive cocci must be performed using the aid of chromatographic analysis of metabolic fatty acids and/or molecular identification methods (5, 9, 11, 12).

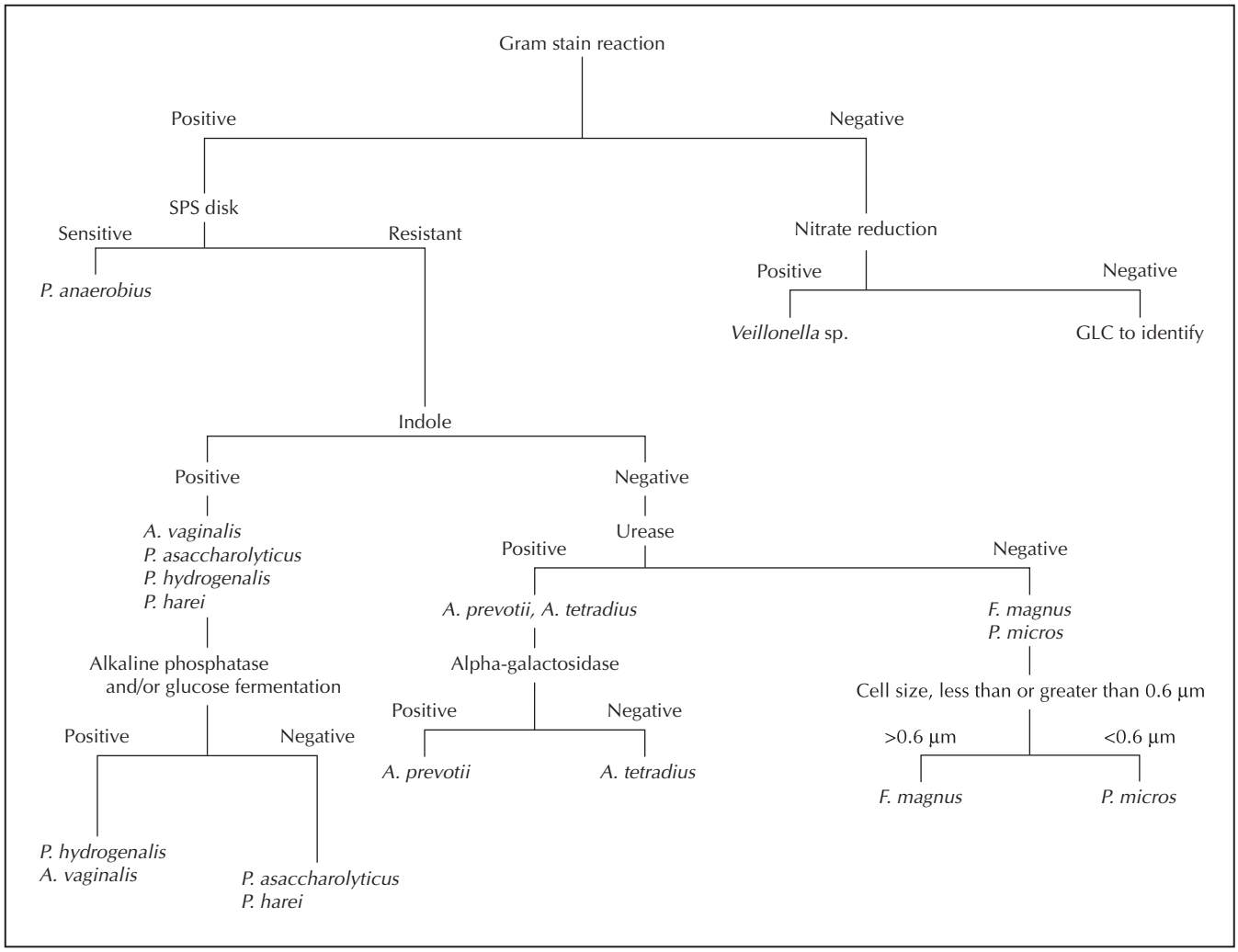


Figure 4.12–1 Flowchart for identification of anaerobic cocci.

V. RESULTS

A. Colony morphology (*see* Table 4.12–1)

1. Growth of anaerobic cocci is usually slower than that of *Bacteroides* or *Clostridium* spp. Small colonies are not apparent on brucella agar until 48 h of incubation. However, *Peptostreptococcus anaerobius* can produce growth within 24 h of incubation.

2. Colonies of gram-positive cocci are typically small, convex, grayish white, and opaque. The edge of most colonies is entire, and the surface may appear stippled or pockmarked. The colony diameter is usually $<$0.5 to 2 mm. Other specific clues are as follows.

a. Colonies of *F. magna* are often $<$0.5 mm in diameter, raised, dull, smooth, and nonhemolytic.

b. Colonies of *P. micros* are $\leq$1 mm, convex, and dull. A halo around the colonies representing partial hemolysis may be seen.

c. Colonies of *P. anaerobius* are larger than those of other anaerobic gram-positive cocci ($>$1 mm) and on enriched media can appear within 24 h of inoculation. The colonies are nonhemolytic, gray to white, shiny, and opaque. Often the colonies will have a pungent sweet odor, like the smell of cantaloupe.

d. Colonies of *Peptoniphilus asaccharolyticus* are minute to 2 mm and may have a yellow pigment on blood agar.

3. *Veillonella* spp. produce small, convex, translucent to transparent colonies with an entire edge. These colonies may show red fluorescence under a long-wave Wood's lamp (366 nm).

B. Gram stain morphology (*see* Table 4.12–1)

1. There are no unique microscopic characteristics to differentiate anaerobic cocci from facultatively anaerobic or aerobic cocci. Some may even resemble staphylococci microscopically. The presence of staphylococcal forms on direct Gram stain with no staphylococci recovered aerobically may suggest an anaerobic coccus (3–6, 9).

2. Microscopically, the anaerobic gram-positive cocci are usually more consistent, although some coccobacillary forms exist, and other clues may be helpful.

a. Anaerobic gram-positive cocci do not always stain fully gram positive; some may actually appear more gram negative.

b. *P. productus* and *P. anaerobius* may be elongated and resemble gram-positive coccobacilli.

c. *F. magna* has cells that are 0.7 to 1.2 μm in diameter and appear in a tightly packed arrangement or in masses.

d. *P. micros* has cells that are smaller, 0.3 to 0.7 μm in diameter, and that usually form short chains. Usually, *F. magna* and *P. micros* can be differentiated by this size difference in their cells on Gram stain.

e. *P. anaerobius* has cells that are 0.5 μm in diameter and that are elongated and in long chains.

f. *Peptoniphilus asaccharolyticus* has cells that are 0.5 to 1.5 μm in diameter and are arranged in pairs, tetrads, or irregular clumps.

g. *A. prevotii* has cells that are 0.6 to 0.9 μm in diameter and that occur in tetrads, irregular groups, and occasionally in short chains (six to eight cells).

3. *Veillonella* spp. are $<$0.5 μm in diameter and are usually seen in clusters or masses and occasionally as diplococci.

C. Special-potency disks (*see* procedure 4.6)

1. If the Gram stain reaction of the organism is difficult to interpret, gram-positive anaerobes can be separated from gram-negative anaerobes by their susceptibility to the vancomycin special-potency antimicrobial agent disk

V. RESULTS *(continued)*

and their resistance to the colistin special-potency antimicrobial agent disk. The susceptibility pattern is the opposite for gram-negative anaerobes.

2. Susceptibility to SPS is a unique characteristic of *P. anaerobius*. A zone size of >12 mm is used to distinguish *P. anaerobius* from something like *P. micros*, which may produce a zone but that zone will be <12 mm (*see* procedure 4.6).

D. Spot indole (*see* procedure 4.6)

Peptoniphilus asaccharolyticus and *P. hydrogenalis* are spot indole positive (see Fig. 4.12–1 and Table 4.12–1 for their separation). *P. asaccharolyticus* is alkaline phosphatase negative, whereas *P. hydrogenalis* is positive. *P. indolicus* is also indole and alkaline phosphatase positive, but it is rarely isolated from clinical specimens and is not described in this procedure (2, 5, 9).

E. Rapid nitrate and urease tests (*see* procedure 4.6)

1. *Veillonella parvula* and *V. montpelliérensis* reduce nitrate (6, 8).
2. Most clinically significant anaerobic gram-positive cocci are nitrate reduction positive.
3. *A. prevotii* and *A. tetradius* can both produce urease. These organisms can be separated by glucose fermentation and α-glucosidase. *P. prevotii* is glucose fermentation negative and α-glucosidase positive. *P. tetradius* is glucose fermentation positive and α-glucosidase negative (Table 4.12–1).

F. Rapid enzymatic biochemical identification (*see* procedure 4.8)

1. Rapid spot and commercially available enzymatic tests can be utilized to complete the identification if the tests described above fail to identify the organism. Depending upon the clinical need, in most instances, a report of anaerobic coccus or a presumptive report using Fig. 4.12–1 as a flowchart as described by Song et al. (10) may be satisfactory.
2. Many of the commercial identification systems described in procedure 4.8 can be used to adequately identify strictly anaerobic *Streptococcus* spp. and other gram-positive cocci which are not included in this procedure.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Send a preliminary report to the physician as soon as the rapid-testing results are available. In some situations, the identification of the anaerobic isolate may be complete by this time.

B. Send the preliminary or final report to the physician after results from further tests, if necessary, are available.

C. Anaerobic isolates that cannot be identified by rapid tests or commercial kit systems, or for which complete species level identification is not warranted, may be reported as anaerobic gram-positive cocci or anaerobic gram-negative cocci, depending upon Gram stain and colonial morphology. Consult with the requesting physician about the anaerobes recovered to ensure that results are clinically significant before pursuing definitive identification. Depending upon the clinical need and the specimen source, a presumptive or group level identification may be satisfactory for the physician to initiate therapy.

D. Appendix 4.12–1 is an example of a report form.

REFERENCES

1. **Downes, J., M. A. Munson, D. A. Spratt, E. Kononen, E. Tarkka, H. Jousimies-Somer, and W. G. Wade**. 2001. Characterisation of *Eubacterium*-like strains isolated from oral infections. *J. Med. Microbiol*. **50:**947–951.

2. **Ezaki, T., Y. Kawamura, N. Li, Z. Y. Li, L. Zhao, and S. Shu.** 2001. Proposal of the genera *Anaerococcus* gen. nov., *Peptoniphilus* gen. nov., and *Gallicola* gen. nov. for members of the genus *Peptostreptococcus*. *Int. J. Syst. Evol. Microbiol.* **51:**1521–1528.

REFERENCES *(continued)*

3. **Finegold, S. M., and W. L. George.** 1989. *Anaerobic Infections in Humans*. Academic Press, Inc., San Diego, CA.
4. **Forbes, B. A., D. F. Sahm, and A. S. Weissfeld (ed.).** 2007. *Bailey and Scott's Diagnostic Bacteriology*, 12th ed. Mosby Elsevier, St. Louis, MO.
5. **Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold.** 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual*, 6th ed. Star Publishing Co., Belmont, CA.
6. **Jumas-Bilak, E., J. P. Carlier, H. Jean-Pierre, C. Teyssier, B. Gay, J. Campos, and H. Marchandin**. 2004. *Veillonella montpellierensis* sp. nov., a novel, anaerobic, gram-negative coccus isolated from human clinical samples. *Int. J. Syst. Evol. Microbiol.* **54:**1311–1316.
7. **Murdoch, D. A.** 1998. Anaerobic gram-positive cocci. *Clin. Microbiol. Rev.* **11:**81–120.
8. **Murdoch, D. A., and N. H. Shah.** 1999. Reclassification of *Peptostreptococcus magnus* (Prevot 1933) Holdeman and Moore 1972 as *Finegoldia magna* comb. nov. and *Peptostreptococcus micros* (Prevot 1933) Smith 1957 as *Micromonas micros* comb. nov. *Anaerobe* **5:**555–559.
9. **Song, Y., and S. M. Finegold.** 2007. *Peptostreptococcus*, *Finegoldia*, *Anaerococcus*, *Peptoniphilus*, *Veillonella*, and other anaerobic cocci, p. 862–871. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
10. **Song, Y., C. Liu, and S. M. Finegold.** 2007. Development of a flow chart for identification of gram-positive anaerobic cocci in the clinical laboratory. *J. Clin. Microbiol.* **45:**512–516.
11. **Song, Y., C. Liu, M. McTeague, and S. M. Finegold.** 2003. 16S ribosomal DNA sequence-based analysis of clinically significant gram-positive anaerobic cocci. *J. Clin. Microbiol.* **41:**1363–1369.
12. **Song, Y., C. Liu, M. McTeague, A. Vu, J. Y. Liu, and S. M. Finegold.** 2003. Rapid identification of Gram-positive anaerobic coccal species originally classified in the genus *Peptostreptococcus* by multiplex PCR assays using genus- and species-specific primers. *Microbiology* **149:**1719–1727.

APPENDIX 4.12–1

Example of an anaerobic culture worksheet. Results of aerobic culture and microscopic examination of the original smear are helpful information in working up the anaerobic culture. LKV, laked kanamycin-vancomycin; PEA, phenylethyl alcohol agar; BBE, *Bacteroides* bile esculin; SPS, sodium polyanethol sulfonate. (*See* procedures 4.6 and 4.8.)

ANAEROBIC CULTURE WORKSHEET

SPECIMEN # _____ SOURCE _____

COMMENT _____

_____

Gram stain of original smear	Aerobic culture results
_____ No organism observed	_____ No growth in 24 hours
_____ Gram-positive cocci	_____ Gram-negative bacilli
_____ Gram-negative cocci	_____ *Pseudomonas* species
_____ Gram-positive bacilli	_____ Staphylococci
_____ Gram-negative bacilli	_____ Streptococci
_____ Gram-positive coccobacilli	_____ Gram-positive bacilli
_____ Gram-variable coccobacilli	_____ Yeast form
_____ Fusiform bacilli	

Anaerobic culture

Col. no.	Medium	Amt	Gram Stain	No Growth	Hemolysis	Fluorescence	Pigment	CHOC	LKV	Brucella	THIO	PEA	BBE	Kanamycin	Colistin	Vancomycin	SPS	Catalase	Indole	Urea	Nitrate	Esculin	Lipase	Lecithinase	Rapid ID	Identification
1.																										
2.																										
3.																										
4.																										
5.																										
6.																										
7.																										

4.13 Suggestions for a Practical Scheme for the Workup of Anaerobic Cultures

When a specimen is sent to the laboratory in an appropriate container and from an appropriate site with a request for anaerobe testing, a Gram stain should be performed and reported. The results of the Gram stain should direct the culture workup to be done as well as provide information to the clinician and the laboratory about the quality of the specimen and the potential for mixed aerobic and anaerobic floras. All anaerobe requests should receive a simultaneous aerobic culture, and these should be worked up in concert before the report is finalized. The scheme for identification of anaerobes can take on many models, depending upon the extent of workup required by the clinicians and the amount of expertise and time that can be allotted to their identification by the clinical microbiologist. Certainly, all single isolates from sterile sites should be completely worked up to genus and species name as much as is possible. It is less consistent among laboratories as to how much is fully identified in nonsterile sites or what should be identified when a "sterile" site, such as ascites fluid or abscess or even tissues, contains a mixed flora, including aerobic and anaerobic organisms, in a quantity of more than three organisms.

Identifying anaerobic bacteria to the species level can be quite complex; however, the extent to which isolates of anaerobic bacteria are identified varies widely and may be limited to basic information that is of clinical utility. For example, the mixed anaerobic microbiota from a site such as a decubitus ulcer, perirectal fistula, or intra-abdominal abscess may simply be reported as a mixed fecal microbiota without specific identification of its components. Determining the species of an isolate may be limited to those present in pure culture from a normally sterile body site and can be readily accomplished by any one of several commercially available biochemical assay-containing kits or by using GLC. One should understand that the clinical value of any report of the presence of anaerobic bacteria is directly related to the speed of reporting such results from the laboratory. Identification procedures that require 1 or 2 weeks to complete are generally of academic interest only.

A tech sample a number of years ago addressed this issue and offered a scheme for limited workup of anaerobes based on site (2). Hence, if the specimen was from a nonsterile site, any gram-positive bacteria would be worked up as shown in Table 4.13–1. Most of the identification would be descriptive, with the exception of ruling out *Clostridium perfringens* specifically. Anaerobic gram-negative cocci would not be identified any further. Based on the growth or lack thereof on a *Bacteroides* bile esculin (BBE) plate, the gram-negative rods would be called consistent with the *Bacteroides fragilis* group if growth occurred and not consistent with the *B. fragilis* group if there was no growth on this screening medium. If the specimen was from a sterile site, then the flowchart in Fig. 4.13–1 can be considered for proceeding with workup. The cocci are worked up as if from a nonsterile site, but a more complete identification is performed on the bacilli, often to the level of biochemical identification in one of the "kit" systems and potentially a GLC, if needed. More recent taxonomic changes and literature on the clinical relevance of additional anaerobes may cause slight modifications in this flow scheme, but for the most part, it allows for a clinically relevant workup of anaerobes.

Another modified scheme was proposed by Baron and Citron (1) and utilizes many spot and rapid tests for gram-negative and gram-positive bacilli along with colonial morphology but not a complete identification in all cases. Both of these proposals attempt to address the issues of how far to work up an anaerobic specimen based on relevance, efficiency, and cost constraints. Whichever you choose, the decision should be made with consultation of infectious diseases physicians, pharmacists, and other colleagues from clinical medicine, keeping in mind clinical relevance and the need for a full identification of mixed microbiotas. Most times it is sufficient for the clinician to know that the specimen contains anaerobes, not their full genus and species name. We as microbiologists need to help in the education process for this as well.

SOME SPECIFIC INSTANCES TO CONSIDER WHEN REVIEWING WORKFLOW

A. For blood cultures that contain an anaerobe, consider a complete identification of all isolates as described above for sterile sites. An exception, however, could be when one finds that only one of many blood draws is positive for the anaerobe. This could represent contamination or transient bacteremia, in particular, if the anaerobe is a usual component of the skin microbiota, such as a gram-positive coccus or *Propionibacterium* sp. (including *Propionibacterium acnes*). This does not mean that these organisms are never related to bacteremia, but in general one would expect growth from many or all of the patients' blood cul-

Table 4.13–1 Identification of isolates of anaerobic bacteria in nonsterile sites

Gram reaction	Reporting for cocci	Reporting for bacilli
Gram negative	Report as anaerobic gram-negative cocci.	If BBE is positive, report as *B. fragilis* group. If BBE is negative, report as anaerobic gram-negative bacillus, not *B. fragilis* group.
Gram positive	Report as anaerobic gram-positive cocci.	If a sporeformer, observe for double-zone hemolysis on BAP. If positive, use motility, reaction on egg yolk agar, and morphology to identify as *C. perfringens*. If negative for above, report as a *Clostridium* sp., not *C. perfringens.* Nonsporeformers can be reported as anaerobic non-spore-forming bacilli.

SOME SPECIFIC INSTANCES TO CONSIDER WHEN REVIEWING WORKFLOW *(continued)*

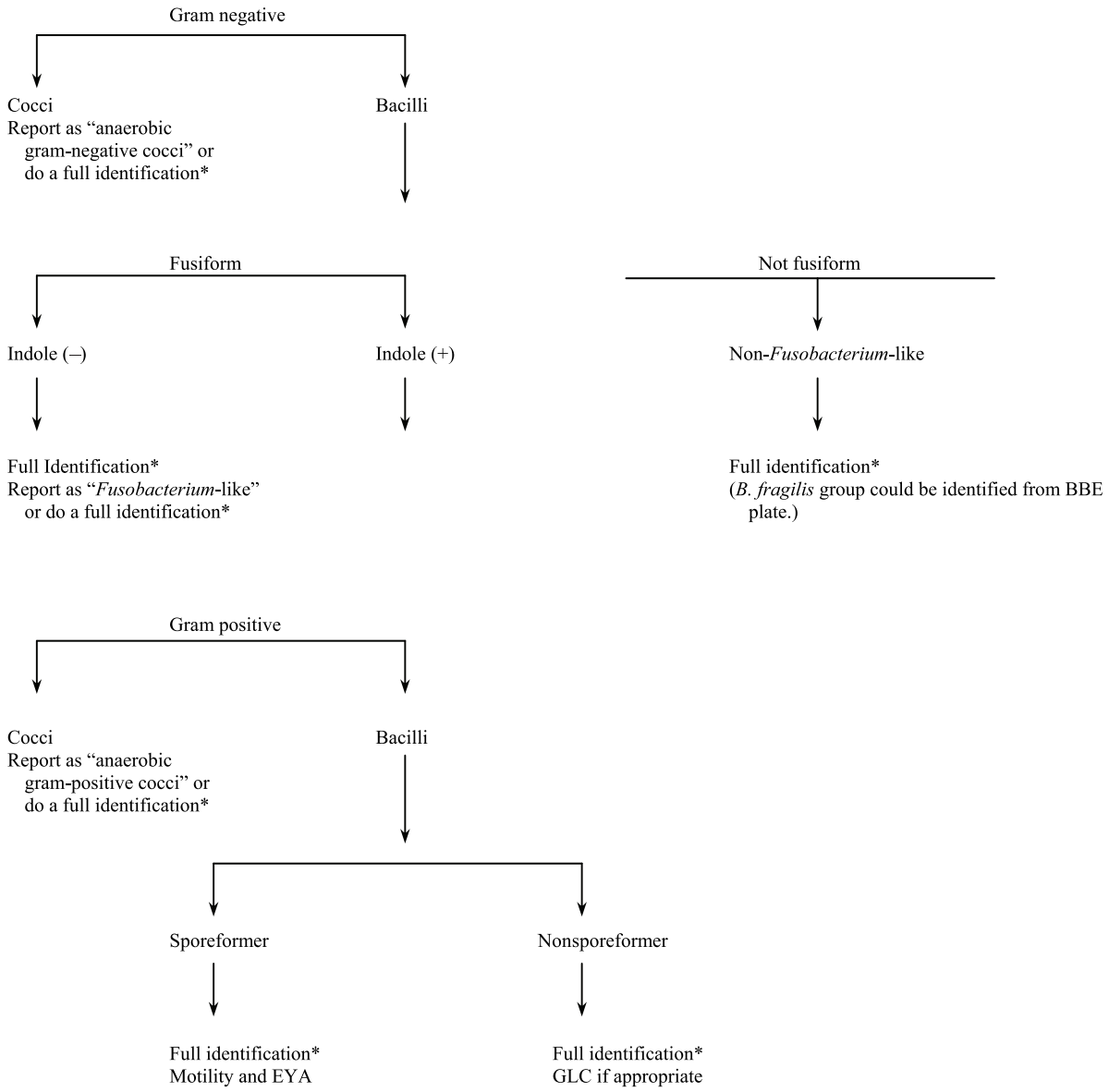
tures, not just an isolated draw, unless the patient has already been placed on broad-spectrum antimicrobial agents. The reasoning here is similar to that used in the handling of a single positive blood culture for coagulase-negative staphylococci or "diphtheroids."

B. CSF should be treated as a sterile site in which everything is worked up according to Fig. 4.13–1. However, planting and processing of thioglycolate (or other) broths are controversial. In routine, nonshunt CSF, the broth usually does not add much to the recovery of clinically relevant anaerobic bacteria since the incidence of anaerobic meningitis is very low (5). Planting and subbing of the broth often results in isolation of contaminants. If specimens are taken from a patient with a ventricular shunt, the requests should be labeled as such and *P. acnes* should be considered as a potential significant pathogen (8, 12). For these specimens, it is appropriate to plant a thioglycolate broth along with solid media and the broth probably should be incubated longer (up to 10 days) to ensure maximal recovery.

C. Consider rejecting specimens sent to the lab with an anaerobic culture request if submitted on swabs. Wound samples in particular, if collected on swabs, are less than optimal specimens, easily contaminated with anaerobic skin microbiota organisms and not appropriate for demonstration of the true pathogen in most situations (4, 7). If accepted, workup of the specimen should be directed in light of the direct Gram stain results, and if the specimen has mixed aerobes and anaerobes, consider ruling out the *B. fragilis* group and *C. perfringens*; if these are present, consider reporting their presences and signing out that they were found in conjunction with mixed aerobic and anaerobic organisms. A comment on the result that swabs are inappropriate for optimal anaerobic recovery would be helpful as an educational tool for your clinical colleagues.

D. If an aspirate or a large fluid of body volume is sent, treat like an abscess. If there are many aerobes and anaerobes, there is rarely a clinical need to work everything up—look for more of the typical pathogens (*B. fragilis* group, *Bilophila wadsworthia*, and *C. perfringens*) and then morphologically describe others. Again, use the Gram stain to determine a correlation to the specimen contents that were sent to the lab initially.

E. Requests for *Actinomyces* spp. should result in a mechanism in the laboratory for holding the culture longer, at least 10 to 14 days, and an indication to the technologist to work up branching gram-positive bacilli resembling *Actinomyces* spp. with this specific request in mind. In addition, the thioglycolate broth that is set up with this specimen should be subbed for *Actinomyces* spp. specifically before the culture is finalized. Sites where one might want to consider looking



*Full identification could include a "kit" biochemical system and/or individual biochemical tests as outlined in other sections of this chapter.

Figure 4.13–1 Workup of isolates from sterile sites. EYA, egg yolk agar.

SOME SPECIFIC INSTANCES TO CONSIDER WHEN REVIEWING WORKFLOW *(continued)*

for *Actinomyces* spp. would include mandibular (jaw) abscess, neck abscess, sinus (collected on other than a swab), lacrimal gland specimens, tonsillar area, intrauterine device-related specimens, and sites where the Gram stain demonstrates "sulfur granules" and the presence of gram-positive branching bacilli (6).

F. Tables 4.10–1, 4.11–1, and 4.12–2 list some of the nomenclature changes that have been suggested as of 2009, and it would be advisable to investigate implementation of these changes where appropriate.

G. There are many laboratories incorporating the use of sequencing methods for the identification of anaerobic bacteria; this will most probably increase, so

SOME SPECIFIC INSTANCES TO CONSIDER WHEN REVIEWING WORKFLOW *(continued)*

taxonomic changes will continue (10). Use of 16S ribosomal DNA sequence-based analysis of gram-positive anaerobic cocci has confirmed the revised taxonomic changes in Tables 4.10–1, 4.11–1, and 4.12–2 for example (9, 11). Likewise, sequencing has been used to identify common clinically relevant gram-negative bacteria (3).

REFERENCES

1. **Baron, E. J., and D. M. Citron.** 1997. Anaerobic identification flowchart using minimal laboratory resources. *Clin. Infect. Dis.* **25:**S143–S146.
2. **Hall, G. S.** 1991. Practical approach to identification and antimicrobial susceptibility testing of anaerobes. Tech Sample Mb-3. American Society for Clinical Pathology, Chicago, IL.
3. **Jousimies-Somer, H., and P. Summanen.** 2002. Recent taxonomic changes and terminology update of clinically significant anaerobic Gram-negative bacteria (excluding spirochetes). *Clin. Infect. Dis.* **35:**S17–S21.
4. **Kessler, L., Y. Piemont, F. Ortega, O. Lesens, C. Boeri, C. Averous, R. Meyer, Y. Hansmann, D. Christmann, J. Gaudias, and M. Pinget.** 2006. Comparison of microbiological results of needle puncture vs. superficial swab in infected diabetic foot ulcer with osteomyelitis. *Diabet. Med.* **23:**99–102.
5. **Meredith, F. T., H. K. Phillips, and L. B. Reller.** 1997. Clinical utility of broth cultures of cerebrospinal fluid from patients at risk for shunt infections. *J. Clin. Microbiol.* **35:**3109–3111.
6. **Mims, C., H. M. Dockrell, R. V. Goering, I. Roitt, D. Wakelin, and M. Zuckerman.** 2004. *Medical Microbiology,* 3rd ed. Mosby, Philadelphia, PA.
7. **Senneville, E., H. Melliez, E. Beltrand, L. Legout, M. Valette, M. Cazaubiel, M. Cordonnier, M. Caillaux, Y. Yazdanpanah, and Y. Mouton.** 2005. Culture of percutaneous bone biopsy specimens for diagnosis of diabetic foot osteomyelitis: concordance with ulcer swab cultures. *Clin. Infect. Dis.* **42:**57–62.
8. **Skinner, P. R., A. J. Taylor, and H. Coakham.** 1978. *Propionibacteria* as a cause of shunt and postneurosurgical infections. *J. Clin. Pathol.* **31:**1085–1090.
9. **Song, Y.** 2004. Anaerobiosis: molecular biology, genetics, and other aspects—minireview. PCR-based diagnostics for anaerobic infections. *Anaerobe* **11:**79–91.
10. **Song, Y., C. Liu, M. McTeague, and S. M. Finegold.** 2003. 16S ribosomal DNA sequence-based analysis of clinically significant gram-positive anaerobic cocci. *J. Clin. Microbiol.* **41:**1363–1369.
11. **Song, Y., C. Liu, M. McTeague, A. Vu, J. Y. Liu, and S. M. Finegold.** 2003. Rapid identification of Gram-positive anaerobic coccal species originally classified in the genus *Peptrostreptococcus* by multiplex PCR assays using genus- and species-specific primers. *Microbiology* **149:**1719–1727.
12. **Thompson, T. P., and A. L. Albright.** 1998. *Propionibacterium acnes* infections of cerebrospinal fluid shunts. *Childs Nerv. Syst.* **14:**378–380.

4.14

Clostridium difficile as a Pathogen Involved in Antimicrobial Agent-Associated Diarrhea, Colitis, and Pseudomembranous Colitis

Clostridium difficile is the major cause of nosocomial diarrhea and the primary pathogen responsible for pseudomembranous colitis. In the United States, there are >300,000 cases per year of antimicrobial agent-associated diarrhea, colitis, or pseudomembranous colitis caused by this organism and its toxins. In a recent review article, one hospital reported that medical expenditures associated with *C. difficile*-associated disease were almost $1,000,000 per year (11). *C. difficile* can also be a rare cause of abscesses, wound infections, osteomyelitis, pleuritis, peritonitis, septicemia, and urogenital tract infections.

Rates of carriage of *C. difficile* and its toxins are high (50% or more) in neonates, but disease is rare in neonates (1, 3). Colonization, with or without toxin production, may be maintained for several months, but when the adult microbiota becomes established at 6 to 12 months of age, colonization rates fall, and only about 3% of healthy adults are thought to be colonized with the organism. *C. difficile* almost always is acquired in the hospital by persons receiving antimicrobial agents via direct or indirect exposure to human or inanimate reservoirs. The presentation of the symptoms, however, may not occur until discharge from the hospital, so one could get requests for and positive results with diarrheic stools collected from "outpatients." Although clindamycin and the penicillins and cephalosporins are implicated most frequently, any antimicrobial agent may trigger *C. difficile*-associated disease. Disease rarely occurs without antimicrobial agent exposure, but cases have been reported following therapy with antineoplastic agents that have antibacterial activity.

Pseudomembranous colitis is a toxin-mediated illness in which microbial invasion of the mucosa is not known to occur. *C. difficile* produces two toxins. Toxin A, a weakly cytopathic toxin, is predominantly responsible for the enterotoxic activity of the organism. Toxin B, a potent cytotoxin, appears to play a minor role in human disease, although toxin A-negative, B-positive strains have been isolated from symptomatic patients (4). Use of a PCR assay in the laboratory for detection of both toxin A and toxin B may increase the sensitivity (7, 10). Recently a third toxin, binary toxin, has been described for some strains of *C. difficile* in addition to toxins A and B. The B1/NAP1 strains that carry binary toxin produce large amounts of toxin which may add to their increased virulence (9).

Stool samples are submitted to the laboratory, in most cases, for the detection of toxin A, toxin B, or both toxins A and B. Initially, the cytotoxin B assay was the most common test performed in laboratories. This consists of inoculation of specific tissue culture panels, usually containing McCoy epithelial cells or human foreskin cells. The panels are inoculated with an aliquot of stool supernatant, and this is incubated overnight, after which the wells are examined for the presence of cytopathic effect (CPE). If CPE is demonstrated, a neutralizing antitoxin is added to the wells at the time of stool addition, and results are read within 24 h for a lack of CPE in wells that showed CPE prior to neutralization. This test has a high sensitivity and, if performed appropriately, high specificity. All strains that possess toxin B were thought initially to also possess toxin A, and a number of EIAs for the detection of toxin A appeared from commercial companies to "replace" the toxin B cytotoxin assay. These were more rapid and less time-consuming and provided good information in formats that allowed for batch or individual sample testing. When a few toxin B-positive/toxin A-negative strains were discovered (4), a number of EIA products that detected both toxin A and toxin B were developed. The sensitivity did not increase as dramatically as one might have imagined, but it makes sense to look for both toxins in all cases, hence the rapid progression in laboratories from toxin A alone to toxin A and B tests. As of 2009, many laboratories were investigating the use of amplification for detection of *C. difficile* toxins directly in stool samples. Laboratories have developed their own home brew real-time PCR assays; two commercial products are also available. The data thus far indicate that these molecular methods will significantly increase the sensitivity and potentially the specificity of detection especially compared to the EIAs (2, 6, 10, 12). Toxigenic culture remains, however, the most sensitive method of detection even compared to PCR (12).

There are at least two commercially available assays that detect glutamate dehydrogenase, an antigen common to *C. difficile* and other *Clostridium* spp. (5, 8, 13). These assays are very highly sensitive but are not specific enough to be used alone. However, they can be used as a negative screen initially for *C. difficile* stool requests and when positive confirmatory tests that detect *C. difficile* toxins specifically can be employed. *C. DIFF CHEK*-60 (TechLab, Blacksburg, VA) is a 96-well ELISA that can be performed in 60 min. The Triage *C. difficile* Panel (Biosite Inc., San Diego, CA) is a rapid visual test that detects both the *C. difficile* common antigen and toxin A in approximately 15 min. Use of the antigen as a negative screen, followed by a cytotoxin B assay on the samples that are positive, has been shown to produce very sensitive results in a cost-effective approach (5, 8, 13).

STEPS FOR PROCESSING STOOL TO REDUCE THE AMOUNT OF NORMAL FECAL ORGANISMS

Culture of stool for *C. difficile*, followed by a toxigenic assay to confirm the presence of the toxins, can be done; however, the time involved in this procedure renders it impractical for many laboratories. Culture is very important if epidemiological studies are being employed. If culture is done, an attempt is made to reduce the amount of normal bacteria present in stool first by processing a portion of the stool as follows.

A. Mix 0.5 g of stool with 0.5 ml of 95% ethanol.
B. Incubate for 1 h at room temperature.
C. Inoculate 2 drops of the suspension onto a CDC brucella blood agar plate.
D. Alternatively, a selective medium, cycloserine-cefoxitin-fructose agar, can be inoculated to even more selectively isolate *C. difficile*.
E. Incubate for up to 48 h at 37°C and examine for typical colonies of *C. difficile*: white, "spready" flat colonies that exhibit a "horse barn" odor. The Gram stain should demonstrate gram-positive bacilli with spores.
F. Identification can be confirmed by use of one of the biochemical kit systems.
G. Once identified as *C. difficile*, the isolate should be tested for the presence of toxin, using the cytotoxin B assay, EIAs for toxins A and B, or a PCR, if available.

REFERENCES

1. **Bartlett, J. G.** 1997. *Clostridium difficile* infection: pathophysiology and diagnosis. *Semin. Gastrointest. Dis.* **8:**12–21.
2. **Bélanger, S. D., M. Boissinot, N. Clairoux, F. J. Picard, and M. J. Bergeron. 2005.** Rapid detection of *Clostridium difficile* in feces by real-time PCR. *J. Clin. Microbiol.* **41:**730–734.
3. **Johnson, S., and D. N. Gerding.** 1998. *Clostridium difficile*-associated diarrhea. *Clin. Infect. Dis.* **26:**1027–1034.
4. **Kader, H. A., D. A. Piccoli, A. F. Jawad, K. L. McGowan, and E. S. Maller.** 1998. Single toxin detection is inadequate to diagnose *Clostridium difficile* diarrhea in pediatric patients. *Gastroenterology* **115:**1329–1334.
5. **Landry, M. L., J. Topal, D. Ferguson, D. Giudetti, and Y. Tang.** 2001. Evaluation of Biosite Triage *Clostridium difficile* Panel for rapid detection of *Clostridium difficile* in stool samples. *J. Clin. Microbiol.* **39:**1855–1858.
6. **Lemee, L., A. Dhalluin, S. Testelin, M. A. Mattrat, K. Maillard, J. F. Lemeland, and J. L. Pons.** 2004. Multiplex PCR targeting *tpi* (triose phosphate isomerase), *tcdA* (toxin A), and *tcdB* (toxin B) genes for toxigenic culture of *Clostridium difficile. J. Clin. Microbiol.* **42:**5710–5714.
7. **Peterson, L. R., R. U. Manson, S. M. Paule, D. M. Hacek, A. Robicsek, R. B. Thomson, and K. L. Kaul.** 2007. Detection of toxigenic *Clostridium difficile* in stool samples by real-time polymerase chain reaction for the diagnosis of *C. difficile*-associated diarrhea. *Clin. Infect. Dis.* **45:**1152–1160.
8. **Reller, M. E., C. A. Lema, T. M. Perl, T. L. Ross, K. A. Speck, and K. C. Carroll.** 2007. Yield of stool culture with isolate toxin testing versus a two-step algorithm including stool toxin testing for detection of toxigenic *Clostridium difficile. J. Clin. Microbiol*. **45:**3601–3605.
9. **Rupnik, M., M. Grabnar, and B. Geric**. 2003. Binary toxin producing *Clostridium difficile* strains. *Anaerobe* **9:**289–294.
10. **Sloan, L. M., B. J. Duresko, D. R. Gustafson, and J. E. Rosenblatt**. 2007. Comparison of real-time PCR for detection of the *tcdC* gene with four toxin immunoassays and culture in diagnosis of *Clostridium difficile* infection. *J. Clin. Microbiol.* **46:**1996–2001.
11. **Song, X., J. G. Bartlett, K. Speck, A. Naegeli, K. Carroll, and T. M. Perl**. 2008. Rising economic impact of *Clostridium difficile*-associated disease in adult hospitalized patient population. *Infect. Control Hosp. Epidemiol.* **29:**823–828.
12. **Stamper, P. D., R. Alcabase, D. Aird, W. Babiker, J. Wehrlin, I. Ikpeama, and K. C. Carroll.** 2009. Comparison of a commercial real-time PCR assay for *tcdB* detection to a cell culture cytotoxicity assay and toxigenic culture for direct detection of toxin-producing *Clostridium difficile* in clinical samples. *J. Clin. Microbiol.* **47:**373–378.
13. **Ticehurst, J. R., D. Z. Aird, L. M. Dam, A. P. Borek, J. T. Hargrove, and K. C. Carroll.** 2006. Effective detection of toxigenic *Clostridium difficile* by a two-step algorithm including tests for antigen and cytotoxin. *J. Clin. Microbiol.* **44:**1145–1149.

THIRD EDITION

Clinical Microbiology Procedures Handbook

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VOLUME 2

Address editorial correspondence to ASM Press, 1752 N St., N.W., Washington, DC 20036-2904, USA

Send orders to ASM Press, P.O. Box 605, Herndon, VA 20172, USA
Phone: 800-546-2416; 703-661-1593
Fax: 703-661-1501
E-mail: books@asmusa.org
Online: http://estore.asm.org

Copyright © 2010 ASM Press
American Society for Microbiology
1752 N St., N.W.
Washington, DC 20036-2904

Library of Congress Cataloging-in-Publication Data

Clinical microbiology procedures handbook.—3rd ed. / editor in chief, third edition and 2007 update, Lynne S. Garcia.
p. ; cm.
"Editor in chief, original and second editions Henry D. Isenberg."
Includes bibliographical references and index.
ISBN-13: 978-1-55581-527-1
ISBN-10: 1-55581-527-8
1. Diagnostic microbiology—Laboratory manuals. I. Garcia, Lynne Shore. II. Isenberg, Henry D.
[DNLM: 1. Microbiological Techniques—methods—Laboratory Manuals. WQ 25 C6415 2010]
QR67.C555 2010
616.9′041—dc22

2010014355

10 9 8 7 6 5 4 3 2 1

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Printed in the United States of America

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5.1 Disk Diffusion Test

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

A standardized inoculum of bacteria is swabbed onto the surface of a Mueller-Hinton agar (MHA) plate. Filter paper disks impregnated with antimicrobial agents are placed on the agar. After overnight incubation, the diameter of the zone of inhibition is measured around each disk. By referring to the tables in the NCCLS disk diffusion standard (1), a qualitative report of susceptible, intermediate, or resistant is obtained.

The NCCLS describes a standard method for disk diffusion testing of nonfastidious bacteria. The NCCLS also describes standardized procedures for testing *Haemophilus influenzae, Neisseria gonorrhoeae, Streptococcus pneumoniae,* and *Streptococcus* spp. The procedures for these bacteria are similar to those for nonfastidious bacteria; however, the test media are supplemented with nutrients that will support the growth of these fastidious organisms. Any bacterium not addressed by the NCCLS in the disk diffusion standard should be tested by an MIC method if testing is required.

II. SPECIMEN

Prepare inoculum from four or five isolated colonies of similar colony morphology.

A. For log-phase-growth inoculum: use colonies grown for 1 or 2 days on nonselective or selective (e.g., MAC) medium.

B. For direct colony suspension inoculum: use colonies grown overnight on nonselective medium (e.g., BAP or CHOC).

C. Subculture stock, frozen, or lyophilized isolates two times prior to testing.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Media and reagents

1. Agar plates (150 or 100 mm; depth, approximately 4 mm)
Store at 2 to 8°C.
  a. MHA
  b. MHA with 5% sheep blood (BMHA)
  c. *Haemophilus* test medium (HTM)
  d. GC agar base with defined supplements

2. Nutrient broth (e.g., Mueller-Hinton, TSB) or 0.9% NaCl (3.0- to 5.0-ml aliquots)
Store at 2 to 30°C.

3. Antimicrobial disks
Store with desiccant at −14 to 8°C.
  a. To maintain activity of beta-lactam class antimicrobial agents, store at −14°C or below in a frost-free freezer. Once opened, discard after 1 week of refrigeration.
  b. Cefaclor, imipenem, and clavulanic acid combinations are the most labile drugs tested. Improved stability may be achieved by storing frozen until the day of testing.

B. Supplies

1. Sterile cotton tipped swabs
2. Sterile plastic pipettes
3. McFarland 0.5 turbidity standard

C. Equipment

1. Forceps
2. Ruler, template, or sliding caliper
3. Movable light source
4. Black nonreflecting surface (e.g., sheet of black paper or black counter surface)
5. Vortex mixer
6. 35°C ambient-air incubator; CO_2 incubation for some organisms (*see* Appendix 5.1–3)
7. Multidisk disk dispensing apparatus and container (optional)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. QC strains (*see* appendixes to this proceedure for QC of specific fastidious organisms)

1. *Escherichia coli* ATCC 25922
2. *Staphylococcus aureus* ATCC 25923
3. *Pseudomonas aeruginosa* ATCC 27853
4. *E. coli* ATCC 35218
5. *Enterococcus faecalis* ATCC 29212

B. Monitoring accuracy

1. Test QC strains by following routine procedure, and record results in QC notebook. Record lot numbers and expiration dates of disks and agar. *See* Appendix 5.1–1.
2. Compare to expected results (*see* NCCLS QC tables [1]). Note any out-of-control result and document; proceed with corrective action, if necessary.

C. Daily QC testing

1. Unless 30-day proficiency has been documented to reduce frequency of testing to weekly (*see* item IV.D), monitor performance of the test system by testing appropriate QC strains each day susceptibility testing is performed on patient isolates.
2. QC testing is in control if, for each antimicrobial agent-organism combination, no more than 1 out of 20 consecutive results is outside acceptable range.
3. If any more than 1 of 20 results is out of control, proceed with corrective action.

D. Weekly QC testing

1. Frequency of QC testing
a. QC testing can be reduced from daily to weekly providing a laboratory can document proficiency in performing this test by performing QC daily (or each day patient tests are performed) until results from 20 consecutive test days have been obtained.
b. Proficiency in performing QC testing is confirmed if for each antimicrobial agent-organism combination no more than 1 of 20 results are outside accuracy limits. Weekly QC testing may then be initiated.
c. If 2 or 3 results of the 20 are out of control, continue testing for a total of 30 days. If no more than 3 of 30 results are outside accuracy limits, proficiency is demonstrated, and weekly testing can be initiated.
2. Weekly QC testing is in control if *all* zones are within specified accuracy limits. If any result is out of control, proceed with corrective action (*see* item IV.E).
3. Document proficiency each time a new antimicrobial agent is added to testing protocols.
◩ Save records documenting proficiency indefinitely.
4. Perform QC testing each time a new lot or new shipment of agar or disks is put into use.

E. Corrective action

1. Out-of-control result due to obvious error (e.g., use of wrong disk, contamination, incorrect organism tested, inadequate incubation conditions, etc.) that can be easily corrected: record reason for out-of-control result and retest the same day. If the repeated result is in range, no further action is needed.
2. Out-of-control result not due to obvious error
a. Test the involved antimicrobial agent(s)-QC strain combination for five consecutive test days. Record all results.

IV. QUALITY CONTROL
(continued)

b. For each antimicrobial agent-QC strain combination, if all of the five zone diameters are within the acceptable QC range, weekly testing may be resumed.

c. If the problem is not resolved (at least one zone diameter is outside the accuracy control limits), continue daily QC testing until the problem is resolved.

d. Investigate possible procedural problems (e.g., review measurement of zones, standardization of inoculum, storage and expiration dates of reagents, incubation conditions, equipment, and maintenance and purity of control strain).

e. In order to return to weekly testing, satisfactory performance for another 20 or 30 consecutive days must be documented (as described in item IV.D. 1.b and c above).

f. During corrective action, carefully evaluate whether patient reports will be affected. It may be necessary to use an alternate test method until the problem is resolved.

g. See Appendix 5.1–2 for additional information regarding corrective action. (Refer to procedure 5.13 for maintenance of QC strains and additional measures for verifying results for bacteria isolated from patients.)

V. PROCEDURE

See Appendix 5.1–3 for summary of testing conditions for various organisms and Appendixes 5.1–4 to 5.1–7 for special procedures for testing *Haemophilus* spp., *N. gonorrhoeae, S. pneumoniae,* and *Streptococcus* spp. and testing for extended-spectrum beta-lactamase (ESBL) production in *E. coli* and *Klebsiella* spp.

A. Bring agar plates and canisters of disks to room temperature before use. Agar plates may be removed from refrigerator and placed in a 35°C ambient-air incubator with lids slightly ajar to evaporate excess moisture. Do not leave in incubator for longer than 30 min.

B. Inoculum preparation

Using a loop or swab, transfer colonies as follows.

1. Direct colony suspension method
Pick several colonies from a *fresh* (18- to 24-h) nonselective agar plate to broth or 0.9% NaCl.

2. Log-phase method

a. Pick four or five isolated colonies to 3.0 to 5.0 ml of broth.

b. Incubate at 35°C for 2 to 8 h until growth reaches the turbidity at or above that of a 0.5 McFarland standard.

3. For either the log phase or direct colony suspension method, vortex well and adjust turbidity visually with sterile broth or 0.9% NaCl to match a 0.5 McFarland standard (1×10^8 to 2×10^8 CFU/ml). Alternatively, standardize suspension with a photometric device.

4. Selection of inoculum preparation method

a. Direct colony suspension

(1) Use for bacteria that grow slowly or unpredictably in broth.

(2) Always use for staphylococci.

(3) Use for any organism when fresh (18- to 24-h) growth is available.

b. Log-phase or stationary-phase method

(1) Use for nonfastidious rapidly growing bacteria when fresh colonies are not available.

(2) If desired, use for nonfastidious rapidly growing bacteria when fresh growth is available.

V. PROCEDURE *(continued)*

C. Inoculation of agar plate
- **1.** Within 15 min of adjusting turbidity, dip a sterile cotton swab into the inoculum and rotate against the wall of the tube above the liquid to remove excess inoculum.
- **2.** Swab entire surface of agar plate three times, rotating plate approximately 60° between streaking to ensure even distribution. Avoid hitting the sides of the plate to avoid aerosols. Finally, run swab around the edge of the agar to remove any excess moisture.
- **3.** Allow inoculated plate to stand for 3 to 15 min before applying disks.

D. Application of disks
- **1.** Apply disks to agar surface with dispenser or manually with a sterile forceps.
- **2.** Apply gentle pressure with sterile forceps or needle to ensure complete contact of disk with agar (some dispensers do this automatically).
- **3.** Do not place disks closer than 24 mm from center to center.
  - **a.** No more than 12 disks on 150-mm plate
  - **b.** No more than 5 disks on 100-mm plate
- **4.** *Do not* relocate a disk once it has made contact with agar surface. Instead, place a new disk in another location on the agar.

E. Incubation
- **1.** Invert plates and incubate within 15 min of disk application.
- **2.** Incubate for 16 to 18 h at 35°C in an ambient-air incubator.
  - **a.** Incubate all staphylococci that are susceptible to penicillinase-stable penicillins (e.g., oxacillin) or to vancomycin after 16 to 20 h for an additional 4 to 8 h (total of 24 h).
  - **b.** Incubate all enterococci that are susceptible to vancomycin after 16 to 20 h for an additional 4 to 8 h (total of 24 h).

F. Reading plates
- **1.** Read plates only if lawn of growth is confluent or nearly confluent.
- **2.** Translucent media (MHA, HTM, GC agar)
  - **a.** Hold inverted plate a few inches above a black nonreflecting surface.
  - **b.** Illuminate plate with reflected light.
  - **c.** Use a sliding caliper or ruler held on the back of the plate to measure the diameter of inhibition zone to nearest whole millimeter.
  - **d.** For staphylococci with penicillinase-stable penicillins (oxacillin, methicillin, nafcillin) and vancomycin and for enterococci with vancomycin
    (1) Use transmitted light; hold plate between your eye and light source.
    (2) Examine closely; consider *any* growth as resistant.
- **3.** Opaque media (BMHA)
  - **a.** Remove cover of agar plate.
  - **b.** Illuminate plate with reflected light.
  - **c.** Measure diameter of inhibition zone at agar surface to nearest whole millimeter.
  - **d.** When testing hemolytic organisms, measure the diameter of the zone of inhibition of growth and not the zone of inhibition of hemolysis.
- **4.** Additional considerations
  - **a.** With the exception of staphylococci and enterococci, when reading vancomycin or oxacillin zones, always disregard minute colonies visible only by viewing with transmitted light or by examining with a magnifying device.
  - **b.** Disregard swarming of *Proteus* spp. and measure the edge of the obvious inhibition under the veil of swarming.
  - **c.** When measuring zones for sulfonamides, trimethoprim, or trimethoprim-sulfamethoxazole, disregard light growth (20% or less of lawn of growth) and measure edge of the more obvious margin of the zone.

V. PROCEDURE *(continued)*

d. Discrete colonies growing within the inhibition zone may represent a mixed culture or resistant variants; subculture a single colony from the primary culture plate, reidentify, and retest for susceptibility. If the discrete colonies are still apparent, measure the colony-free inner zone.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Interpretation

Use criteria specified by the NCCLS (1) to interpret the zones of inhibition for each antimicrobial agent.

B. Reporting

Report categorical result as either susceptible (S), intermediate (I), or resistant (R).

Example: *E. coli*

Drug	Result
Ampicillin	R
Cefazolin	S
Piperacillin	I
Gentamicin	S
Trimethoprim-sulfamethoxazole	S

C. Important reporting rules

1. Report methicillin-, oxacillin-, or nafcillin-resistant staphylococci as resistant to all beta-lactam antimicrobial agents (including beta-lactam–beta-lactamase inhibitor combinations, all cephems, all penicillins, and carbapenems) regardless of results of in vitro susceptibility testing.

2. If test organisms require a nonstandardized method, add notation to report that susceptibility testing has been performed by a nonstandardized method (e.g., small-colony variant of *S. aureus* performed on BMHA, or if CO_2 incubation is used for organisms other than *Haemophilus* spp., *N. gonorrhoeae, S. pneumoniae,* or *Streptococcus* spp.).

3. Misleading results

Specific organism-antimicrobial agent combinations should not be reported, since susceptible results could be *dangerously misleading:*

a. First- and second-generation cephalosporins and aminoglycosides against *Salmonella* spp. and *Shigella* spp.

b. Cephalosporins, trimethoprim-sulfamethoxazole, clindamycin, and aminoglycosides (except for high-level disk testing for aminoglycosides) against enterococci

c. Beta-lactams against oxacillin-resistant *Staphylococcus* spp.

d. Cephalosporins, penicillins, and aztreonam against ESBL-producing *E. coli* and *Klebsiella* spp.

VII. PROCEDURE NOTES

A. Oxacillin (methicillin)-resistant staphylococci

1. Oxacillin is the recommended penicillinase-stable penicillin for testing, and oxacillin results can represent all penicillinase-stable penicillins (oxacillin, methicillin, and nafcillin and also cloxacillin, dicloxacillin, and flucloxacillin). Testing of oxacillin with staphylococci should be performed in cation-adjusted Mueller-Hinton broth (CAMHB) supplemented with 2% NaCl (other drugs should not be tested in 2% NaCl supplemented CAMHB).

VII. PROCEDURE NOTES *(continued)*

2. Clues to detection of methicillin (oxacillin)-resistant *S. aureus*

a. Multiple resistance to other antimicrobial agents (beta-lactams, aminoglycosides, erythromycin, clindamycin, and tetracycline). Recently, strains of oxacillin-resistant *S. aureus* that are not multiply resistant have been encountered among isolates from patients with community-acquired infections and nosocomial infections.

b. Presence of haze of growth within the zone of inhibition around an oxacillin, methicillin, nafcillin, or cephalothin disk

B. Materials

1. Disks

Working supplies of disks (properly stored as described above) can be utilized for at least 1 week; verify acceptability by QC methods

2. Media

a. Thymidine can interfere with performance of sulfonamides and trimethoprim. Monitor by testing *E. faecalis* ATCC 29212 and trimethoprim-sulfamethoxazole (zone of $\leq$20 mm indicates acceptable performance). Blood components (other than horse blood) contain thymidine and should not be used (except sheep blood for *S. pneumoniae* and *Streptococcus* spp. as described in this procedure).

b. An increase in the cation content (Ca^{2+}, Mg^{2+}) of the medium results in decreased zone sizes with aminoglycosides and increased zone sizes with tetracycline for *P. aeruginosa.* A decrease in cation content has the opposite effect. Monitor cation content by testing aminoglycosides with *P. aeruginosa* ATCC 27853 and making sure results are within defined QC limits.

c. Increased zinc ions may cause decreased zone sizes with carbapenems.

d. Variation in calcium ions affects the results of daptomycin tests.

VIII. LIMITATIONS

A. This method is standardized only for rapidly growing aerobes, including the *Enterobacteriaceae, P. aeruginosa, Acinetobacter* spp., *Staphylococcus* spp., and *Enterococcus* spp. Modifications have been made to standardize testing of some fastidious organisms, such as *Haemophilus* spp., *N. gonorrhoeae, S. pneumoniae,* and *Streptococcus* spp (*see* appendixes to this procedure). For other organisms an MIC test is recommended.

B. Numerous factors can affect results, including inoculum size, rate of growth, formulation and pH of media, incubation environment and length of incubation, disk content and drug diffusion rate, and measurement of endpoints. Therefore, strict adherence to protocol is required to ensure reliable results.

C. Emergence of resistance

Some bacteria may become resistant during antimicrobial therapy. Performing susceptibility testing on subsequent isolates after 3 or 4 days is recommended for the following.

1. *Enterobacter, Citrobacter,* and *Serratia* for cephalosporins

2. *P. aeruginosa* for all antimicrobial agents

3. Staphylococci for quinolones

D. Additional QA measures

Following recommended QC procedures alone does not guarantee prevention of reporting erroneous results for isolates from patient specimens. A mechanism to flag atypical results is an integral component of an effective QA program. A protocol for verification of unusual results should be included in the susceptibility testing procedures.

REFERENCE

1. **NCCLS.** 2003. *Performance Standards for Antimicrobial Disk Susceptibility,* 8th ed. Approved standard M2-A8. NCCLS, Wayne, Pa.

SUPPLEMENTAL READING

Barry, A. L., M. D. Coyle, C. Thornsberry, E. H. Gerlach, and R. W. Hawkinson. 1979. Methods of measuring zones of inhibition with Bauer-Kirby disk susceptibility test. *J. Clin. Microbiol.* **10:**885–889.

Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Turck. 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* **45:**493–496.

Bradford, P. A. 2001. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin. Microbiol. Rev.* **14:**933–951.

Jorgensen, J. H. and M. J. Ferraro. 2000. Antimicrobial susceptibility testing: special needs for fastidious organisms and difficult-to-detect resistance mechanisms. *Clin. Infect. Dis.* **30:**799–808.

Jorgensen, J. H. 2003. Susceptibility test methods: dilution and disk diffusion methods. p. 1108–1127. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.

Miller, J. M., C. Thornsberry, and C. N. Baker. 1984. Disk diffusion susceptibility test troubleshooting guide. *Lab Med.* **15:**183–185.

APPENDIX 5.1–1

Disk diffusion susceptibility testing QC (*see* p. 5.1.15)

APPENDIX 5.1–2

Troubleshooting guide for disk diffusion susceptibility testing[a,b]

Aberrant result	Probable cause	Corrective action[b]	Effect on current day's reports
Tetracycline zone too large and clindamycin zone too small with *E. coli* or *S. aureus* control strains	pH of medium too low	Adjust pH to 7.2–7.4 before pouring medium. Commercial media should not have pH problems. Get a new lot (incubation in CO_2 may alter agar surface pH).	*Do not* report test results until corrective action has been taken and new batch of medium demonstrates acceptable results.
Tetracycline zone too small and clindamycin zone too large with *S. aureus* or *E. coli* control strains	pH of medium too high	Adjust pH to 7.2–7.4 before pouring medium. Commercial media should not have pH problems. Get a new lot (incubation in CO_2 may alter agar surface pH).	*Do not* report test results until corrective action has been taken and new batch of medium demonstrates acceptable results.
Aminoglycoside zone too small with *P. aeruginosa* control strain	Ca^{2+} and/or Mg^{2+} level too high in medium	Acquire new lot of agar medium that will meet QC criteria.	*Do not* report aminoglycoside results on *P. aeruginosa* or *Acinetobacter* until zone sizes meet QC standard.
Aminoglycoside zone too large with *P. aeruginosa* control strain	Ca^{2+} and/or Mg^{2+} level too low in medium	Acquire new lot of agar medium that will meet QC criteria.	*Do not* report aminoglycoside results on *P. aeruginosa* or *Acinetobacter* until zone sizes meet QC standard.
Zones universally too large on control plates	Inoculum too light	Adjust inoculum to McFarland 0.5 turbidity standard.	Hold results until repeat QC is within limits (6-h reading).
	Nutritionally poor medium	Use only MHA medium.	*Do not* report until MHA is used.
	Slow-growing organism (not seen with controls)	Use MIC procedure only.	*Do not* report any slow-grower disk results.
	Improper medium depth (too thin)	Use 4- to 5-mm depth	*Do not* use this batch or lot of medium for testing.
Zones universally too small on control plates	Inoculum too heavy	Adjust inoculum to 0.5 McFarland standard.	Hold results until repeat QC is within limits (6-h reading).
	Agar depth too thick (minor)	Use 4- to 5-mm depth.	*Do not* use this batch or lot of medium for testing.
Methicillin zone decreasing over days or wk with control organisms	Methicillin degrading during refrigerator storage	Change methicillin disks or use oxacillin or nafcillin as routine test disk.	Report methicillin only if within QC range.
Carbenicillin zone disappears with *Pseudomonas* control	Resistant mutant has been selected for testing.	Change *Pseudomonas* control strain every 2 wk and whenever resistant mutants appear within carbenicillin zone.	Repeat carbenicillin disk only. Read at 6 h. Report other disks as usual.
Single disk result above or below control limit	Error in reading, fuzzy zone edge, transcription error, bad disk	Note error. Recheck reading and ask for second opinion.	Report other disks by following standard protocol. Repeat test for out-of-control disk before reporting actual test results. Read repeat QC at 6 h.
	Disk may not be pressed firmly onto agar surface (bad disks usually demonstrate trend toward being out of control).	Statistically, one may expect occasional out-of-range result. Values usually fall within range on retesting.	
Colonies within zone of inhibition	Mixed culture	Isolate, identify, and retest pure culture only.	*Do not* report results of this plate.
	Resistant mutants within zone (*see* carbenicillin above)	Gram stain or do other test to rule out contaminant.	Report as resistant.
Very large zones with anaerobes		*Do not* use disk agar diffusion procedure to test anaerobes!	
S. aureus from patient resistant to methicillin one day and sensitive the next	*May be two different organisms;* temp shift from 37 to 35°C can dramatically alter zone size in this case.	Check testing temp. Test must be performed at 35°C for methicillin (oxacillin or nafcillin) and *S. aureus.*	Report result obtained at 35°C.
	Low methicillin content in disk	Use new disks.	

APPENDIX 5.1–2 *(continued)*

Aberrant result	Probable cause	Corrective action[b]	Effect on current day's reports
Zones overlap	Disks too close together	Use no more than 12 disks on a 150-mm plate and 4 to 5 on a 100-mm plate. Place disks no closer than 15 mm to edge of plate.	Repeat test.
Zones indistinct with single colonies noted on plate	Poorly streaked plate, inadequate inoculum	Use properly adjusted inoculum, and repeat test.	Repeat test before reporting.
Zone-within-zone phenomenon	Swarming *Proteus* sp.	Read wide, distinct zone, and disregard growth that swarmed over	Report *Proteus* spp. from outer distinct zone.
	Feather edges of zones around penicillin or ampicillin disks usually with beta-lactamase-negative strains of *S. aureus*	Take half distance from inner zone to outermost zone as measure mark.	Report zone as described under "Corrective action" column.
	Sulfonamides	Disregard growth from disk margin to major inner zone.	Report outer zone
	Beta-lactamase-positive *H. influenzae* with penicillin or ampicillin	Use inside zone.	Call physician if meningitis.
Indistinct zones with sulfamethoxazole ± trimethoprim or with trimethoprim alone	Thymidine in medium inhibits action of these antimicrobials	Use commercial thymidine-free plates. Disregard small amount of growth within zone as with sulfonamides.	Report as usual if confident of results.

[a] Reprinted from **J. M. Miller, C. Thornsberry, and C. N. Baker.** 1984. Disk diffusion susceptibility test troubleshooting guide. *Lab. Med.* **15:**183–185, with permission of the American Society of Clinical Pathologists.

[b] Record and note all errors.

Supplemental Reading

NCCLS. 2003. *Performance Standards for Antimicrobial Disk Susceptibility Tests*, 8th ed. Approved standard M2-A8. NCCLS, Wayne, Pa.

APPENDIX 5.1–3

Quick reference list for performing disk diffusion tests[a]

Organism(s)	Agar	Inoculum method	Incubation length (h)	Incubation atmosphere[b]	Notes
Enterobacteriaceae	MHA	DCS/LPG	16–18	Ambient air	
Pseudomonas aeruginosa and *Acinetobacter* spp.	MHA	DCS/LPG	16–18	Ambient air	
Haemophilus spp.	HTM	DCS	16–18	CO_2	Beta-lactamase test
Neisseria gonorrhoeae	GC	DCS	20–24	CO_2	Beta-lactamase test
Vibrio cholerae	MHA	DCS/LPG	16–18	Ambient air	
Staphylococcus spp.	MHA	DCS	16–18 24 (oxacillin[c] and vancomycin)	Ambient air	Read oxacillin and vancomycin using transmitted light; determine MIC if vancomycin zone = 14 mm or less.
Enterococcus spp.	MHA	DCS/LPG	16–18 24 (vancomycin)	Ambient air	Beta-lactamase and high-level aminoglycoside screen on blood and CSF isolates; read vancomycin using transmitted light and determine MIC for any vancomycin "I" result.
Nonenterococcal streptococci	BMHA	DCS	20–24	CO_2	Determine penicillin MIC for viridans group streptococci from endocarditis patients
Streptococcus pneumoniae	BMHA	DCS	20–24	CO_2	Oxacillin disk for penicillin resistance

[a] Disk diffusion testing is not standardized by the NCCLS for organisms other than those in this table. If testing is required, perform by MIC method. Abbreviations: DCS, direct colony suspension—standardize to McFarland 0.5 turbidity standard; DCS/LPG, direct colony suspension or log-phase growth—standardize to McFarland 0.5 turbidity standard; GC, GC agar with defined supplement; LPG, log-phase growth.

[b] All incubation temperatures are 35°C.

[c] And other penicillinase-stable penicillins.

APPENDIX 5.1–4

Haemophilus spp.

PREANALYTICAL CONSIDERATIONS

A. SPECIMEN

Isolated colonies of similar colony morphology grown overnight on CHOC

B. SUPPLEMENTAL MATERIALS

1. CHOC
   Store at 2 to 8°C.
2. HTM plates
   Store at 2 to 8°C.
3. Photometric standardizing device (e.g., bench top photometer) for 0.5 McFarland turbidity and test tubes that will fit this device

ANALYTICAL CONSIDERATIONS

C. QC STRAINS

1. *H. influenzae* ATCC 49247
2. *H. influenzae* ATCC 49766
3. *E. coli* ATCC 35218 (for beta-lactam–beta-lactamase inhibitor combinations)
4. Refer to NCCLS *Haemophilus* QC table for acceptable QC ranges (1).

D. PROCEDURE

1. Prepare inoculum by using a direct colony suspension in Mueller-Hinton broth or 0.9% NaCl from a fresh (20- to 24-h) CHOC plate.
2. Use a photometric device to adjust turbidity to match a 0.5 McFarland turbidity standard (1×10^8 to 4×10^8 CFU/ml). This step is recommended to avoid erroneous results due to under- or overinoculation.
3. Without delay, inoculate agar, and apply disks.
4. Because *Haemophilus* spp. show large zones with some drugs (e.g., third-generation cephalosporins), apply no more than nine disks per 150-mm plate or no more than four disks per 100-mm plate.
5. Incubate in 5% CO_2 for 16 to 18 h at 35°C.

E. READING RESULTS

Measure zones of inhibition from back of plate.

POSTANALYTICAL CONSIDERATIONS

F. REPORTING RESULTS

1. Refer to the appropriate NCCLS interpretive table for zone interpretation for *Haemophilus* spp. (1).
2. Only the susceptible interpretation is defined in NCCLS tables for several cephalosporins, carbapenems, aztreonam, macrolides, and fluoroquinolones because no (or very few) resistant strains have been reported to date. Confirm identification and susceptibility results for all isolates which are not susceptible to these agents, save isolate, and submit to a reference laboratory for testing using an NCCLS dilution reference method.

G. NOTES

1. Beta-lactamase-positive *Haemophilus* spp. are resistant to ampicillin and amoxicillin regardless of the MICs.
2. Occasional isolates are beta-lactamase negative and ampicillin resistant. Consider these resistant to amoxicillin-clavulanic acid, ampicillin-sulbactam, cefaclor, cefamandole, cefetamet, cefonicid, cefprozil, cefuroxime, loracarbef, and piperacillin-tazobactam despite in vitro results.
3. For life-threatening *Haemophilus* infections (e.g., meningitis, bacteremia), report only ampicillin, a third-generation cephalosporin, chloramphenicol, and meropenem.

Reference

1. **NCCLS.** 2003. *Performance Standards for Antimicrobial Disk Susceptibility,* 8th ed. Approved standard M2-A8. NCCLS, Wayne, Pa.

APPENDIX 5.1–5

Neisseria gonorrhoeae

PREANALYTICAL CONSIDERATIONS

A. SPECIMEN

Isolated colonies of similar colony morphology grown overnight in 5% CO_2 on CHOC

B. SUPPLEMENTAL MATERIALS

1. CHOC
   Store at 2 to 8°C.
2. GC agar base and 1% defined growth supplement (GC medium). Cysteine-free growth supplement is not required for disk testing. Refer to procedure 5.14. Store at 2 to 8°C.

ANALYTICAL CONSIDERATIONS

C. QC STRAIN

1. *N. gonorrhoeae* ATCC 49226
2. Refer to NCCLS *N. gonorrhoeae* QC table for QC ranges (1).

D. PROCEDURE

1. Prepare inoculum by using a direct colony suspension in Mueller-Hinton broth or 0.9% NaCl from a fresh (18- to 24-h) CHOC plate.
2. Adjust turbidity visually to a 0.5 McFarland turbidity standard or use a photometric device.
3. Inoculate agar within 15 min, and apply disks.
4. Apply no more than nine disks per 150-mm plate and four disks per 100-mm plate.
5. Incubate in 5% CO_2 for 20 to 24 h at 35°C.

E. READING RESULTS

Measure zones of inhibition from back of plate.

POSTANALYTICAL CONSIDERATIONS

F. REPORTING RESULTS

1. Refer to the appropriate NCCLS interpretive table for zone interpretations for *N. gonorrhoeae* (1).
2. Only the susceptible interpretation is defined for several cephalosporins because resistant strains have not been reported to date. Confirm identification and susceptibility results for all isolates which are not susceptible to these agents, save isolate, and submit to a reference laboratory for testing using an NCCLS dilution reference method.

G. NOTES

1. *N. gonorrhoeae* organisms with zone diameters of ≤19 mm with the 10-U penicillin disk generally are beta-lactamase producers. Beta-lactamase tests are preferred for detecting this plasmid-mediated resistance.
2. *N. gonorrhoeae* organisms with zone diameters of ≤19 mm with the 30-μg tetracycline disk usually indicate plasmid-mediated resistance to tetracycline. Confirm by a dilution MIC test.
3. Strains interpreted as susceptible have a >95% clinical cure rate, whereas those isolates which are intermediate have a lower cure rate, 85 to 95%.

Reference

1. **NCCLS.** 2003. *Performance Standards for Antimicrobial Disk Susceptibility*, 8th ed. Approved standard M2-A8. NCCLS, Wayne, Pa.

APPENDIX 5.1–6

Streptococcus pneumoniae and *Streptococcus* spp.

PREANALYTICAL CONSIDERATIONS

A. SPECIMEN

Isolated colonies of similar colony morphology grown overnight on BAP

B. SUPPLEMENTAL MATERIALS

1. BAP
   Store at 2 to 8°C.
2. Agar plates: use BMHA plates. Store at 2 to 8°C.

ANALYTICAL CONSIDERATIONS

C. QC STRAINS

1. *S. pneumoniae* ATCC 49619
2. Refer to NCCLS *S. pneumoniae* and *Streptococcus* QC table for acceptable QC ranges (1).

D. PROCEDURE

1. Prepare inoculum by using a direct colony suspension in Mueller-Hinton broth or 0.9% NaCl from fresh (16- to 18-h) growth on BAP.
2. Adjust turbidity visually to a 0.5 McFarland turbidity standard or use a photometric device.
3. Without delay, inoculate the plate as previously described.
4. Incubate in 5% CO_2 for 20 to 24 h at 35°C.

E. READING RESULTS

1. Remove lid and measure zones of inhibition from top of plate.
2. Only the susceptible interpretation is defined for vancomycin and linezolid for all streptococci and for penicillin, ampicillin, and extended-spectrum cephalosporins for beta-hemolytic streptococci because resistance has not been observed. Confirm identification and susceptibility results for all isolates which are not susceptible to these agents, save isolate, and submit to a reference laboratory for testing using an NCCLS dilution reference method.

POSTANALYTICAL CONSIDERATIONS

F. REPORTING RESULTS

1. Refer to the appropriate NCCLS interpretive table for zone interpretations for *S. pneumoniae* and the separate table for *Streptococcus* spp. (1).
2. Penicillin and *S. pneumoniae* (test 1-μg oxacillin disk to determine penicilin susceptibility)
   a. Susceptible: oxacillin zone $\geq$ 20 mm.
      Report results for *penicillin,* not *oxacillin.* Penicillin-susceptible pneumococci are also considered susceptible to all other beta-lactam antimicrobial agents which have approved indications for pneumococci, and these drugs need not be tested.
   b. Penicillin resistant or intermediate: oxacillin zone $\leq$ 19 mm.
      Isolates with zones of $\leq$ 19 mm may occur with penicillin-resistant or intermediate strains or certain susceptible strains. Perform an MIC test with penicillin, meropenem, and either cefotaxime or ceftriaxone on isolates with oxacillin zones of $\leq$19 mm.

G. NOTES

1. *S. pneumoniae*
   a. Pneumococci may lyse rapidly in liquid (particularly water). Therefore, follow the inoculum preparation procedure described here precisely and inoculate the BMHA plate immediately after standardization of the inoculum suspension.
   b. Penicillin-resistant *S. pneumoniae* does not produce beta-lactamase; do not use a beta-lactamase test to predict penicillin resistance.
2. Penicillin and viridans group streptococci
   Penicillin (and oxacillin) disk testing is not reliable for viridans group streptococci. Perform penicillin MIC testing on viridans group streptococci isolated from normally sterile body sites (e.g., blood, CSF, bone, etc.).

Reference

1. **NCCLS.** 2003. *Performance Standards for Antimicrobial Disk Susceptibility,* 8th ed. Approved standard M2–A8. NCCLS, Wayne, Pa.

APPENDIX 5.1–7

Extended-Spectrum Beta-Lactamase Testing for *Escherichia coli, Klebsiella pneumoniae,* and *Klebsiella oxytoca*

PREANALYTICAL CONSIDERATIONS

A. PRINCIPLE

ESBLs are derived from point mutations in the genes that code for common beta-lactamases such as TEM-1, TEM-2, or SHV-1. In contrast to TEM-1, TEM-2, and SHV-1, ESBLs hydrolyze or inactivate extended-spectrum cephalosporins, aztreonam, and expanded-spectrum penicillins. They do not hydrolyze carbapenems and generally do not hydrolyze cephamycins. ESBLs are blocked by beta-lactamase inhibitors such as clavulanic acid, and laboratory tests for detecting ESBL-producing bacteria are based on this property.

The screening test for ESBL production in *E. coli* and *Klebsiella* spp. utilizes interpretive criteria for certain third-generation cephalosporins and aztreonam that are different from those used for routine testing of *Enterobacteriaceae.* Once an isolate is deemed screen positive and therefore suspicious for ESBL production, a phenotypic confirmatory test is performed. The confirmatory test requires four disk tests: (i) ceftazidime alone, (ii) cefotaxime alone, (iii) ceftazidime plus clavulanic acid, and (iv) cefotaxime plus clavulanic acid. If the zone diameter of either ceftazidime or cefotaxime (or both) in the presence of clavulanic acid is ≥5 mm larger than the zone diameter of the respective agent alone, the test is considered positive for ESBL production.

B. SPECIMEN

Colonies as described in item II of procedure 5.1 for routine disk diffusion testing of rapidly growing nonfastidious bacteria

C. SUPPLEMENTAL MATERIALS

1. Materials as stated in item III of procedure 5.1 for routine disk diffusion testing of rapidly growing nonfastidious bacteria
2. Disks for the following agents at the concentrations specified
   a. Ceftazidime, 30 μg
   b. Ceftazidime-clavulanic acid, 30/10 μg
   c. Cefotaxime, 30 μg
   d. Cefotaxime-clavulanic acid, 30/10 μg

ANALYTICAL CONSIDERATIONS

D. QC STRAINS

1. *Klebsiella pneumoniae* ATCC 700603 (ESBL-producing strain)
2. *E. coli* ATCC 25922
3. Refer to current NCCLS QC and ESBL tables for acceptable QC ranges (1).

E. PROCEDURE

Follow standard disk diffusion testing recommendations for inoculum preparation, inoculation, and incubation as indicated in item V of procedure 5.1.

1. Initial screen test
   a. Perform disk diffusion tests with one or more of the following agents and include the disk concentration listed below. If the zone of inhibition is below that listed (positive screen test), proceed with the ESBL phenotypic confirmatory test.

Antimicrobial agent	Disk potency (μg)	Zone diam (mm) for positive screen
Cefpodoxime	10	≤17
Ceftazidime	30	≤22
Cefotaxime	30	≤27
Ceftriaxone	30	≤25
Aztreonam	30	≤27

   b. If the screen test is positive, suppress any susceptible results for penicillins, cephalosporins, or aztreonam from the patient report.
   c. If the screen test is negative, consider the isolate ESBL negative.

APPENDIX 5.1–7 *(continued)*

2. Phenotypic confirmatory test
   a. Perform a routine disk diffusion test and include four disks at the concentrations listed.
      (1) Ceftazidime, 30 μg
      (2) Ceftazidime-clavulanic acid, 30/10 μg
      (3) Cefotaxime, 30 μg
      (4) Cefotaxime-clavulanic acid, 30/10 μg
   b. Measure zones as described above for testing rapidly growing nonfastidious bacteria. See procedures 5.2 and 5.8 for alternative ESBL confirmatory testing methods.

POSTANALYTICAL CONSIDERATIONS

F. REPORTING RESULTS

1. Positive for ESBL production: a ≥5-mm increase in the zone diameter for either ceftazidime or cefotaxime tested in combination with clavulanic acid versus its zone diameter when tested alone
   a. Demonstration of enhanced activity (≥5-mm increase in the zone diameter) in the presence of clavulanic acid with either one or both pairs of antimicrobial agents is a positive result.
   b. Report all penicillins, cephalosporins (excluding the cephamycins such as cefoxitin or cefotetan), and aztreonam as resistant, even if the in vitro susceptibility test results are intermediate or susceptible.
2. Negative for ESBL production: a ≤5-mm increase in the zone diameter for either ceftazidime or cefotaxime tested in combination with clavulanic acid versus its zone diameter when tested alone

G. NOTES

1. At least 130 different types of ESBL enzymes have been described for *E. coli* and *Klebsiella* spp. Among the bacteria that produce these, a wide variety of susceptibility profiles may be noted with extended-spectrum beta-lactam antimicrobial agents, including the ESBL test screening agents. Use of more than one screening agent increases the likelihood of detecting the various types of ESBLs.
2. Extended-spectrum beta-lactam resistance in isolates that are screen test positive but confirmatory test negative is generally due to a mechanism other than ESBL production. ESBL screening tests have high specificity for *K. pneumoniae* but low specificity for *E. coli.* Many ESBL screen-positive *E. coli* strains are resistant to extended-spectrum antimicrobial agents due to a resistance mechanism such as hyperproduction of AmpC beta-lactamase. As a screening agent for *E. coli,* cefpodoxime is the most sensitive but the least specific.
3. Generally, the hierarchy of activity of the screening agents in detecting ESBL production is cefpodoxime > ceftazidime > cefotaxime > ceftriaxone > aztreonam.
4. The genes for ESBL production are typically located on plasmids, and genes for resistance to other antimicrobial agents are often found on the same plasmid. Consequently, isolates that produce ESBLs often (but not always) demonstrate multiple resistance to other classes of antimicrobial agents (e.g., aminoglycosides, fluoroquinolones, or trimethoprim-sulfamethoxazole).
5. ESBLs do not hydrolyze carbapenems, and ESBL-producing *E. coli* and *Klebsiella* spp. remain susceptible to these antimicrobial agents. Carbapenems are considered the beta-lactams of choice for treating infections due to ESBL-producing bacteria.

H. LIMITATIONS

1. Some organisms with ESBLs may contain other beta-lactamases or other resistance mechanisms (e.g., porin alterations) that can mask ESBL production in the phenotypic confirmatory test, resulting in a false-negative result.
2. Other members of *Enterobacteriaceae* produce ESBLs. However, practical clinical laboratory methods for screening and detection of these isolates have not been determined as yet.

Reference

1. **NCCLS.** 2003. *Performance Standards for Antimicrobial Disk Susceptibility,* 8th ed. Approved standard M2-A8. NCCLS, Wayne, Pa.

Disk Diffusion Susceptibility Testing Quality Control

ORGANISM _____ MEDIA TYPE _____

ANTIMICROBIC CONTENT (µg)													MEDIA		DATE	
ACCEPTABLE RANGE (mm)[a]													Lot #	Manuf.	Disks Changed	COMMENTS
Lot # Disks																
Exp. Date Disks																
DATE TECH 1.																
Lot # Disks																
Exp. Date Disks																
DATE TECH 2.																
Lot # Disks																
Exp. Date Disks																
DATE TECH 3.																
Lot # Disks																
Exp. Date Disks																
DATE TECH 4.																
Lot # Disks																
Exp. Date Disks																
DATE TECH 5.																

[a]Acceptable ranges of accuracy are given in **NCCLS**. 2003. *Performance Standards for Antimicrobial Susceptability Testing*. Thirteenth informational supplement M100-S13 (M2). NCCLS, Wayne, Pa.

5.2 Broth Microdilution MIC Test

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The broth microdilution MIC method is used to measure (semiquantitatively) the in vitro activity of an antimicrobial agent against a bacterial isolate. A sterile plastic tray containing various concentrations of antimicrobial agents is inoculated with a standardized number of test bacteria. After overnight incubation at 35°C, the MIC is determined by observing the lowest concentration of an antimicrobial agent which will inhibit visible growth of the bacterium. MICs obtained are interpreted as susceptible, intermediate, or resistant, based on the criteria listed in the NCCLS MIC standard (1).

For full-range MIC testing, five to eight concentrations representing a therapeutically achievable range for each agent are usually tested. Breakpoint MIC testing represents a modification of microdilution MIC testing in which generally one to three concentrations of each agent are tested and results are reported qualitatively. This enables more agents to be tested per tray.

The NCCLS describes a standard method for broth microdilution MIC testing of nonfastidious bacteria which utilizes cation-adjusted Mueller-Hinton broth (CAMHB). The NCCLS also describes standardized broth microdilution MIC procedures for testing *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Streptococcus* spp. The procedures for these bacteria are similar to those for nonfastidious bacteria; however, the test media are supplemented with nutrients that will support the growth of these fastidious organisms.

II. SPECIMEN

Prepare inoculum from four or five isolated colonies of similar colony morphology.

A. Log-phase or stationary-phase growth inoculum
Use colonies grown for 1 or 2 days on nonselective or selective (e.g., MAC) medium.

B. Direct colony suspension inoculum
Use colonies grown overnight on nonselective medium (e.g., BAP or CHOC).

C. Subculture stock, frozen, or lyophilized isolates two times prior to testing.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Media and reagents

1. Prepared broth microdilution trays containing an antimicrobial agent in CAMHB in volumes of 0.1 ml solution per well. Store at −70°C.
2. Nutrient broth (e.g. Mueller-Hinton, TSB) or 0.9% NaCl (3.0- to 5.0-ml aliquots)
   Store at 2 to 30°C.
3. BHI broth (0.5-ml aliquots)
   Store at 2 to 30°C.
4. Water diluent containing 0.02% Tween 80 dispensed in aliquots of 40 ml in screw-cap tubes
   Store at 25°C.
5. BAP
   Store at 2 to 8°C.

B. Supplies

1. Sterile cotton tipped swabs
2. Sterile plastic pipettes
3. McFarland 0.5 turbidity standard (McFarland 4.0 standard for stationary-phase inoculum)
4. Sterile disposable plastic multipronged inoculator sets (include inoculum reservoir)

C. Equipment

1. Vortex mixer
2. Ambient-air incubator (34 to 35°C), CO_2 incubation for some organisms (*see* Appendix 5.2–1)

III. MATERIALS *(continued)*

3. Adjustable micropipette and sterile pipette tips
4. Calibrated loop (0.001 ml [1 µl])
5. Viewing device to read inoculated MIC trays after incubation
6. Microdilution tray storage containers (e.g., Tupperware-type containers or plastic bags)
7. Freezer (−70°C; sometimes a −20°C, non-frost-free freezer is acceptable)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. QC strains (*see* appendixes to this procedure for QC of specific fastidious organisms)

1. *Escherichia coli* ATCC 25922
2. *Pseudomonas aeruginosa* ATCC 27853
3. *Staphylococcus aureus* ATCC 29213
4. *E. coli* ATCC 35218
5. *Enterococcus faecalis* ATCC 29212

B. Monitoring accuracy

1. Test QC strains by following routine procedures and record results in a QC notebook. Record lot numbers and expiration dates of broth microdilution trays.
2. Compare with expected results listed in the most recent NCCLS standard tables (1). Note any out-of-control result and document; proceed with corrective action, if necessary.
3. Record all results on QC worksheet (Appendix 5.2–2)

C. Additional controls

1. Growth controls
   A growth control well must show 3+ to 4+ growth; a purity plate must show good growth without contaminating organisms.
2. The sterility control well should be free of any growth.
3. Inoculum controls
   An inoculum count verification plate (checked once per month) should show approximately 50 colonies.

D. Daily QC testing

1. QC testing is in control if, for each drug-organism combination, no more than 1 out of 20 consecutive results is outside acceptable range.
2. If any more than 1 of 20 results is out of control, proceed with corrective action.

E. Weekly QC testing

1. Frequency of QC testing
   a. QC testing can be reduced from daily to weekly providing a laboratory can document proficiency in performing this test by performing QC daily (or each day patient tests are performed) until results from 20 consecutive days of testing have been obtained.
   b. Proficiency in performing QC testing is confirmed if for each drug-organism combination no more than 1 of 20 results are outside accuracy limits. Weekly QC testing may then be initiated.
   c. If 2 or 3 of the 20 results are out of control, continue testing for a total of 30 days. If no more than 3 of 30 results are outside accuracy limits, proficiency is demonstrated, and weekly testing can be initiated.
2. Weekly QC testing is in control if all MICs are within specified accuracy limits. If any result is out of control, proceed with corrective action.

IV. QUALITY CONTROL *(continued)*

3. Document proficiency each time a new drug is added to testing protocols.
◩ Save records documenting proficiency indefinitely.

4. Perform QC testing each time a new lot or new shipment of materials (broth microdilution trays) is put into use.

5. Perform colony counts on QC strains at least once per month.

F. Corrective action

1. Out-of-control result due to obvious error (e.g., contamination, incorrect organism tested, inadequate incubation conditions, etc.) that can be easily corrected: record reason for out-of-control result and retest the same day. If the repeated result is in range, no further action is needed.

2. Out-of-control result not due to obvious error

a. Test the panel with the QC strain that demonstrated out-of-control results for five consecutive test days. Record results. It is up to each laboratory to decide whether to record results from all agents tested or just those for the "problematic" agent.

b. If all five results for the problematic antimicrobial agent are now within the acceptable QC range, weekly testing may be resumed.

c. If the problem is not resolved (at least one MIC is outside the accuracy control limits), continue daily QC testing until the problem is resolved.

d. Investigate possible procedural problems (e.g., review reading of MICs, standardization of inoculum, storage and expiration dates of reagents, incubation conditions, equipment, and maintenance and purity of control strain).

e. In order to return to weekly testing, satisfactory performance for another 20 or 30 consecutive days must be documented (as described above).

f. During corrective action, carefully evaluate whether patient reports will be affected. It may be necessary to use an alternate test method until the problem is resolved. (Refer to procedure 5.13 for maintenance of QC strains and additional measures for verifying results for bacteria isolated from patients.)

V. PROCEDURE

See Appendix 5.2–1 for a summary of testing conditions for various organisms and Appendixes 5.2–3 to 5.2–5 for special procedures for testing *Haemophilus* spp., *S. pneumoniae*, and *Streptococcus* spp. and testing for extended-spectrum beta-lactamase (ESBL) production in *E. coli* and *Klebsiella* spp. See Appendix 5.2–6 for breakpoint MIC panels.

A. Allow frozen trays to thaw at room temperature.

B. Inoculum preparation

1. Using a loop or swab, transfer colonies as follows.

a. Direct colony suspension method
Pick several colonies from a fresh (18- to 24-h) nonselective agar plate to broth or 0.9% NaCl.

b. Log-phase method

(1) Pick four or five isolated colonies to 3.0 to 5.0 ml of broth.

(2) Incubate at 35°C for 2 to 8 h until growth reaches the turbidity at or above that of a 0.5 McFarland standard.

c. For either the log-phase or direct colony suspension method, vortex well and adjust turbidity visually with sterile broth or 0.9% NaCl to match that of a 0.5 McFarland standard (1×10^8 to 2×10^8 CFU/ml). Alternatively, standardize suspension with a photometric device.

V. PROCEDURE *(continued)*

d. Stationary-phase method
(1) Transfer several isolated colonies to 0.5 ml of BHI.
(2) Incubate at 35°C to the stationary phase (visual inspection must show very dense turbidity [4.0 McFarland standard] corresponding to 10^9 CFU/ml).
(a) *Enterobacteriaceae*, 2 to 8 h
(b) *Pseudomonas*, 4 to 8 h
(c) Others, 4 to 8 h

e. Selection of inoculum preparation method
(1) Direct colony suspension
(a) Use for bacteria that grow slowly or unpredictably in broth.
(b) Always use for staphylococci.
(c) Use for any organism when fresh (18- to 24-h) growth is available.
(2) Log-phase or stationary-phase method
(a) Use for nonfastidious rapidly growing bacteria when fresh colonies are not available.
(b) If desired, use for nonfastidious rapidly growing bacteria when fresh growth is available.

2. For the intermediate dilution, calculate the volume of standardized suspension to be added to 40 ml of water-Tween 80 diluent to obtain a final organism concentration of approximately 5×10^5 CFU/ml in each microdilution well. For prongs that deliver 0.01 ml, use the following.

Inoculum preparation method	Equivalent organism concn (CFU/ml)	Amt (ml of organism suspension to add to 40-ml water blank)	Dilution	Organism concn/ml in 0.1-ml well
Log-phase growth or direct colony suspension	1.5×10^8 (McFarland 0.5)	2.0	1:20	5×10^5–8×10^5
Stationary-phase growth	10^9 (McFarland 4 or higher)	0.2	1:200	5×10^5–6×10^5

C. Inoculation
1. Transfer the appropriate volume of well-mixed organism suspension to the water-Tween 80 diluent with a micropipette (or other pipette).
2. Gently mix by inverting five or six times (try to avoid producing air bubbles).
3. Within 1 h (immediately if testing fastidious organisms), inoculate the MIC tray.
4. Inoculation with disposable plastic inoculators that deliver 0.01 ml
a. Remove inoculator-reservoir set from its plastic packaging.
b. Pour diluted inoculum into inoculum tray reservoir.
c. Dip prongs into the inoculum suspension.
(1) Orient the sterility well so that it does not get inoculated.
(2) Press down on the inoculator firmly to ensure that all prongs have come in contact with the inoculum in the reservoir tray.
(3) Carefully remove prongs from inoculum.
d. Inoculate the MIC tray by dipping the filled prongs carefully into the MIC tray. Press down on the inoculator firmly to ensure that all prongs have come in contact with the antimicrobial solutions in the tray.
e. Discard the prongs as biohazardous waste.

V. PROCEDURE *(continued)*

f. Inoculate a purity plate by subculturing 0.001 ml of inoculum from the reservoir onto a BAP. Discard liquid and reservoir tray as biohazardous waste.

5. Prepare an inoculum count verification plate monthly on each QC strain routinely tested as follows.

a. Immediately after inoculation, transfer 0.01 ml from the positive growth control well into 10 ml of sterile water or saline.

b. Vortex the mixture well, and then transfer 0.1 ml to a BAP.

c. Using a loop, thoroughly spread the inoculum over the surface of the plate by streaking in several directions.

D. Incubation

1. Stack inoculated MIC trays in stacks of no more than four high and place them in a plastic bag or microdilution tray storage container with the top loose. Alternatively, place an empty microdilution tray on top of the stack.

2. Incubate MIC trays for 16 to 20 h at 35°C in an ambient-air incubator; incubate purity plates and inoculum count verification plates in CO_2.

a. Incubate all staphylococci that are susceptible to penicillinase-stable penicillins (e.g., oxacillin) or to vancomycin after 16 to 20 h for an additional 4 to 8 h (total of 24 h).

b. Incubate all enterococci that are susceptible to vancomycin or high concentrations of gentamicin (synergy screen) after 16 to 20 h for an additional 4 to 8 h (total of 24 h). Incubate the streptomycin synergy screen for 48 h before reporting a susceptible result (*see* procedure 5.5).

E. Reading MICs

1. Examine purity control plates. If mixed, repeat the corresponding MIC test. When available, examine the inoculum count verification plate, which should show approximately 50 colonies.

2. Place tray on the tray reading device.

3. Read the MIC tray only if the growth control well is turbid (3+ to 4+ growth).

a. ± to 1+, tiny button of very light haze in well

b. 2+, light haze in well

c. 3+ to 4+, heavy turbidity or fine granular growth throughout the well or a dense white button at bottom of well

◩ Do not read the MICs if there is inadequate growth in the growth control well (often there are separate control wells if different broths are used, e.g., BHI for *Enterococcus* synergy screens).

4. Examine the uninoculated broth sterility control well. Compare questionable growth to this well when necessary.

5. The MIC is the lowest concentration of antimicrobial agent showing complete inhibition of growth, or, for sulfonamides and trimethoprim, the MIC is the lowest concentration that inhibits 80% of the growth compared to the growth control well.

a. For trimethoprim-sulfamethoxazole, ignore very slight hazes and/or a pinpoint button (≤2 mm) that persists through several dilutions.

b. When skipped wells occur, record the MIC only if the true MIC is apparent. If >1 drug skips or a skip occurs with a reportable drug, repeat the MIC test.

6. For staphylococci and penicillin, if the MIC is 0.06 to 0.12 μg/ml, perform an induced-beta-lactamase test to determine whether the isolate is a low-level beta-lactamase producer.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Interpretation

Interpret MICs based on criteria specified by the NCCLS (1).

B. Reporting

Report the MIC along with its categorical interpretation: susceptible (S), intermediate (I), or resistant (R).

Example: *E. coli*

Drug	MIC (μg/ml)	Interpretation
Ampicillin	>32	R
Cefazolin	1	S
Piperacillin	32	I
Gentamicin	1	S
Trimethoprim-sulfamethoxazole	≤0.5/9.5	S

C. Important reporting rules

1. Report methicillin-, oxacillin-, or nafcillin-resistant staphylococci as resistant to all beta-lactam drugs (including beta-lactam–beta-lactamase inhibitor combinations, all cephems, all penicillins, and carbapenems) regardless of results of in vitro susceptibility testing.

2. If test organisms require a nonstandardized method, or if organisms other than the *Enterobacteriaceae, P. aeruginosa, Acinetobacter* spp., *Stenotrophomonas maltophilia, Pseudomonas* spp., *Staphylococcus* spp., *Enterococcus* spp., *Haemophilus* spp., *S. pneumoniae,* and *Streptococcus* spp. are tested by these methods, add a comment to the report stating that susceptibility testing has been performed by a nonstandardized method (e.g., for *Corynebacterium* spp. tested in lysed horse blood MIC panels).

3. Misleading results

Specific organism-drug combinations should not be reported, since susceptible results could be *dangerously misleading:*

- **a.** First- and second-generation cephalosporins and aminoglycosides against *Salmonella* spp. and *Shigella* spp.
- **b.** Cephalosporins, trimethoprim-sulfamethoxazole, clindamycin, and aminoglycosides (except for high-level-synergy testing for aminoglycosides) against enterococci
- **c.** Cephalosporins against *Listeria* spp.
- **d.** Beta-lactams against oxacillin-resistant *Staphylococcus* spp.
- **e.** Cephalosporins, penicillins, and aztreonam against ESBL-producing *E. coli* and *Klebsiella* spp.

4. For staphylococci and penicillin

- **a.** MIC of 0.03 μg/ml, report penicillin susceptible
- **b.** MIC of 0.25 μg/ml, report penicillin resistant
- **c.** MIC of 0.06 to 0.12 μg/ml and beta-lactamase negative, report penicillin susceptible
- **d.** MIC of 0.06 to 0.12 μg/ml and beta-lactamase positive, report penicillin resistant

VII. PROCEDURE NOTES

A. Oxacillin (methicillin)-resistant staphylococci

1. Oxacillin is the recommended penicillinase-stable penicillin for testing, and oxacillin results can represent all penicillinase-stable penicillins (oxacillin, methicillin, and nafcillin and also cloxacillin, dicloxacillin, and flucloxacillin). Testing of oxacillin with staphylococci should be performed in CAMHB supplemented with 2% NaCl (other drugs should not be tested in 2% NaCl-supplemented CAMHB).
2. Clues to detection of methicillin (oxacillin)-resistant *S. aureus*
   a. Multiple resistance to other antimicrobial agents (beta-lactams, aminoglycosides, erythromycin, clindamycin, and tetracycline). Recently, strains of oxacillin-resistant *S. aureus* that are not multiply resistant have been encountered among isolates from patients, primarily with community-acquired infections.
   b. Subtle growth in wells containing oxacillin, methicillin, or nafcillin

B. Media

1. Thymidine can interfere with performance of sulfonamides and trimethoprim. Monitor media by testing *E. faecalis* ATCC 29212 and trimethoprim-sulfamethoxazole (MIC of $\leq$0.5/9.5 μg/ml indicates acceptable performance). Blood components (other than horse blood) contain thymidine and should not be used.
2. An increase in the cation content (Ca^{2+}, Mg^{2+}) of the medium results in an increase in the MICs of aminoglycosides for *P. aeruginosa* and an increase in the MICs of tetracycline for all organisms. A decrease in cation content has the opposite effect. Monitor cation content by testing aminoglycosides with *P. aeruginosa* ATCC 27853 and making sure results are within defined QC limits.
3. Increased zinc ions may cause decreased zone sizes with carbapenems.
4. Variation in calcium ions affects the results of daptomycin tests.

C. Materials

1. When supplements (e.g., lysed horse blood) are added to individual wells of antimicrobial broth, the diluting effect need not be considered providing the amount added is less than 10% of the total volume in the well.
2. Although it is preferable to store MIC trays at -70°C, it is often acceptable to store frozen panels without imipenem and combinations with clavulanic acid at -20°C. Imipenem and clavulanic acid are more temperature labile than many other drugs and require -70°C storage. Follow storage recommendations of manufacturer.

D. Inoculum preparation

1. The stationary-phase method of inoculum preparation is not an NCCLS-recommended method; however, this method is suggested as an alternative for several commercial systems. Since the critical factor in inoculum preparation is the final concentration of viable organisms in each microdilution well (approximately 5×10^5 CFU/ml), the stationary-phase method will produce satisfactory results for nonfastidious rapidly growing organisms providing the correct number of organisms is obtained. It is assumed that incubation to stationary phase will produce a suspension containing approximately 10^9 CFU/ml.
2. Alternative inoculating devices, such as semiautomated inoculators, may be used to inoculate MIC trays. Follow the manufacturer's instructions to achieve a final concentration of approximately 5×10^5 CFU/ml.

E. Reading MICs

1. With bacteriostatic antimicrobial agents such as chloramphenicol, erythromycin, and clindamycin, very slight hazes may persist through several di-

VII. PROCEDURE NOTES
(continued)

lutions. Similarly, very slight hazes and/or pinpoint buttons (≤2 mm) may persist through all dilutions of trimethoprim-sulfamethoxazole. These should be ignored. Hazes should not be ignored for resistance that may be subtle, such as that encountered with oxacillin or vancomycin and staphylococci or vancomycin with enterococci.

2. When skipped wells occur, it may be necessary to repeat the MIC test. Skipped wells are indicated by growth at higher concentrations of an antimicrobial agent and no growth at one or more of the lower concentrations. This may occur as a result of the following.
   - **a.** Contamination at higher dilutions
   - **b.** Inadequate numbers or organisms inoculated into the wells
   - **c.** Wells not inoculated properly owing to improper alignment of inoculating pins or prongs
   - **d.** Peculiarity of the test organism (e.g., it might contain a resistant subpopulation)
   - **e.** Improper concentrations of antimicrobial agents in the wells

   Each skipped well case must be evaluated individually to determine if the test should be repeated.
3. Contamination may not always be detected using a purity plate since only a very small volume of the inoculum is sampled. The appearance of growth in the microdilution tray wells must be examined closely, and peculiar growth patterns, skipped wells, and/or atypical antibiograms should be investigated.
4. Reproducibility of broth microdilution MIC testing is generally within ±1 twofold dilution.

VIII. LIMITATIONS

A. The basic procedure described here has been standardized for testing commonly isolated bacteria that grow well after overnight incubation in CAMHB. Various modifications have been made for testing some of the more fastidious and special problem pathogens such as *Haemophilus* spp., *S. pneumoniae*, and *Streptococcus* spp. (see appendixes to this procedure).

B. Because of the small number of organisms tested in each well (approximately 5×10^4 CFU/well), resistance by a small subpopulation may not always be detected with the broth microdilution MIC method.

C. NCCLS M7-A6 (1) establishes MIC breakpoints based in part on blood levels following standard dosing. For optimal use of MICs, the MIC should be correlated with the presumed antimicrobial agent concentration at the infection site.

D. Numerous factors can affect results, including inoculum size, rate of growth, formulation and pH of media, incubation environment and length of incubation, drug concentration, and measurement of endpoints. Strict adherence to protocol is required to ensure reliable results.

E. Emergence of resistance

Some bacteria may become resistant during antimicrobial therapy. Performing susceptibility testing on subsequent isolates after 3 or 4 days is recommended for the following.

1. *Enterobacter, Citrobacter*, and *Serratia* for cephalosporins
2. *P. aeruginosa* for all antimicrobials
3. Staphylococci for quinolones

F. Additional QA measures

Following recommended QC procedures alone does not guarantee prevention of reporting erroneous results for isolates from patient specimens. A mechanism to flag atypical results is an integral component of an effective QA program. A protocol for verification of unusual results should be included in the susceptibility testing procedures (*see* procedure 5.13).

REFERENCE

1. **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.

SUPPLEMENTAL READING

Barry, A. L., and L. E. Braun. 1981. Reader error in determining minimal inhibitory concentrations with microdilution susceptibility test panels. *J. Clin. Microbiol.* **13:**228–230.

Bradford, P. A. 2001. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin. Microbiol. Rev.* **14:**933–951.

Jorgensen, J. H. 2003. Susceptibility test methods: dilution and disk diffusion methods, p. 1108–1127. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.

Jorgensen, J. H., and M. J. Ferraro. 2000. Antimicrobial susceptibility testing: special needs for fastidious organisms and difficult-to-detect resistance mechanisms. *Clin. Infect. Dis.* **30:**799–808.

APPENDIX 5.2–1

Quick reference list for performing broth microdilution MIC tests[a]

Organism (s)	Medium	Inoculum method	Incubation length (h)	Incubation atmosphere[b]	Notes
Enterobacteriaceae	CAMHB	DCS/LPG	16–20	Ambient air	
Pseudomonas aeruginosa and *Acinetobacter* spp.	CAMHB	DCS/LPG	16–20	Ambient air	
Other *Pseudomonas* spp.	CAMHB	DCS/LPG	16–20	Ambient air	
Haemophilus spp.	HTM broth	DCS	20–24	Ambient air	Perform beta-lactamase test.
Neisseria meningitidis[c]	CAMHB with 2–5% LHB	DCS	24	CO_2	Presumptive
Staphylococcus spp.	CAMHB; CAMHB with 2% NaCl for oxacillin	DCS	16–20; 24 (oxacillin[d] and vancomycin)	Ambient air	
Enterococcus spp.	CAMHB; BHI for GM 500 and STR 1,000	DCS/LPG	16–20; 24 (vancomycin and GM 500); 48 (STR 1,000)	Ambient air	Perform beta-lactamase and high-level amino-glycoside screen on blood and CSF isolates.
Streptococcus pneumoniae	CAMHB with 2–5% LHB	DCS	20–24	Ambient air (CO_2 if needed)	Determine penicillin (not oxacillin) and cefotaxime or ceftriaxone MIC when ≤19-mm oxacillin zones appear on the disk diffusion test.
Streptococcus spp.	CAMHB with 2–5% LHB	DCS	20–24	Ambient air (CO_2 if needed)	Determine penicillin MIC for viridans group streptococci from endocarditis patients.
Listeria[c]	CAMHB with 2–5% LHB	DCS	16–20	Ambient air	Presumptive
Others[c]	CAMHB or CAMHB with 2–5% LHB	DCS	16–24	Ambient air (CO_2 if needed)	Presumptive

[a] DCS, direct colony suspension standardized to a 0.5 McFarland turbidity standard; DCS/LPG, direct colony suspension or log-phase growth standardized to a 0.5 McFarland turbidity standard; HTM, *Haemophilus* test medium; LHB, lysed horse blood; GM, gentamicin; STR, streptomycin.

[b] All incubation temperatures are 35°C.

[c] Tests for these species not fully addressed by the NCCLS; "presumptive" indicates that at least parts of the procedure are nonstandardized.

[d] And other penicillinase-stable penicillins.

APPENDIX 5.2–2

Broth microdilution QC (*see* p. 5.2.16)

APPENDIX 5.2–3

Haemophilus spp.

PREANALYTICAL CONSIDERATIONS

A. SPECIMEN

Isolated colonies of similar colony morphology grown overnight on CHOC

B. SUPPLEMENTAL MATERIALS

1. CHOC
   Store at 2 to 8°C.
2. Prepared broth microdilution trays utilizing *Haemophilus* test medium (HTM)
   Store at −70°C.
3. Photometric standardizing device (e.g., bench top spectrophotometer) for 0.5 McFarland turbidity and test tubes that will fit this device

ANALYTICAL CONSIDERATIONS

C. QC STRAINS

1. *H. influenzae* ATCC 49247
2. *H. influenzae* ATCC 49766
3. *E. coli* ATCC 35218 (for beta-lactam–beta-lactamase inhibitor combinations)
4. Refer to NCCLS *Haemophilus* QC table for acceptable QC ranges (1).

D. PROCEDURE

1. Prepare inoculum by using a direct colony suspension in Mueller-Hinton broth or 0.9% NaCl from a fresh (18- to 24-h) CHOC plate.
2. Use a photometric device to adjust turbidity to match a 0.5 McFarland turbidity standard (1×10^8 to 2×10^8 CFU/ml). This step is recommended to avoid erroneous results due to under- or overinoculation.
3. Without delay, prepare intermediate dilution and inoculate the MIC tray and purity plate as previously described.
4. Incubate for 20 to 24 h at 35°C in ambient air.

E. READING RESULTS

Read MICs as described for nonfastidious organisms.

POSTANALYTICAL CONSIDERATIONS

F. REPORTING RESULTS

1. Refer to NCCLS *Haemophilus* interpretive table for MIC interpretation; refer to NCCLS *Haemophilus* QC table for QC ranges (1).
2. Only the susceptible interpretation is defined in NCCLS tables for several cephalosporins, carbapenems, aztreonam, macrolides, and fluoroquinolones because no (or very few) resistant strains have been reported to date. Confirm identification and susceptibility results for all isolates which are not susceptible to these agents, save isolate, and submit to a reference laboratory for testing using an NCCLS dilution reference method.

G. NOTES

1. Beta-lactamase-positive *Haemophilus* spp. are resistant to ampicillin and amoxicillin regardless of the MICs.
2. Occasional isolates are beta-lactamase negative and ampicillin resistant. Consider these resistant to amoxicillin-clavulanic acid, ampicillin-sulbactam, cefaclor, cefamandole, cefetamet, cefonicid, cefprozil, cefuroxime, loracarbef, and piperacillin-tazobactam despite in vitro results.
3. For life-threatening *Haemophilus* infections (e.g., meningitis, bacteremia), report only ampicillin, a third-generation cephalosporin, chloramphenicol, and meropenem.

Reference

1. **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.

APPENDIX 5.2–4

Streptococcus pneumoniae and *Streptococcus* spp.

PREANALYTICAL CONSIDERATIONS

A. SPECIMEN

Isolated colonies of similar colony morphology grown overnight on BAP

B. SUPPLEMENTAL MATERIALS

Prepared broth microdilution trays utilizing CAMHB supplemented with 2 to 5% lysed horse blood

Store at −70°C.

ANALYTICAL CONSIDERATIONS

C. QC STRAIN

1. *S. pneumoniae* ATCC 49619
2. Refer to NCCLS *S. pneumoniae* and *Streptococcus* QC table for acceptable QC ranges (1).

D. PROCEDURE

1. Prepare inoculum by using a direct colony suspension in Mueller-Hinton broth or 0.9% NaCl from fresh (16- to 18-h) growth on BAP.
2. Adjust turbidity visually to a 0.5 McFarland turbidity standard or use a photometric device.
3. Without delay, prepare intermediate dilution and inoculate MIC tray and purity plate as previously described.
4. Incubate for 20 to 24 h at 35°C in ambient air (CO_2 only if necessary).

E. READING RESULTS

1. Read MICs as described above for nonfastidious organisms.
2. Only the susceptible interpretation is defined for vancomycin and linezolid for all streptococci and for penicillin, ampicillin, and extended-spectrum cephalosporins for beta-hemolytic streptococci because resistance has not been observed. Confirm identification and susceptibility results for all isolates which are not susceptible to these agents, save isolate, and submit to a reference laboratory for testing using an NCCLS dilution reference method.

POSTANALYTICAL CONSIDERATIONS

F. REPORTING RESULTS

Refer to NCCLS *S. pneumoniae* and *Streptococcus* interpretive tables for MIC interpretation (1).

G. NOTES

1. *S. pneumoniae*
   a. Pneumococci may lyse rapidly in liquid (particularly water). Therefore, follow the inoculum preparation procedure described here precisely and inoculate the MIC tray immediately after standardization of the inoculum suspension.
   b. Penicillin-resistant *S. pneumoniae* does not produce beta-lactamase; do not use a beta-lactamase test to predict penicillin resistance.
2. Nutritionally variant streptococci (*Abiotrophia adiacens* and *Abiotrophia defectiva*)
   a. When supplementing with pyridoxal (for nutritionally variant streptococci), the final concentration should equal 0.001% or 1 μg/ml (pyridoxal stock, 1,000 μg/ml; add 0.2 ml to 9.8 ml of broth [20 μg/ml]; add 0.005 ml to each well).
   b. Wells in a panel with CAMHB can be converted to lysed horse blood wells by adding 0.005 ml of lysed horse blood (50%, vol/vol) to each well containing 0.1 ml of antimicrobial agent (final concentration is 2.5% lysed horse blood).

Reference

1. **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.

APPENDIX 5.2–5

Extended-Spectrum Beta-Lactamase Testing for *E. coli, Klebsiella pneumoniae,* and *Klebsiella oxytoca*

PREANALYTICAL CONSIDERATIONS

A. PRINCIPLE

ESBLs are derived from point mutations in the genes that code for common beta-lactamases such as TEM-1, TEM-2, or SHV-1. In contrast to TEM-1, TEM-2, and SHV-1, ESBLs hydrolyze or inactivate extended-spectrum cephalosporins, aztreonam, and expanded-spectrum penicillins. They do not hydrolyze carbapenems and generally do not hydrolyze cephamycins. ESBLs are blocked by beta-lactamase inhibitors such as clavulanic acid, and laboratory tests for detecting ESBL-producing bacteria are based on this property.

The MIC screening test for ESBL production in *E. coli* and *Klebsiella* spp. utilizes MIC interpretive criteria for certain third-generation cephalosporins and aztreonam that are different from those used for routine testing of *Enterobacteriaceae.* Once an isolate is deemed screen positive and therefore suspicious for ESBL production, a phenotypic confirmatory test is performed. The confirmatory test requires four MIC tests: (i) ceftazidime alone, (ii) cefotaxime alone, (iii) ceftazidime plus clavulanic acid, and (iv) cefotaxime plus clavulanic acid. If the MIC of either ceftazidime or cefotaxime (or both) in the presence of clavulanic acid is three or more twofold dilutions lower than the MIC of the respective agent alone, the test is considered positive for ESBL production.

B. SPECIMEN

Colonies as described in item II of procedure 5.2 for routine MIC testing of rapidly growing nonfastidious bacteria

C. SUPPLEMENTAL MATERIALS

MIC tests for the following agents at the concentrations specified

1. Ceftazidime, 0.25 to 128 μg/ml
2. Ceftazidime-clavulanic acid, 0.25/4 to 128/4 μg/ml
3. Cefotaxime, 0.25 to 64 μg/ml
4. Cefotaxime-clavulanic acid, 0.25/4 to 64/4 μg/ml

ANALYTICAL CONSIDERATIONS

D. QC STRAINS

1. *Klebsiella pneumoniae* ATCC 700603 (ESBL-producing strain)
2. *E. coli* ATCC 25922
3. Refer to current NCCLS QC and ESBL tables for acceptable QC ranges (1).

E. PROCEDURE

Follow standard MIC testing recommendations for inoculum preparation, inoculation, and incubation as indicated in item V of procedure 5.2.

1. Initial screen test
   a. Perform MIC tests with one or more of the following agents and include the concentration listed below. If the MIC exceeds that listed (positive screen test), proceed with the ESBL phenotypic confirmatory test.

Antimicrobial agent	MIC (μg/ml)
Cefpodoxime	4
Ceftazidime	1
Cefotaxime	1
Ceftriaxone	1
Aztreonam	1

   b. If the screen test is positive, suppress any susceptible results for penicillins, cephalosporins, or aztreonam from the patient report.
   c. If the screen test is negative, consider the isolate ESBL negative.

APPENDIX 5.2–5 *(continued)*

2. Phenotypic confirmatory test
   a. Perform four MIC tests to include the concentrations listed.
      (1) Ceftazidime, 0.25 to 128 μg/ml
      (2) Ceftazidime-clavulanic acid, 0.25/4 to 128/4 μg/ml
      (3) Cefotaxime, 0.25 to 64 μg/ml
      (4) Cefotaxime-clavulanic acid, 0.25/4 to 64/4 μg/ml
   b. Read MICs as described above for testing rapidly growing nonfastidious bacteria.

POSTANALYTICAL CONSIDERATIONS

F. REPORTING RESULTS

1. Positive for ESBL production: at least a three-twofold decrease in an MIC for either ceftazidime or cefotaxime tested in combination with clavulanic acid versus its MIC when tested alone
   a. Demonstration of enhanced activity (at least three-twofold decrease in an MIC) in the presence of clavulanic acid with either one or both pairs of agents is a positive result.
   b. Report all penicillins, cephalosporins (excluding the cephamycins such as cefoxitin or cefotetan), and aztreonam as resistant, even if the in vitro susceptibility test results are intermediate or susceptible.
2. Negative for ESBL production: less than a three-twofold decrease in an MIC for either antimicrobial agent tested in combination with clavulanic acid versus its MIC when tested alone

G. NOTES

1. At least 130 different types of ESBL enzymes have been described for *E. coli* and *Klebsiella* spp. Among the bacteria that produce these, a wide variety of susceptibility profiles may be noted with extended-spectrum beta-lactam agents, including the ESBL test screening agents. Use of more than one screening agent increases the likelihood of detecting the various types of ESBLs.
2. Extended-spectrum beta-lactam resistance in isolates that are screen test positive but confirmatory test negative is generally due to a mechanism other than ESBL production. ESBL screening tests have high specificity for *K. pneumoniae* but low specificity for *E. coli.* Many ESBL screen positive *E. coli* strains are resistant to extended-spectrum antimicrobial agents due to a resistance mechanism such as hyperproduction of AmpC beta-lactamase. As a screening agent for *E. coli,* cefpodoxime is the most sensitive but the least specific.
3. Generally, the hierarchy of activity of the screening agents in detecting ESBL production is cefpodoxime > ceftazidime > cefotaxime > ceftriaxone > aztreonam.
4. The genes for ESBL production are typically located on plasmids, and genes for resistance to other agents are often found on the same plasmid. Consequently, isolates that produce ESBLs often (but not always) demonstrate multiple resistance to other classes of antimicrobial agents (e.g., aminoglycosides, fluoroquinolones, or trimethoprim-sulfamethoxazole).
5. ESBLs do not hydrolyze carbapenems, and ESBL-producing *E. coli* and *Klebsiella* spp. remain susceptible to these agents. Carbapenems are considered the beta-lactams of choice for treating infections due to ESBL-producing bacteria.

H. LIMITATIONS

1. Some organisms with ESBLs may contain other beta-lactamases or other resistance mechanisms (e.g., porin alterations) that can mask ESBL production in the phenotypic confirmatory test, resulting in a false-negative result.
2. Other members of *Enterobacteriaceae* produce ESBLs. However, practical clinical laboratory methods for screening and detection of these isolates have not been determined as yet.

Reference

1. **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically,* 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.

APPENDIX 5.2–6

Breakpoint MIC Panels

I. PRINCIPLE

The breakpoint MIC test is a variation of the broth microdilution MIC test. Generally, one to three concentrations of each drug are tested, and these represent the categorical breakpoint concentrations specified in NCCLS documents. These selected concentrations allow interpretation of results as susceptible (S), intermediate (I), and resistant (R). When two concentrations are tested, growth at both indicates resistance, growth in the low but not the high concentration indicates intermediate results, and a lack of growth in either well indicates susceptibility. Sometimes a single high concentration is tested for urinary tract agents; growth indicates resistance and a lack of growth indicates susceptibility. Results are reported in qualitative terms; however, the bracketed MIC can also be included in the report.

The major advantage of breakpoint panels is that numerous drugs can be tested on a single panel. The extra wells are often used for biochemical tests so that identification and susceptibility tests can be performed at the same time. The primary disadvantage is that a precise MIC is not obtained.

The information provided in this appendix addresses the unique aspects of performing breakpoint MIC tests. The details that are identical to those described for full-range broth microdilution MIC tests have been omitted.

II. MATERIALS

Broth microdilution MIC trays usually contain 0.1 ml of drug per well. An example of a portion of an MIC breakpoint panel for gram-negative bacilli is shown in Appendix 5.2–7.

III. QUALITY CONTROL

A. Ideally, the QC strains for each drug tested should include the following.
   1. An isolate for which the MIC is right below the lowest concentration tested
   2. An isolate for which the MIC is right above the highest concentration tested

   ◩ Although such strains are often utilized by commercial manufacturers of breakpoint panels, this does not represent a practical approach for clinical laboratories.

B. Current recommendations for QC of breakpoint panels are the same as those for full-range MIC panels. Weekly QC is acceptable once a laboratory documents satisfactory daily QC testing.

IV. REPORTING RESULTS

A. Interpretation

Interpret MICs based on criteria specified by the NCCLS (1).

B. Reporting

Report interpretive category result with or without bracketed MIC result. It is inappropriate to report an MIC alone when three or fewer drug concentrations are tested.

Example: *Klebsiella pneumoniae*

Drug	MIC (μg/ml)	Interpretation
Ampicillin	>16.0	R
Cefazolin	≤8.0	S
Gentamicin	≤4.0	S
Piperacillin	32	I

V. LIMITATIONS

A. The QC method used for full-range MIC panels currently represents the only practical approach for clinical laboratories. However, because endpoints of these strains are often off the scale (lower than the lowest concentration tested or higher than the highest concentrations tested), the value of this approach is limited for breakpoint MIC panels.

B. Because the ability of current QC procedures to identify problems with breakpoint testing is limited, use extreme caution when performing breakpoint MIC testing.

C. Because only one to three drug concentrations are tested, skipped wells often go undetected.

APPENDIX 5.2–6 *(continued)*

Reference

1. **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.

APPENDIX 5.2–7

Example of a MIC breakpoint panel (*see* p. 5.2.17)

Broth Microdilution QC

LOT NO. _____

ANTIMICROBIAL	EXPECTED RANGE (µg/ml)	*											

* Date/Tech

ORGANISM _____

Example of a breakpoint MIC panel. Remaining wells may contain additional antimicrobial agents or biochemical tests. Including biochemical tests allows identification and antimicrobial susceptibility testing on a single tray. Abbreviations: AMP, ampicillin; PIPER, piperacillin; GENT, gentamicin; TOBRA, tobramycin; AMIK; amikacin; CEFAZ, cefazolin; CFTIZ, ceftizoxime; CFTAZ, ceftazidime; CFTRX, ceftriaxone; CIPRO, ciprofloxacin; IMIP, imipenem; AM-SUL, ampicillin-sulbactam; TM-SMZ, trimethoprim-sulfamethoxazole.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Neg growth control	Pos growth control										
B	AMP 8.0	AM-SUL 8.0/4.0	PIPER 16.0	GENT 4.0	TOBRA 4.0	AMIK 16.0	CEFAZ 8.0	CFTIZ 8.0	CFTAZ 8.0	CFTRX 8.0	CIPRO 1.0	IMIP 4.0
C	AMP 16.0	AM-SUL 16.0/8.0	PIPER 64.0	GENT 8.0	TOBRA 8.0	AMIK 32.0	CEFAZ 16.0	CFTIZ 16.0	CFTAZ 16.0	CFTRX 16.0	CIPRO 2.0	IMIP 8.0
D		TM-SMZ 2/38										
E												
F												
G												
H												

5.3 Beta-Lactamase Tests

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Beta-lactamases are enzymes produced by many clinically significant bacteria and are major mediators of bacterial resistance to beta-lactam agents. Routine beta-lactamase tests are based on visual detection of the end products of beta-lactamase hydrolysis, which is demonstrated with a colorimetric reaction. These tests primarily include the chromogenic cephalosporin method, the acidimetric method, and the iodometric method and are summarized in Appendix 5.3–1. Not every method is satisfactory for detecting beta-lactamase produced by all of the bacteria for which the test is useful.

II. SPECIMEN

A. Several colonies of similar colony morphology grown overnight (18 to 24 h) on nonselective medium (e.g., BAP or CHOC)

B. Some staphylococci may require induction (exposure to a beta-lactam agent) to increase production of enzyme to measurable levels.

1. Streak isolate onto a nonselective medium such as BAP and drop an oxacillin disk (1 μg) onto the area where the inoculum was streaked (it is not necessary to standardize the inoculum, as the goal is to obtain cells that have come in contact with beta-lactam molecules).
2. Incubate overnight.
3. Test growth from the periphery of the zone of inhibition around the disk.
4. Alternatively, grow the isolate in broth overnight containing a subinhibitory concentration of a beta-lactam agent (e.g., 0.25 μg of cefoxitin per ml), and perform the test on this suspension.

ANALYTICAL CONSIDERATIONS

III. QUALITY CONTROL

A. QC strains

1. *Staphylococcus aureus* ATCC 29213—positive
2. *Haemophilus influenzae* ATCC 10211—negative

B. Perform QC each day the test is performed and record results on QC form (*see* Appendix 5.3–2).

IV. CHROMOGENIC CEPHALOSPORIN METHOD

Include QC information on reagent container and in QC records.

A. Materials

1. Media and reagents

a. Cefinase disks (Becton Dickinson Microbiology Systems, Cockeysville, Md.; other products commercially available; follow manufacturer's instructions)
Store at 2 to 8°C.

b. Sterile distilled water
Store at 25°C.

2. Supplies

a. Glass slides or empty petri plates

b. Sterile Pasteur pipettes

c. Sterile wooden applicator sticks or inoculating loops

B. Procedure

1. Dispense the required number of disks onto a clean microscope slide or an empty petri plate.

2. Moisten each disk with 1 drop of sterile distilled water.

3. With a sterile loop or applicator stick, smear several colonies onto the disk surface.

4. Observe disk for color change. Positive results usually appear within 15 s to 5 min. If no color change occurs within 5 min, the test is negative. However, positive reactions for some staphylococci may take up to 1 h.

C. Reading reactions

1. Positive: yellow changes to red.

2. Negative: no change in color occurs.

V. ACIDIMETRIC METHOD

A. Disk or strip method

1. Materials

a. Media and reagents

(1) Acidimetric disk or strip (commercially available from several suppliers; store according to manufacturer's instructions)

(2) Sterile distilled water
Store at 25°C.

b. Supplies

(1) Glass slides or empty petri plates

(2) Sterile Pasteur pipettes

(3) Sterile wooden applicator sticks or inoculating loops

Include QC information on reagent container and in QC records.

2. Procedure

a. Dispense the required number of disks or strips onto a clean microscope slide or a sterile petri plate.

b. Moisten with 1 or 2 drops of sterile distilled water.

c. With a sterile loop or applicator stick, smear several colonies onto the disk or strip surface.

d. Observe for color change. A positive result will occur within 10 min. If no color change occurs within 10 min, the test is negative. However, positive reactions for some staphylococci may take up to 1 h. When dry, the color remains for up to 24 h.

3. Reading reactions

a. Positive: violet changes to yellow.

b. Negative: no change in color occurs.

V. ACIDIMETRIC METHOD *(continued)*

Include QC information on reagent container and in QC records.

B. Tube test

1. Materials

a. Media and reagents

(1) 0.5% Phenol red solution
Add 0.5 g of phenol red to 100 ml of water. Store at 25°C. Shelf life is 6 months. Heat may be needed to dissolve dye.

(2) Crystalline potassium penicillin G (vial containing 20 million U)
Store as indicated by manufacturer.

(3) 1 N NaOH
Add 4 g of NaOH crystals to 100 ml of water.
Caution: *This will cause heat production.*
Store at 25°C. Shelf life is 6 months.

(4) Substrate preparation

(a) Add 2 ml of the 5% phenol red solution to 16.6 ml of sterile distilled water. Mix.

(b) Add the phenol red-water solution (18.6 ml) to the vial of crystalline benzylpenicillin G.

(c) Remove solution from vial and place in sterile container.

(d) Add 1 N NaOH dropwise to this acidic solution until it develops a violet color (pH 8.5).

(e) Dispense in 0.1-ml aliquots into sterile tubes and freeze at −20°C or lower in *non*-frost-free freezer.

(f) Thaw tubes and use as needed. Typical shelf life is 6 months. If the solution turns yellow, the penicillin has deteriorated; discard the tubes.

b. Supplies

(1) Sterile 1- and 10-ml pipettes and pipette bulb

(2) Sterile polystyrene capped tubes (12 by 75 mm)

(3) Sterile wooden applicator sticks or inoculating loops

2. Procedure

a. Remove desired number of reagent tubes from freezer and allow to thaw at room temperature (one tube per organism).

b. With a sterile loop or applicator stick, add four or five colonies to the test solution to make an opaque, milky suspension.

c. Observe for color change. A positive reaction will occur in less than 15 min. If no color change occurs within 15 min, the test is negative. A color change after 15 min usually indicates deterioration of the substrate not related to the presence of beta-lactamase and should not be considered positive. Since positive reactions for some staphylococci may take up to 1 h, results that turn positive after 15 min may not be reliable for these bacteria.

3. Reading reactions

a. Positive: violet (red) changes to yellow.

b. Negative: no change in color occurs.

VI. IODOMETRIC METHOD

Include QC information on reagent container and in QC records.

A. Materials

1. Media and reagents

a. Penicillin (6,000 μg/ml) dissolved in phosphate buffer (pH 6.0, 0.05 to 1 M)
Store at 2 to 8°C. Shelf life is 24 h.

b. Starch reagent
Add 1 g of soluble starch to 100 ml of distilled water and heat in a boiling water bath until starch dissolves. Store at 2 to 8°C. Shelf life is 1 week.

VI. IODOMETRIC METHOD *(continued)*

c. Iodine reagent
Dissolve 2.03 g of iodine and 53.2 g of potassium iodide in a small volume of distilled water and q.s. to 100 ml. Store at 2 to 8°C in dark bottle; replace if precipitate is apparent. Shelf life is 2 months.

2. Supplies
- **a.** Empty sterile microdilution tray or small test tube
- **b.** Sterile 1.0-ml pipettes and pipette bulb
- **c.** Sterile wooden applicator sticks or inoculating loops

B. Procedure

1. Dispense 0.1 ml of the penicillin solution into a well of a microdilution tray (or a small test tube).
2. Add test organism to make an opaque, milky suspension.
3. Add 2 drops of the starch solution and mix.
4. Let sit at room temperature (approximately 25°C) for 30 to 60 min.
5. Add 1 drop of the iodine reagent.
6. Shake or stir the mixture for 1 min.
7. Observe for color change. Decolorization (to white) in less than 10 min indicates a positive reaction. If no color change occurs within 10 min, the test is negative. However, positive reactions for some staphylococci may take up to 1 h.

C. Reading reactions

1. Positive: fading of blue color to colorless
2. Negative: blue or purple color

POSTANALYTICAL CONSIDERATIONS

VII. RESULTS

A. Interpretation
Interpret the color reaction for the respective test as described above.

B. Reporting suggestion

1. *Haemophilus influenzae*—beta-lactamase positive (amoxicillin and ampicillin resistant)
2. *Haemophilus influenzae*—beta-lactamase negative

VIII. PROCEDURE NOTES

A. For some bacteria there is a direct correlation between a positive beta-lactamase reaction and resistance to specific beta-lactam drugs that might be prescribed for treatment of infections caused by them.
1. *H. influenzae:* amoxicillin, ampicillin
2. *Moraxella catarrhalis:* amoxicillin, ampicillin, penicillin
3. *Neisseria gonorrhoeae:* amoxicillin, ampicillin, penicillin
4. *Staphylococcus* spp.: amoxicillin, ampicillin, penicillin, carbenicillin, ticarcillin, mezlocillin, piperacillin

B. For organisms other than those listed above (with the exception of some anaerobes), beta-lactamase production cannot fully predict resistance to the various beta-lactam drugs that may be considered for use. Beta-lactamase testing as described here should not be used for these (e.g., *Enterobacteriaceae, Pseudomonas* spp.) in a clinical laboratory setting.

C. Beta-lactamase testing of staphylococci
1. Staphylococci may require induction to demonstrate a positive beta-lactamase reaction. A negative test should not be reported unless the test has been performed on cells that have been subjected to an inducing agent.
2. Microdilution MIC tests with penicillin may fail to detect penicillin-resistant (beta-lactamase-positive) staphylococci among some isolates that produce

VIII. PROCEDURE NOTES
(continued)

small amounts of beta-lactamase. An induced-beta-lactamase test is needed to confirm an isolate as penicillin susceptible (1, 2) (*see* item II).

3. Beta-lactam agents other than oxacillin and cefoxitin can be used as inducing agents providing it can be demonstrated that they perform satisfactorily.

4. Staphylococci may require up to 1 h to show a positive beta-lactamase test.

D. Production of beta-lactamase is not the only mechanism of beta-lactam resistance in the species commonly tested (although it is the most common mechanism). Consequently, a positive beta-lactamase test by definition indicates resistance to the penicillinase-labile penicillins. However, a negative test *does not* guarantee susceptibility to these agents, and a conventional susceptibility test must be performed.

E. The current NCCLS recommendations for routine testing of *M. catarrhalis* include performance of a beta-lactamase test only. This is because the incidence of resistance to other commonly prescribed agents is very low, as is the incidence of ampicillin, amoxicillin, and penicillin resistance due to a mechanism other than beta-lactamase production (1, 2).

REFERENCES

1. **NCCLS.** 2003. *Performance Standards for Antimicrobial Disk Susceptibility*, 8th ed. Approved standard M2-A8. NCCLS, Wayne, Pa.

2. **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.

SUPPLEMENTAL READING

Becton Dickinson Microbiology Systems. 1996. BBL paper disks for the detection of beta-lactamase enzymes. Product 88-0973-1. Becton Dickinson Microbiology Systems, Cockeysville, Md.

Livermore, D. M., and D. F. Brown. 2001. Detection of beta-lactamase-mediated resistance. *J. Antimicrob. Chemother.* **48:**59–64.

Swenson, J. M., J. A. Hindler, and J. H. Jorgensen. 2003. Special phenotypic methods for detecting antibacterial resistance, p. 1178–1195. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.

APPENDIX 5.3–1

Summary of beta-lactamase testing methods

Test detail(s)	Acidimetric	Nitrocefin (chromogenic cephalosporin)	Iodometric
Substrate	Citrate-buffered penicillin plus phenol red	Nitrocefin	Phosphate-buffered penicillin plus starch-iodine complex
Reaction	Penicilloic acid produces pH decrease.	Color change when beta-lactam ring opened	Penicilloic acid reduces iodine and prevents it from combining with starch.
Results	Positive: yellow Negative: violet (red)	Positive: red Negative: no color change	Positive: colorless Negative: blue/purple

Method(s) generally satisfactory for various organisms

Organism(s)	Testing method		
	Acidimetric	Nitrocefin	Iodometric
Haemophilus spp.	×	×	
Neisseria gonorrhoeae	×	×	×
Staphylococcus spp.	×	×	×
Moraxella catarrhalis		×	
Bacteroides spp.		×	

APPENDIX 5.3–2

Beta-Lactamase Test QC

Reagent _____ Year _____

Test Date	Tech	Manufacturer Lot #	Date Prep/ Rec'd	Exp. Date	*S. aureus* ATCC 29213 (+)	*H. influenzae* ATCC 10211 (–)	Comments/Corrective Action

5.4 Oxacillin Salt-Agar Screen Test To Detect Oxacillin (Methicillin)-Resistant *Staphylococcus aureus*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

A standard number of bacteria is inoculated onto Mueller-Hinton agar (MHA) containing 6 μg of oxacillin per ml and 4% NaCl. Following overnight incubation, the appearance of growth indicates that the *Staphylococcus aureus* isolate is resistant to oxacillin and other penicillinase-stable penicillins (methicillin, nafcillin, cloxacillin, and dicloxacillin) (1).

II. SPECIMEN

Prepare inoculum from four or five isolated colonies of similar colony morphology grown overnight (18 to 24 h) on nonselective medium (e.g., BAP or CHOC).

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Media and reagents

1. MHA with 4% NaCl and oxacillin (6 μg/ml)
   Store at 2 to 8°C.
2. Nutrient broth (e.g., Mueller-Hinton, TSB) or 0.9% saline (3.0- to 5.0-ml aliquots)
   Store at 2 to 30°C.

B. Supplies

1. Sterile cotton-tipped swabs
2. Sterile plastic pipettes
3. McFarland 0.5 turbidity standard

C. Equipment

1. Calibrated loop (1 μl [0.001 ml])
2. Vortex mixer
3. Ambient-air incubator (34 to 35°C)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. QC strains

1. *S. aureus* ATCC 29213—oxacillin susceptible
2. *S. aureus* ATCC 43300—oxacillin resistant

B. Perform QC with each test run and record results on QC form (Appendix 5.4–1).

V. PROCEDURE

A. Inoculum preparation

Using a loop or swab, transfer colonies to broth or saline to obtain an organism suspension that matches a McFarland 0.5 turbidity standard (1×10^8 to 2×10^8 CFU/ml); vortex thoroughly.

B. Inoculation and incubation

1. Method 1
   Using a 1-μl loop that has been dipped in the standardized suspension, spot inoculate onto the surface of the plate to cover an area that is 10 to 15 mm in diameter.

V. PROCEDURE *(continued)*

2. Method 2
   a. Dip a fresh sterile cotton swab into the standardized suspension and express any excess fluid against the side of the tube.
   b. Spot inoculate onto the surface of the plate over an area that is 10 to 15 mm in diameter or streak an entire quadrant of the plate.
3. Several organisms can be tested on each plate.
4. Allow the inoculum to be absorbed into the agar.
5. Invert plates and incubate at 35°C (no higher) in an ambient-air incubator.
6. Examine after overnight incubation. If any isolates are susceptible, reincubate the plate until a full 24 h of incubation has occurred and examine again.
7. Do not reuse plates.

C. Reading plates

Examine plates closely with transmitted light.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Interpretation
1. No growth or a single colony: oxacillin susceptible
2. >1 colony or a light film of growth: oxacillin resistant

B. For oxacillin-resistant *S. aureus*, report all beta-lactams as resistant regardless of in vitro results. These beta-lactams include all penicillins, all cephems, all carbapenems, and all beta-lactam–beta-lactamase inhibitor combination drugs.

VII. PROCEDURE NOTES

A. Procedure
1. Improper storage or exposure of oxacillin screen plates to higher temperatures may result in rapid deterioration of oxacillin, which is temperature labile.
2. Incubation at temperatures of >35°C may adversely affect detection of oxacillin-resistant *S. aureus.*
3. Plates must be examined very carefully using transmitted light. Although some resistant isolates may demonstrate confluent growth, isolates expressing a low frequency of resistance may appear as a few small colonies or fine haze of growth. Detection of growth may be enhanced using a dissection microscope or hand lens.
4. Since other organisms may grow on oxacillin screen plates, care must be taken to use pure cultures.
5. Resistant results can be reported after overnight incubation (16 to 20 h). Isolates susceptible after overnight incubation must be incubated for a full 24 h before final reading.

B. Other
1. The typical or intrinsically oxacillin (methicillin)-resistant *S. aureus* isolates possess the *mecA* gene, are associated with high oxacillin MICs (>16 μg/ml), and usually demonstrate resistance to multiple classes of antimicrobial agents, including clindamycin and erythromycin and sometimes chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, and the aminoglycosides. However, methicillin-resistant *S. aureus* (MRSA) organisms which are not multiresistant have been isolated from outpatients and inpatients.
2. Borderline oxacillin (methicillin)-resistant *S. aureus* organisms lack the *mecA* gene and are usually not multiply resistant, and oxacillin MICs for these are often at or just above the susceptible breakpoint (>2.0 μg/ml), in contrast to MICs for intrinsically resistant isolates (>16.0 μg/ml). Occasionally, these organisms may grow on the oxacillin salt-agar screen plate. The

VII. PROCEDURE NOTES
(continued)

clinical significance of borderline resistant isolates has not been established, but some have suggested that infections caused by these are treatable with beta-lactam agents. Borderline resistant isolates are infrequently encountered.

3. Because the oxacillin screen plate method is reliable (and easy and inexpensive), it can be used to establish and evaluate the accuracy of other systems that have on occasion been shown to be less reliable in detecting MRSA (e.g., some of the rapid automated methods).

4. Tests for the *mecA* gene or for PBP2a, the gene product, are available and can be used for rapid detection of MRSA. They are also sometimes useful for definitive identification when borderline resistant results are encountered. There is currently a commercial latex agglutination test for PBP2a (2).

VIII. LIMITATIONS

The oxacillin salt-agar screen plate detects intrinsically oxacillin (methicillin)-resistant *S. aureus* but usually does not detect borderline oxacillin (methicillin)-resistant *S. aureus.*

REFERENCES

1. **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 6th ed. Approved standard. M7-A6. NCCLS, Wayne, Pa.
2. **Swenson, J. M., P. P. Williams, G. Killgore, C. M. O'Hara, and F. C. Tenover.** 2001. Performance of eight methods, including two new rapid methods, for detection of oxacillin resistance in a challenge set of *Staphylococcus aureus* organisms. *J. Clin. Microbiol.* **39:**3785–3788.

SUPPLEMENTAL READING

Jorgensen, J. H. 2003. Susceptibility test methods: dilution and disk diffusion methods, p. 1108–1127. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.

Swenson, J. M., J. A. Hindler, and J. H. Jorgensen. 2003. Special phenotypic methods for detecting antimicrobial resistance, p. 1178–1195. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.

Swenson, J. M., J. Spargo, F. C. Tenover, and M. J. Ferraro. 2001. Optimal inoculation methods and quality control for the NCCLS oxacillin agar screen test for detection of oxacillin resistance in *Staphylococcus aureus. J. Clin. Microbiol.* **39:**3781–3784.

APPENDIX 5.4–1

Staphylococcus aureus
Oxacillin Agar Screen Test QC

Year _____

Test Date	Tech	Manufacturer Lot #	Date Prep/ Rec'd	Exp. Date	*S. aureus* ATCC 29213 ≤1 Colony	*S. aureus* ATCC 43300 >1 Colony	Comments/Corrective Action

5.5 Screen Tests To Detect High-Level Aminoglycoside Resistance in *Enterococcus* spp.

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

High-level aminoglycoside resistance (HLAR) in enterococci is most commonly detected by assessing growth at high concentations of gentamicin (500 μg/ml) and sometimes streptomycin (1,000 μg/ml in broth and 2,000 μg/ml in agar) (1). Strains that show HLAR to gentamicin will not be synergistically killed with combinations of cell wall-active drugs (generally ampicillin, penicillin, or vancomycin) plus gentamicin. HLAR to gentamicin also means HLAR to tobramycin, netilmicin, amikacin, and kanamycin. HLAR to streptomycin equals resistance to combinations of cell wall-active drugs and streptomycin. See additional information in Appendix 5.5–1. The agar screen test is described in detail here, and reference is made to the disk diffusion and the broth microdilution MIC test procedures for detecting HLAR.

II. SPECIMEN

Prepare inoculum from four or five isolated colonies of similar colony morphology.

1. Log-phase or stationary-phase growth inoculum
   Use colonies grown for 1 or 2 days on nonselective (e.g., BAP) or selective medium.
2. Direct colony suspension inoculum
   Use colonies grown overnight on nonselective medium (e.g., BAP).
3. Subculture stock, frozen, or lyophilized isolates two times prior to testing.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Media and reagents

1. BHI agar screen plates prepared in quadrant plates for convenience
   - **a.** Quadrant 1, BHI agar control
   - **b.** Quadrant 2, 500 μg of gentamicin per ml
   - **c.** Quadrant 3, 2,000 μg of streptomycin per ml

   Store at 2 to 8°C.
2. Nutrient broth (e.g., Mueller-Hinton, TSB) or 0.9% NaCl (3.0 to 5.0-ml aliquots)
   Store at 2 to 30°C.

B. Supplies

1. Sterile cotton-tipped swabs
2. Sterile plastic pipettes
3. 0.5 McFarland turbidity standard

C. Equipment

1. Calibrated loop (10 μl [0.01 ml]) or 10-μl micropipette with sterile tips
2. Vortex mixer
3. Ambient-air incubator (34 to 35°C)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. QC strains
- **1.** *Enterococcus faecalis* ATCC 29212—susceptible to high concentrations of gentamicin and streptomycin
- **2.** *E. faecalis* ATCC 51299—resistant to high concentrations of gentamicin and streptomycin

B. QC can be performed weekly if proficiency in performing daily QC has been documented (*see* procedure 5.2) and if the laboratory performs this test routinely. Always perform QC when new reagents are put into use.

C. Record all results on QC worksheet (Appendix 5.5–2).

V. PROCEDURE

A. Inoculum preparation
- **1.** Direct colony suspension method
  Pick several colonies from a fresh (18- to 24-h) nonselective agar plate to broth or 0.9% NaCl.
- **2.** Log phase method
  - **a.** Pick four or five isolated colonies to 3.0 to 5.0 ml of broth.
  - **b.** Incubate at 35°C for 2 to 8 h until growth reaches the turbidity at or above that of a 0.5 McFarland standard.
- **3.** For either the log phase or direct colony suspension method, vortex well and adjust turbidity visually with sterile broth or 0.9% NaCl to match a 0.5 McFarland standard (1×10^8 to 2×10^8 CFU/ml). Alternatively, standardize suspension with a photometric device.

B. Inoculation and incubation
- **1.** Use a 10-μl micropipette with a sterile tip or calibrated loop to spot the inoculum onto the agar surface of each quadrant. The final concentration should be approximately 10^6 CFU.
- **2.** Allow the inoculum to be absorbed into the agar.
- **3.** Invert plates and incubate at 35°C in an ambient-air incubator for 24 h; if there is no growth at 24 h for streptomycin, reincubate streptomycin tests for an additional 24 h (total of 48 h).

C. Reading plates
- **1.** Examine the control quadrant (no drug) for adequate growth.
- **2.** Examine drug quadrants for presence of growth (>1 colony indicates resistance).

POSTANALYTICAL CONSIDERATIONS

VI. RESULTS

A. Interpretation
- **1.** No growth (or one colony): no high-level resistance
- **2.** Growth at gentamicin (500 μg/ml): high-level gentamicin resistance (also resistance to tobramycin, amikacin, netilmicin, and kanamycin)
- **3.** Growth on streptomycin (2,000 μg ml): high-level streptomycin resistance

B. Reporting suggestion
Add comment to report: "Serious enterococcal infections require combination therapy with ampicillin, penicillin, or vancomycin plus an aminoglycoside. Bactericidal synergy occurs only when the bacterium under scrutiny is susceptible to both drugs."

VII. PROCEDURE NOTES

A. Agar method

1. Uninterpretable results may result from improper inoculation or the inability of the organism to grow on the particular agar base medium. The test should be repeated before reporting as uninterpretable.
2. Some isolates with streptomycin HLAR may not demonstrate resistance until after 48 h of incubation.
3. Most *Enterococcus faecium* organisms produce an aminoglycoside-modifying enzyme (6′-acetyltransferase) that makes them inherently resistant to amikacin, kanamycin, netilmicin, and tobramycin; this may not be expressed as HLAR (e.g., MIC may be ≤2,000 μg ml), but synergy will not occur with these agents.
4. Standard petri plates may be used as an alternative to quadrant petri plates. Three separate plates including one containing BHI, one containing gentamicin at 500 μg/ml, and one containing streptomycin at 2,000 μg/ml are required.

B. Other methods

1. HLAR can also be determined using the standard disk diffusion method with special high-content disks (120 μg of gentamicin and 300 μg of streptomycin). Zones of 6 mm (no zone) indicate HLAR. Zones of 7 to 9 mm are inconclusive and require that isolates be retested by an agar dilution or broth dilution HLAR screen method. Zones of ≥10 mm indicate no HLAR (2).
2. HLAR can also be determined using the standard broth dilution MIC method by testing 500 μg of gentamicin and 1,000 μg of streptomycin per ml diluted in BHI broth (1). Growth is indicative of high-level resistance.
3. To detect HLAR to amikacin, use 2,000 μg of kanamycin per ml for the agar screen or broth dilution method.
4. Since gentamicin is the most widely used aminoglycoside (in combination with a cell wall-active drug) for treatment of serious enterococcal infections, an HLAR screen for gentamicin is often sufficient. However, if the isolate demonstrates high-level gentamicin resistance, screening for high-level streptomycin resistance is needed to identify those strains that might be high-level gentamicin resistant but not high-level streptomycin resistant. In this case, streptomycin could be used therapeutically.
5. Dextrose phosphate agar and broth have been shown to perform comparably to BHI in broth and agar screen tests.

VIII. LIMITATIONS

Occasional isolates that lack HLAR to gentamicin and streptomycin but show HLAR to amikacin and kanamycin may occur. This resistance would be detected only with a kanamycin screen test, which is generally not available in clinical laboratories and currently not addressed by the NCCLS.

REFERENCES

1. **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.
2. **NCCLS.** 2003. *Performance Standards for Antimicrobial Disk Susceptibility Tests*, 8th ed. Approved standard M2-A8. NCCLS, Wayne, Pa.

SUPPLEMENTAL READING

Chow, J. W. 2000. Aminoglycoside resistance in enterococci. *Clin. Infect. Dis.* **31:**586–589.

Swenson, J. M., J. A. Hindler, and J. H. Jorgensen. 2003. Special phenotypic methods for detecting antibacterial resistance, p. 1178–1195. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.

APPENDIX 5.5–1

Aminoglycoside-modifying enzymes that confer HLAR on *Enterococcus* spp.

Enzyme	Inactivation[a]				
	Gentamicin	Streptomycin	Tobramycin	Amikacin/kanamycin	Netilmicin
6′-Adenylyltransferase [AAD (6′)]	N	Y	N	N	N
3′-Phosphotransferase [APH (3′)]	N	N	N	Y	N
2″-Phosphotransferase/6′-acetyltransferase [APH (2″)/AAC (6′)]	Y	N	Y	Y	Y
6′-Acetyltransferase [AAC (6′)][b]	N	N	Y	Y	Y

[a] Y, modifies the activity of the respective aminoglycoside; no synergy achievable with a cell wall-active agent. N, does not modify the activity of the respective aminoglycoside; synergy achievable.

[b] Found in virtually all *E. faecium* organisms.

APPENDIX 5.5–2

***Enterococcus* HLAR Agar Screen Test QC**

Year _____

Test Date	Tech	Manufacturer Lot #	Date Prep/ Rec'd	Exp. Date	*E. faecalis* ATCC 29212		*E. faecalis* ATCC 51299		Comments/Corrective Action
					GM 500 (–)	STR 2000 (–)	GM 500 (+)	STR 2000 (+)	

5.6 Agar Screen Test To Detect Vancomycin Resistance in *Enterococcus* spp.

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The vancomycin agar screen is used to detect vancomycin-resistant enterococcal colonies that have been isolated from clinical or surveillance cultures. A standard number of bacteria is inoculated onto BHI agar containing 6 μg of vancomycin per ml. Following incubation, the appearance of growth indicates that the enterococcal isolate is likely to be resistant to vancomycin. MIC and species identification tests are subsequently required to determine if the isolate is a true vancomycin-resistant enterococcus (VRE) (1, 2).

II. SPECIMEN

Prepare inocula from four or five isolated colonies of similar colony morphology grown overnight (18 to 24 h) on agar medium (e.g., BAP, CAP, or enterococcal surveillance agar).

III. MATERIALS

A. Media and reagents

1. BHI agar plates with 6 μg of vancomycin per ml
Store at 2 to 8°C.

2. Nutrient broth (e.g., Mueller-Hinton, TSB) or 0.9% NaCl (3.0- to 5.0-ml aliquots)
Store at 2 to 8°C.

B. Supplies

1. Sterile cotton-tipped swabs

2. Sterile plastic pipettes

3. McFarland 0.5 turbidity standard

C. Equipment

1. 1.0-μl (0.001 ml) or 10-μl (0.01 ml) calibrated loop

2. Vortex mixer

3. 34 to 35°C ambient-air incubator

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. QC strains

1. *Enterococcus faecalis* ATCC 29212—vancomycin susceptible

2. *E. faecalis* ATCC 51299—vancomycin resistant

B. Frequency of testing QC strains

1. Weekly
If the vancomycin screen test is performed at least once a week and criteria for converting from daily to weekly testing have been met (*see* procedure 5.2)

2. Daily
If vancomycin screen test is performed less frequently than once a week

C. Record all results on QC worksheet (Appendix 5.6–1).

V. PROCEDURE

A. Inoculum preparation

Using a loop or swab, transfer colonies to a nutrient broth or saline to obtain an organism suspension that matches a McFarland 0.5 turbidity standard (1.0 $\times$ 10^8 to 2 $\times$ 10^8 CFU/ml); vortex thoroughly.

B. Inoculation and incubation

1. Using a 1- or 10-μl calibrated loop that has been dipped in the inoculum suspension, inoculate a section of the agar plate surface in a single streak (test up to six isolates/plate).
2. Allow the inoculum to be absorbed into the agar.
3. Invert plates and incubate at 35°C in an ambient-air incubator.
4. Examine after overnight incubation. If any isolates are susceptible, reincubate the plate until a full 24 h of incubation has occurred and examine again.

C. Reading plates

Examine plate for presence of growth (>1 colony indicates presumptive resistance).

D. Supplemental testing if the vancomycin screen plate demonstrates growth

1. Perform a vancomycin MIC test.
2. Perform tests for motility and pigment production to distinguish species with acquired resistance (VanA or VanB) from those with intrinsic, low-level resistance to vancomycin (VanC) such as *Enterococcus gallinarum* or *Enterococcus casseliflavus.* (*See* procedure 3.18.1 for procedures for species identification of enterococci.)

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Interpretation

1. No growth or 1 colony: vancomycin susceptible
2. Growth of >1 colony: presumptively vancomycin resistant

B. Reporting

Report presumptive results.

Example: Presumptive VRE based on screen test; confirmatory tests pending.

VII. PROCEDURE NOTES

A. The three common phenotypes of VRE are as follows.

1. VanA or high-level resistance, with vancomycin MICs of ≥64 μg/ml, and resistance to teicoplanin (MICs of ≥16 μg/ml)
2. VanB or low- to high-level resistance, with vancomycin MICs of 16 to 64 μg/ml, and usually without teicoplanin resistance
3. VanC or intrinsic low-level resistance, with vancomycin MICs of 4 to 32 μg/ml, without teicoplanin resistance and typically associated with *E. gallinarum* and *E. casseliflavus*

B. On occasion, *E. faecalis* ATCC 29212 may show slight growth on vancomycin screen plates.

C. The medium described here is to be used for testing enterococci that have been isolated in culture and not as a primary plating medium for surveillance specimens (e.g., rectal swabs).

D. Acquired vancomycin resistance (VanA or VanB) is most common in *Enterococcus faecium,* although it has been noted in *E. faecalis.* The epidemiologic significance of isolates with acquired vancomycin resistance is great, and patients colonized or infected with VRE require strict infection control precautions.

VII. PROCEDURE NOTES *(continued)*

E. Low-level intrinsic vancomycin resistance (VanC) found in *E. gallinarum* and *E. casseliflavus* has not been shown to be of epidemiologic significance. Therefore, these are not reported as VRE for infection control purposes.

F. The vancomycin screen plate as described here has been used to screen for vancomycin-intermediate *Staphylococcus aureus* (VISA). Any growth should be considered suspicious for VISA or vancomycin-resistant *S. aureus* (VRSA). For this purpose, include QC strains *E. faecalis* ATCC 51299 (resistant control) and *S. aureus* ATCC 29213 (susceptible control) (3).

VIII. LIMITATIONS

A. The vancomycin screen plate does not determine the level of vancomycin resistance or the vancomycin phenotype.

B. Vancomycin-resistant bacteria other than enterococci (e.g., gram-negative bacteria, *Leuconostoc* spp., *Lactobacillus* spp., *Pediococcus* spp., *Erysipelothrix rhusiopathiae*) may grow on the screen plates. Make certain that the test organism is *Enterococcus* spp. prior to testing.

REFERENCES

1. **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.
2. **Swenson, J. M., J. F. Hindler, and J. H. Jorgensen.** 2003. Special phenotypic methods for detecting antimicrobial resistance, p. 1178–1195. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
3. **Tenover, F. C., M. V. Lancaster, B. C. Hill, C. D. Steward, S. A. Stocker, G. A. Hancock, C. M. O'Hara, S. K. McAllister, N. C. Clark, and K. Hiramatsu.** 1998. Characterization of staphylococci with reduced susceptibilities to vancomycin and other glycopeptides. *J. Clin. Microbiol.* **36:**1020–1027.

SUPPLEMENTAL READING

Cetinkaya, Y., P. Falk, and C. G. Mayhall. 2000. Vancomycin-resistant enterococci. *Clin. Microbiol. Rev.* **13:**686–707.

APPENDIX 5.6–1

Enterococcus **Vancomycin Agar Screen Test QC**

Year _____

Test Date	Tech	Manufacturer Lot #	Date Prep/ Rec'd	Exp. Date	*E. faecalis* ATCC 29212 ≤1 Colony	*E. faecalis* ATCC 51299 >1 Colony	Comments/Corrective Action

5.7 Broth Microdilution MIC Test for Anaerobic Bacteria

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The broth microdilution MIC method can be used to measure (semiquantitatively) the in vitro activity of an antimicrobial agent against an anaerobic bacterial isolate. This procedure is very similar to the broth microdilution method used for aerobic bacteria. Various concentrations of antimicrobial agents are dispensed into a multiwell plastic tray and inoculated with a test isolate. After 48 h of incubation at 35°C in an anaerobic environment, the MIC is determined by observing the lowest concentration of an antimicrobial agent which will inhibit visible growth of the test bacterium. MICs obtained are interpreted as susceptible, intermediate, or resistant, based on criteria defined by the NCCLS (1).

The anaerobic broth microdilution MIC method is described in detail in the NCCLS M11 standard (1). The recommended medium is supplemented brucella broth (hemin, vitamin K_1, and 5% lysed horse blood). Anaerobic bacteria that grow poorly in supplemented brucella broth will not produce satisfactory results with this method and must be tested by an alternative method such as agar dilution. To date, the NCCLS method has been validated only for testing the *Bacteroides fragilis* group species.

II. SPECIMEN

Prepare inoculum directly from four or five isolated colonies of similar colony morphology grown anaerobically for 24 to 48 h on an anaerobic brucella blood agar plate.

A. Use 24-h growth for rapid growers (e.g., *B. fragilis*), if sufficient growth is available; 48-h growth is required for most other organisms.
B. Subculture stock, frozen, or lyophilized isolates two times prior to testing.
C. Do not expose agar plates containing test isolates to air for longer than 20 to 30 min.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Media and reagents
See Appendix 5.7–1 for additional information.

1. Prepared broth microdilution panels containing antimicrobial agents diluted in brucella broth supplemented with (final concentrations) vitamin K_1 (1 µg/ml), hemin (5 µg/ml), and lysed horse blood (5%) in volumes of 0.1 ml of solution per well. Store at ≤ -60°C. See Appendix 5.7–2.
2. Brucella broth, supplemented with vitamin K_1, hemin, and 5% lysed horse blood dispensed in aliquots of 5 ml. Store at 2 to 8°C.
3. THIO, supplemented with vitamin K_1, hemin, and either a marble chip or sodium bicarbonate, dispensed in aliquots of 5 ml. Store at ambient temperature, protected from light.
4. Sterile 0.9% NaCl dispensed in aliquots of 28 ml in screw-cap tubes. Store at 2 to 8°C.
5. Sterile 0.9% NaCl dispensed in aliquots of 10 ml in screw-cap tubes. Store at 2 to 8°C.
6. Brucella agar supplemented with vitamin K_1, hemin, and sheep blood. Store at 2 to 8°C.
7. BAP. Store at 2 to 8°C.

III. MATERIALS *(continued)*

B. Supplies

1. Sterile cotton-tipped swabs
2. Sterile plastic pipettes
3. 0.5 McFarland turbidity standard
4. Sterile, disposable, plastic multipronged inoculator sets (include inoculum reservoir) or other inoculating device
5. Plastic bags
6. Microdilution tray lids or empty microdilution trays

C. Equipment

1. Vortex mixer
2. Adjustable micropipette and sterile pipette tips
3. 35°C anaerobic atmosphere (in container large enough to hold panels) containing 5 to 10% H_2, 5 to 10% CO_2, and 80 to 90% N_2 (e.g., anaerobe chamber, anaerobe pouch, or anaerobic jar)
4. Viewing device to read inoculated MIC panels after incubation
5. Freezer (≤ -60°C; sometimes a -20°C non-frost-free freezer is acceptable)
6. 35°C ambient-air incubator (CO_2)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. QC strains

1. *B. fragilis* ATCC 25285
2. *Bacteroides thetaiotaomicron* ATCC 29741
3. *Eubacterium lentum* ATCC 43055

B. Monitoring accuracy

1. Test QC isolates by following routine procedures, and record results on a QC log sheet. Record lot numbers and expiration dates of microdilution panels. *See* Appendix 5.7–3.
2. Compare with expected results listed in the most recent NCCLS standards tables (1). Note any out-of-control results and document; proceed with corrective action if necessary.

C. Testing recommendations

1. Test all three QC strains with each new lot or each new shipment of panels.
2. Test all three QC strains with each run. However, if only a few panels are to be set on patients' isolates, it is acceptable to test one QC strain with the run. Select the strain that gives the greatest number of on scale endpoints for the drugs on the panel.

D. Additional controls

1. Growth control well must demonstrate good growth; anaerobic purity plate must show good growth and be free of contaminating organisms.
2. Sterility control well and aerobic purity plate must be free of any growth.
3. Inoculum count verification plate should show approximately 50 to 200 colonies/plate.

V. PROCEDURE

A. Organizational considerations

Inoculum suspensions and the inoculation of the panels can be done outside of the anaerobic chamber. However, the time between isolate exposure to air and placement of the inoculated trays in an anaerobic environment should not exceed 30 min. When testing 10 or more isolates at one time, remove isolates from the anaerobic chamber in sets of 10 in order to minimize exposure of the isolates to oxygen during the procedure.

B. Allow frozen trays to thaw at room temperature (this can be done outside of the chamber).

V. PROCEDURE *(continued)*

C. Inoculum preparation

1. Using a loop or swab, transfer colonies as follows.

a. Growth method

(1) Pick colonies from a supplemented brucella blood agar plate 24 to 48 h old to THIO or supplemented brucella broth. (Prior to using THIO, prereduce by boiling for 5 min and then cool.)

(2) Incubate at 35°C under anaerobic conditions for 4 to 6 h (but no longer than 24 h) or until the turbidity is equivalent to that of a 0.5 McFarland turbidity standard (1.5×10^8 CFU/ml). Ambient-air incubation is acceptable when using prereduced THIO. If turbidity is not reached by 24 h, use the direct colony suspension method.

b. Direct colony suspension method

(1) Pick colonies from a supplemented brucella blood agar plate 24 to 48 h old and suspend in brucella broth.

(2) Gently invert the tube or vortex to obtain a homogeneous suspension.

(3) Adjust the turbidity to match that of a 0.5 McFarland standard, which has been vortexed.

2. Prepare inoculum suspensions no more than 15 min before inoculation of panels.

3. For the intermediate dilution, calculate the volume of standardized suspension to be added to a 28-ml aliquot of 0.85% NaCl to obtain a final organism concentration of 10^5 CFU in each 0.1 ml of antimicrobial solution per well (10^6 CFU/ml).

Example: The following applies when using disposable plastic inoculator with prongs that deliver 0.01 ml per well.

a. Prepare suspension equivalent to a 0.5 McFarland standard.

b. Add 2.0 ml of 0.5 McFarland-standardized suspension to 28 ml of prereduced saline (1:15 dilution = 10^7 CFU/ml). Mix tubes gently by inversion or vortexing.

c. Prongs deliver 0.01 ml into 0.1 ml of drug solution (1:10 dilution).

4. The final organism concentration is 1×10^6 CFU/ml, compared to 5×10^5 CFU/ml for aerobes when using broth microdilution.

D. Inoculation and incubation

1. Within 15 min of preparing the inoculum, inoculate the MIC tray.

2. Use sterile disposable plastic inoculators that deliver 0.01 ml.

a. Remove inoculator-reservoir set from its plastic packaging.

b. Pour diluted inoculum suspension into inoculum tray reservoir.

c. Dip prongs into the inoculum suspension.

(1) Orient the sterility well so that it does not get inoculated.

(2) Press down on the inoculator firmly to ensure that all prongs have come in contact with the inoculum in the reservoir tray.

(3) Carefully remove prongs from inoculum.

d. Inoculate the MIC tray by dipping the filled prongs carefully into the MIC tray. Press down on the inoculator firmly to ensure that all prongs have come in contact with the antimicrobial solutions in the tray.

e. Discard the prongs as biohazardous waste.

f. Prepare purity plates by subculturing separate 0.001 ml of inoculum from the reservoir onto an anaerobic BAP and an aerobic BAP. Discard liquid and reservoir tray as biohazardous waste.

3. Cover panels with empty microdilution tray or tray lid.

4. Incubate inoculated MIC panels and anaerobic purity plates at 35°C under anaerobic conditions for 48 h. Include an indicator of anaerobiasis.

a. Incubate in stacks of four trays or fewer, with an empty tray or lid on top of the stack.

V. PROCEDURE *(continued)*

b. If incubating panels in an anaerobic chamber and there is a tendency for the trays to dry out during incubation, place trays into plastic bags containing wet paper towels under the trays. Do not seal bag in order to maintain an anaerobic environment. Panels can be stacked four trays high with a cover tray on top if a large enough bag is used.

5. Incubate aerobic purity plate at 35°C in CO_2 for 24 to 48 h.

E. Reading MICs

1. After 46 to 48 h of incubation, examine purity plates. If a mixed culture is apparent, repeat the test.

2. Wipe off the bottom of the MIC tray with a lint-free tissue to remove any condensation or debris.

3. Place tray on appropriate reading device.

4. Examine growth control well for adequate growth.

a. Growth control well should demonstrate turbidity or a distinct button in the bottom of the well. The size of the button will depend on the species tested. Organisms that grow as small colonies will generally yield a small button.

b. If the results of the growth control are in question because of a pinpoint button, slight turbidity, or a faint haze, repeat the test.

5. Examine uninoculated sterility control well. Compare questionable growth with this well when necessary.

6. The MIC is the lowest concentration of antimicrobial agent showing complete inhibition of visible growth.

a. Some isolates may exhibit a trailing phenomenon, which appears as a gradual diminishing in the amount of growth at successively higher concentrations of the antimicrobial agent; this makes the endpoint difficult to determine. Read the MIC as the lowest concentration at which the most significant reduction of growth is observed.

b. NCCLS document M11-A5 contains photographic examples of endpoints (1).

7. Record results.

F. Inoculum count verification plates

1. Colony counts should be performed periodically to verify the inoculum concentration. The frequency of testing is up to the individual laboratory and should be based on the frequency of use of the test method. For laboratories that infrequently perform this procedure, colony counts should be done every time the test is performed. For laboratories that perform this procedure more frequently (e.g., at least once a week), colony counts can be performed monthly.

2. Remove 10 µl from the positive control well immediately after inoculation and add it to 10 ml of 0.9% saline (1:1,000 dilution). Mix by at least three gentle inversions.

3. Plate 0.1 ml onto a brucella blood agar plate (or other medium that adequately supports the growth of the organism being tested).

4. Using a loop or bent glass tube ("hockey stick"), thoroughly spread the inoculum over the surface of the plate by streaking in several directions.

5. Incubate under anaerobic conditions for 48 h.

6. The plate count should be approximately 10^2 organisms (50 to 200 colonies per plate).

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Interpretation

Interpret MIC based on criteria specified by the NCCLS (1).

B. Reporting

Report the MIC along with its categorical interpretation: susceptible (S), intermediate (I), or resistant (R).

Example: *B. fragilis*

Drug	MIC (μg/ml)	Interpretation
Ampicillin-sulbactam	8/4	S
Cefoxitin	8	S
Clindamycin	4	I
Metronidazole	1	S
Piperacillin-tazobactam	0.25	S

C. Important reporting rules

The majority of *B. fragilis* group isolates are beta-lactamase producers; therefore, these isolates do not require beta-lactamase testing and should always be reported as resistant to penicillin and ampicillin regardless of the MIC (1).

VII. PROCEDURE NOTES

A. See aerobic broth microdilution procedure (procedure 5.2) for additional record-keeping tips.

B. CHOC can be used in place of BAP for aerobic purity plates.

C. Occasionally, the skipping phenomenon occurs. Skipped wells are evidenced by growth at higher concentrations of an antimicrobial agent and a lack of growth at one or more of the lower concentrations. Evaluate each skipped well individually when determining if it is necessary to repeat MIC testing. In general, if more than two consecutive wells with no growth are followed by additional wells with growth, the results are questionable and the test should be repeated. See procedure 5.2 for a detailed discussion of this subject.

D. Most anaerobic isolates can be stored at $\leq -60°C$ in 20% glycerol or 20% sterile skim milk.

VIII. LIMITATIONS

A. On rare occasions, clinical isolates of *B. fragilis* group organisms may not grow within 48 h in brucella broth supplemented with vitamin K_1, hemin, and 5% lysed horse blood. If no growth occurs after repeat testing, the agar dilution method should be used.

B. As with any procedure, experience is important. Those who perform broth microdilution susceptibility tests on anaerobes should have experience in anaerobic bacteriology. Laboratories with few requests for susceptibility tests should consider sending them to a referral laboratory with experience in anaerobic susceptibility testing.

C. This method has been validated by the NCCLS only for testing members of the *B. fragilis* group.

D. Because many infections involving anaerobes are polymicrobial and successful treatment often involves a combination of surgical intervention and the use of empirical broad-spectrum, antimicrobial therapy, the relative importance of the susceptibility of a single organism to predict a favorable clinical outcome is difficult to determine.

E. Anaerobic organisms differ markedly in size and shape; therefore, a standardization of inocula by using a 0.5 McFarland standard may not yield 1.5×10^8 CFU/ml for all organisms.

VIII. LIMITATIONS *(continued)*

F. Beta-lactamase testing with a chromogenic cephalosporin-based method can be performed with anaerobic species. A beta-lactamase-producing isolate should be reported as resistant to penicillin and ampicillin, regardless of the MIC. Some anaerobic species are known to be resistant to beta-lactam antimicrobial agents by mechanisms other than beta-lactamases. Therefore, a negative beta-lactamase test does not ensure susceptibility to all beta-lactam antimicrobial agents.

REFERENCE

1. **NCCLS.** 2001. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Anaerobically.* Approved standard M11-A5. NCCLS, Wayne, Pa.

SUPPLEMENTAL READING

Citron, D. M., and D. W. Hecht. 2003. Susceptibility test methods: anaerobic bacteria, p. 1141–1148. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.

Finegold, S. M., and W. L. George. 1989. *Anaerobic Infections in Humans.* Academic Press, Inc., San Diego, Calif.

Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.). 1977. *Anaerobe Laboratory Manual*, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.

Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold. 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual*, 6th ed. Star Publishing Co., Belmont, Calif.

APPENDIX 5.7–1

Preparation of Media and Reagents

A. Supplemented brucella broth (1, 2)

1. Use the following formulation of brucella broth powder.

pancreatic digest of casein	10 g
peptic digest of animal tissue	10 g
dextrose	1 g
yeast extract	2 g
sodium chloride	5 g
sodium bisulfite	0.1 g

2. Combine the following in 900 ml of distilled water.

brucella broth powder	28 g
hemin stock solution	1 ml
vitamin k_1 working solution	1 ml

3. Boil to dissolve.
4. Dispense in convenient-size aliquots.
5. Autoclave broth at 121°C for 15 min.
6. Cool to ≤4°C.
7. Add 100 ml of sterile lysed horse blood to 900 ml of broth (or appropriate amount if dispensed in smaller volumes).
8. Cap tightly, and store at 2 to 8°C for up to 1 month.
9. If broth is to be used for inoculum suspension preparation, it is not necessary to add blood.

B. Enriched THIO (1, 2)

1. Use the following formulation of enriched THIO powder:

pancreatic digest of casein	17 g
papaic digest of soybean meal	3 g
dextrose	6 g
sodium chloride	2.5 g
sodium thioglycolate	0.5 g
agar	0.7 g
L-cystine	0.25 g
sodium sulfite	0.1 g

2. Combine the following in 1,000 ml of distilled water.

THIO powder without indicator	30 g
hemin stock solution	1 ml
vitamin k_1 working solution	1 ml

APPENDIX 5.7–1 *(continued)*

3. Boil to dissolve agar (THIO broth contains a small amount of agar).
4. Dispense in 5-ml amounts into screw-cap tubes (13 by 100 mm).
5. Autoclave at 121°C for 15 min.
6. Cool to 48 to 50°C.
7. Add 0.25 ml of filter-sterilized sodium bicarbonate stock solution per 5 ml of THIO.
8. Alternatively, substitute three or four marble chips per 5 ml of THIO for sodium bicarbonate. Add before autoclaving.
9. Cap tightly, and store at 2 to 8°C in the dark for up to 6 months.
10. For optimal performance of broth, boil THIO broth for 5 min and cool it to room temperature prior to use.

C. Hemin stock solution (final concentration, 5 mg/ml)
1. Dissolve 0.1 g of hemin in 2 ml of 1.0 N sodium hydroxide (American Chemical Society certified).
2. Bring volume to 20 ml with distilled water. Hemin must be completely dissolved before the addition of water.
3. Sterilize at 121°C for 15 min.
4. Store at 2 to 8°C in a tightly closed container, protected from light, for up to 1 month.

D. Vitamin K_1 stock solution (final concentration, 10 mg/ml)
1. Add 20 ml of 95% ethanol to 0.2 ml of vitamin K_1 (3-phytylmenadione), and mix thoroughly.
2. Store at 2 to 8°C in a tightly closed dark container for up to 1 year.

E. Vitamin K_1 working solution (final concentration, 1 mg/ml)
1. Add 1 ml of vitamin K_1 stock solution to 9 ml of sterile distilled water, and mix thoroughly.
2. Store at 2 to 8°C in tightly closed dark container for up to 1 month.

F. Lysed horse blood (50%)
1. Aseptically mix equal volumes of defibrinated horse blood and sterile distilled water.
2. Freeze at −20°C or lower and thaw the horse blood five to seven times until the cells are thoroughly lysed (blood will be translucent and dark red).
3. Clarify blood by centrifuging at 12,000 × *g* for 20 min.
4. Decant supernatant and check for clarity. Recentrifuge if necessary. Discard sediment.
5. Store at −20°C for up to 6 months in conveniently sized aliquots.
6. Bring to room temperature before use.
7. Ready-to-use lysed horse blood is commercially available.

References

1. **Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold.** 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual,* 6th ed. Star Publishing Co., Belmont, Calif.
2. **NCCLS.** 2001. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Anaerobically.* Approved standard M11-A5. NCCLS, Wayne, Pa.

APPENDIX 5.7–2

Commercially Available Anaerobe Broth Microdilution MIC Panels

Frozen

PML Microbiological
27120 S.W. 95th Ave.
Wilsonville, OR 97070
(800) 628-7014

Remel (IDS)
12076 Santa Fe Dr.
Lenexa, KS 66215
(800) 255-6730

Dried

BBL Sceptor
Becton Dickinson Microbiology Systems
250 Schilling Circle, P.O. Box 243
Cockeysville, MD 21030
(800) 638-8663
(410) 771-0100

Trek
29299 Clemens Rd.
Suite 1-K
Westlake, OH 44145
(800) 871-8909
(440) 808-0000

The test medium used in these panels may vary from the NCCLS recommendations. If so, it is the manufacturer's responsibility to prove equivalence to a reference method and provide any variation in QC values for the recommended QC strains.

APPENDIX 5.7–3

Anaerobe Broth Microdilution QC

LOT NO. AND EXPIRATION DATE _____

ANTIMICROBIAL	EXPECTED RANGE (µg/ml)	*											

* Date/Tech QC ORGANISM _____

5.8 Etest

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

A standardized inoculum of bacteria is swabbed onto the surface of a Mueller-Hinton agar (MHA) plate. Etest strips containing a continuous gradient of antimicrobial concentrations are placed on the agar surface. After overnight incubation, an elliptical zone of inhibition forms as the antimicrobial agent inhibits growth. The MIC is read where growth intersects the Etest strip. MICs can be interpreted as susceptible, intermediate, or resistant based on the tables in the NCCLS MIC standard (1).

The Etest is a commercial antimicrobial gradient product and is not specifically addressed in NCCLS standards. However, the NCCLS does acknowledge Food and Drug Administration (FDA)-cleared antimicrobial gradient tests that perform comparably to NCCLS reference methods as acceptable for use in clinical laboratories (1).

II. SPECIMEN

Prepare inocula from four or five isolated colonies of similar colony morphology.

A. Log-phase growth inoculum
Use colonies grown for 1 or 2 days on nonselective or selective (e.g., MAC) medium

B. Direct colony suspension inoculum
Use colonies grown overnight on nonselective medium (e.g., BAP or CHOC).

C. Subculture stock, frozen, or lyophilized isolates two times prior to testing.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Media and reagents

1. Agar plates (approximately 4 mm deep)
- **a.** MHA
  ◩ **NOTE:** When testing oxacillin or methicillin with *Staphylococcus* spp., use MHA supplemented with 2% NaCl.
- **b.** MHA with 5% sheep blood (BMHA)
- **c.** *Haemophilus* test medium
- **d.** GC agar base with defined supplements

Store at 2 to 8°C.

2. Nutrient broth (e.g., Mueller-Hinton, TSB) or 0.9% NaCl (3.0- to 5.0-ml aliquots)
Store at 2 to 30°C.

3. Etest strips (AB Biodisk North America, Inc., Piscataway, N.J.)
Store with desiccant at −20°C and handle strips according to the manufacturer's instructions.

B. Supplies

1. Sterile cotton-tipped swabs
2. Sterile plastic pipettes
3. McFarland 0.5 and 1.0 turbidity standards

C. Equipment

1. Forceps
2. Scissors
3. Sterile petri plate
4. Movable light source
5. Vortex mixer
6. 34- to 35°C ambient-air incubator; CO_2 incubation for some organisms
7. Etest applicator (optional)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. QC strains

1. *Escherichia coli* ATCC 25922
2. *Staphylococcus aureus* ATCC 29213
3. *Pseudomonas aeruginosa* ATCC 27853
4. *E. coli* ATCC 35218
5. *Enterococcus faecalis* ATCC 29212
6. *E. faecalis* ATCC 51299
7. *Neisseria gonorrhoeae* ATCC 49226
8. *Haemophilus influenzae* ATCC 49247
9. *H. influenzae* ATCC 49766
10. *Streptococcus pneumoniae* ATCC 49619
11. Additional QC strains as described by the manufacturer

B. Monitoring accuracy

1. Test QC strains by following routine procedure, and record results in QC notebook. Record lot numbers and expiration dates of Etest strips and agar. See Appendix 5.8–1.
2. Compare to expected results (*see* NCCLS QC tables) (1). Note any out-of-control result and document; proceed with corrective action, if necessary.

C. Daily QC testing

1. QC testing is in control if no greater than 1 out of 20 consecutive results is outside specified accuracy limits.
2. If any more than 1 of 20 results is out of control, corrective action is required.

D. Weekly QC testing

1. Frequency of QC testing
   - **a.** QC testing can be reduced from daily to weekly providing a laboratory can document proficiency in performing this test by performing QC daily (or each day patient tests are performed) until 20 consecutive days of testing have been obtained.
   - **b.** Proficiency in performing QC tests is confirmed if, for each antimicrobial agent-QC organism combination, no more than 1 of 20 results are outside accuracy limits.
   - **c.** If 2 or 3 of 20 results are out of control, continue testing until 30 days of testing have been accomplished. If no more than 3 of 30 results are outside the accuracy limits, weekly QC testing can begin.
   - **d.** Document proficiency each time a new antimicrobial agent is added to the testing protocols.
2. Weekly QC testing is in control if all MICs are within specified accuracy limits.
3. *If any* weekly result is out of control, return to daily QC to define the problem, as explained in item IV.E below.
4. Perform QC testing each time a new lot or new shipment of materials (MHA, Etest strips, etc.) is put into use.

E. Corrective action

1. Out of control due to obvious error (e.g., use of wrong Etest strip or QC strain, obvious contamination, or incorrect incubation conditions): record the reason for the out-of-control result and retest the same day. If the repeat result is in range, no further action is needed.
2. Out of control not due to obvious error
   - **a.** Test the antimicrobial agent(s) with appropriate QC strain(s) for five consecutive test days. Record all results.

IV. QUALITY CONTROL *(continued)*

b. For each antimicrobial agent-QC strain combination, all of the five MICs must be within the acceptable control limits. Weekly testing may be resumed.

c. If the problem is not resolved (one or more MICs are outside the accuracy control limits), continue daily QC until the problem can be resolved.

d. Investigate possible systemic problems (e.g., review reading of Etest strips, standardization of inoculum, storage and expiration of reagents, incubation conditions, equipment, and maintenance and purity of QC strain).

e. In order to return to weekly testing, satisfactory performance for another 20 or 30 consecutive days must be documented as described above.

f. During corrective action, carefully evaluate whether patient reports will be affected. It may be necessary to use an alternate test method until the problem is resolved.

V. PROCEDURE

See Appendix 5.8–2 for a summary of testing conditions for various organisms.

A. Bring agar plates and containers of Etest strips to room temperature before use. Ensure that the surface of the agar is dry before use.

B. Inoculum preparation

Using a loop or swab, transfer colonies as follows.

1. Standard method (log-phase growth)

a. Pick four or five isolated colonies to 3.0 to 5.0 ml of broth.

b. Incubate at 35°C for 2 to 8 h until growth reaches the turbidity at or above that of a 0.5 McFarland standard.

2. Direct colony suspension method

Pick several colonies from a *fresh* (18- to 24-h) nonselective agar plate to broth or 0.9% NaCl.

3. For either method, vortex well and adjust turbidity visually with sterile broth or 0.9% NaCl to match a 0.5 McFarland standard (1×10^8 to 2×10^8 CFU/ml). Alternatively, standardize suspension with a photometric device.

C. Inoculation of agar plate

1. Within 15 min of adjusting turbidity, dip a sterile cotton swab into the inoculum and rotate against the wall of the tube above the liquid to remove excess inoculum.

2. Swab entire surface of agar plate three times, rotating plate approximately 60° between streaking to ensure even distribution. Finally, run swab around the edge of the agar to remove any excess moisture.

3. Allow inoculated plate to stand for approximately 10 to 15 min. This is critical for maximum performance of the Etest.

D. Application of Etest strips

1. Make certain MIC scale faces upward; do not touch the underside (antimicrobial side) of the strip.

2. Using forceps or your fingers, grab the end of the strip labeled "E"; use care to take one strip only.

3. If an applicator is used, follow manufacturer's directions for this apparatus.

4. Apply strips to the agar surface with the highest concentration near the edge of the petri dish.

a. For a 100-mm plate, use only one or two strips per plate.

b. For a 150-mm plate, place one to six strips on the plate equal distances apart, radiating from the center of the plate. The "E" end of the strip should point to the rim of the petri dish. Templates are provided by the manufacturer (optional).

V. PROCEDURE *(continued)*

5. Once the strip is in place, remove large air bubbles underneath by using forceps and gently pressing on the strip, beginning at the lower edge of the bubble and moving up the concentration gradient of the strip toward the E. Small bubbles will not interfere.
6. *Do not* relocate an Etest strip once it has landed on the agar surface. The antimicrobial agent is immediately released into the agar. If the strip is accidentally placed upside down, carefully pick it up, turn it over, and place it on the agar surface. If the strip touches the counter or another object, it can still be used providing it does not contact moisture.

E. Incubation

1. Incubate plates within 1 h of Etest application (except for anaerobes which should be incubated immediately). See Appendix 5.8–2 for incubation recommendations and special considerations.
2. Invert plates and stack plates no more than five high.
3. Incubate for 16 to 20 h at 35°C in an ambient-air incubator.
   a. Incubate all staphylococci that are susceptible to penicillinase-resistant penicillins (oxacillin, methicillin, nafcillin) for an additional 4 to 8 h (total of 24 h).
   b. Incubate all staphylococci and all enterococci that are susceptible to vancomycin for an additional 4 to 8 h (total of 24 h).

F. Reading plates

1. Read plates only if lawn of growth is confluent or nearly confluent.
2. Remove the cover of the petri dish, hold plate to a transmitted-light source, and read MIC at the point where growth intersects the Etest strip. If using opaque media (e.g., BMHA), use reflected light; a hand lens may help. Read for complete inhibition of all growth, including haze and isolated colonies.
3. If there is no inhibition of growth, report the MIC as greater than the highest concentration on the Etest strip.
4. If the zone does not intersect the strip (zone is below the strip), report MIC as less than the lowest concentration.
5. For MICs that fall between markings, use the higher value.
6. When testing hemolytic organisms, measure the diameter of the zone of inhibition of growth and not the zone of inhibition of hemolysis.
7. Refer to the AB Biodisk technical guide, which contains photographs of equivocal intersections.
8. Strip placement and reading results
   a. Application of a strip to a wet surface often results in growth that begins at the zone-strip intersection and continues up the side of the strip. Ignore this when reading the MIC. Excessively wet swabs and incomplete swabbing of plates may result in jagged edges and an uneven intersection at the MIC.
   b. The organism and antimicrobial agent tested may have an effect on the appearance of the zone edge where growth intersects the Etest strip. AB Biodisk has extensive reading guidelines (including photographic examples) which must be used.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Interpretation

Interpret MICs based on criteria specified by the NCCLS (1).

B. Reporting

Report the MIC along with its categorical interpretation: susceptible (S), intermediate (I), or resistant (R).

Example: *S. pneumoniae*

Drug	MIC (μg/ml)	Interpretation
Penicillin	>2	R

C. If the Etest MIC falls between the twofold dilutions, round up to the next highest twofold dilution and then interpret the MIC.

D. Important reporting rules

1. Report methicillin-, oxacillin-, or nafcillin-resistant staphylococci as resistant to all beta-lactam drugs (including beta-lactam–beta-lactamase inhibitor combinations, all cephems, all penicillins, and carbapenems) regardless of results of in vitro susceptibility testing.

2. Misleading results

Specific organism-drug combinations should not be reported, since susceptible results could be *dangerously misleading.*

a. First- and second-generation cephalosporins and aminoglycosides against *Salmonella* spp. and *Shigella* spp.

b. Cephalosporins, trimethoprim-sulfamethoxazole, clindamycin, and aminoglycosides (except for high-level-synergy testing for aminoglycosides) against enterococci

c. Cephalosporins against *Listeria* spp.

d. Beta-lactams against oxacillin-resistant *Staphylococcus* spp.

e. Cephalosporins, penicillins, and aztreonam against extended-spectrum beta-lactamase (ESBL)-producing *E. coli* and *Klebsiella* spp.

3. For ESBL confirmatory testing, there is a single strip configured with cefotaxime on one end and cefotaxime with clavulanic acid on the other end. A similar strip with ceftazidime and ceftazidime-clavulanic acid is also available. Both are FDA cleared.

VII. PROCEDURE NOTES

A. Oxacillin (methicillin)-resistant staphylococci

1. Oxacillin is the recommended penicillinase-stable penicillin for testing, and oxacillin results can represent all penicillinase-stable penicillins (oxacillin, methicillin, and nafcillin and also cloxacillin dicloxacillin, and flucloxacillin). Testing of oxacillin with staphylococci should be performed in MHA supplemented with 2% NaCl (other drugs should not be tested on 2% NaCl-supplemented MHA).

2. Clue to detection of methicillin (oxacillin)-resistant *S. aureus:* multiple resistance to other antimicrobial agents (beta-lactams, aminoglycosides, erythromycin, clindamycin, and tetracycline). Recently, strains of oxacillin-resistant *S. aureus* that are not multiply resistant have been encountered in isolates primarily from patients with community-acquired infections.

B. Media

1. Thymidine can interfere with performance of sulfonamides and trimethoprim. Monitor media by testing *E. faecalis* ATCC 29212 and trimethoprim-sulfamethoxazole (MIC of 0.5/9.5 μg/ml indicates acceptable performance). Blood components (other than horse blood) contain thymidine and may interfere with testing of these agents.

VII. PROCEDURE NOTES
(continued)

2. An increase in the cation content (Ca^{2+}, Mg^{2+}) of the medium results in an increase in the MICs of aminoglycosides for *P. aeruginosa* and an increase in MICs of tetracycline for all organisms. A decrease in cation content has the opposite effect. Monitor cation content by testing aminoglycosides with *P. aeruginosa* ATCC 27853 and making sure results are within defined QC limits.
3. Increased zinc ions may cause increased MICs with carbapenems.
4. Variation in calcium ions affects the results of daptomycin tests.

VIII. LIMITATIONS

A. The Etest (an agar-based technique) correlates with the reference agar dilution procedure. However, certain discrepancies between Etest MICs and MICs from non-agar-based systems such as broth microdilution and from other automated systems based on different technical principles may occur as a consequence of the different characteristics inherent in these methods.

B. Numerous factors can affect Etest MICs, such as inoculum size, rate of growth, formulation and pH of media, incubation environment and length of incubation, drug diffusion rate, and measurement of endpoints. Therefore, strict adherence to protocol is required to ensure reliable results.

C. The Etest has been evaluated for other organisms in addition to those listed in Appendix 5.8–2. It is the responsibility of the laboratory to ensure that there are sufficient data to warrant use of Etest for testing bacteria isolated from patients' specimens, particularly for organism-Etest antimicrobial agent combinations not yet FDA cleared. Contact AB Biodisk for additional information.

REFERENCE

1. **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.

SUPPLEMENTAL READING

Baker, C. N., S. A. Stocker, D. H. Culver, and C. Thornsberry. 1991. Comparison of the Etest to agar dilution, broth microdilution, and agar diffusion susceptibility testing techniques by using a special challenge set of bacteria. *J. Clin. Microbiol.* **29:**533–538.

Jorgensen, J. H., and J. D. Turnidge. 2003. Antibacterial susceptibility tests: dilution and disk diffusion methods, p. 1108–1127. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.

APPENDIX 5.8–1

Etest QC (*see* p. 5.8.8)

APPENDIX 5.8–2

Quick reference list for performing Etest[a]

Organism	Agar	Inoculum method	Incubation length (h)	Incubation atmosphere[b]
Testing cleared by FDA				
Enterobacteriaceae	MHA	DCS/LPG	16–20	Ambient air
Pseudomonas aeruginosa	MHA	DCS/LPG	16–20	Ambient air
Stenotrophomonas maltophilia	MHA	DCS/LPG	24	Ambient air
Acinetobacter spp.	MHA	DCS/LPG	24	Ambient air
Haemophilus spp.[c]	HTM	DCS	20–24	CO_2
Neisseria gonorrhoeae	GC (1% defined supplement)	DCS	20–24	CO_2
Staphylococcus spp.	MHA (add 2% NaCl to media for oxacillin)[d]	DCS	16–18; 24 (oxacillin[d] and vancomycin)	Ambient air
Enterococcus spp.	MHA	DCS/LPG	24 (HLAR at 48)	Ambient air
Streptococcus spp.	BMHA	DCS	24	CO_2
Streptococcus pneumoniae[e]	BMHA	DCS	20–24	CO_2
Anaerobes[c,g]	Brucella blood agar supplemented with vitamin K and hemin	DCS/LPG	24–72	Anaerobic environment
Testing not cleared by FDA as of August 2002				
Neisseria meningitidis	BMHA (or CMHA)	DCS	24	CO_2
Moraxella spp.[f]	MHA	DCS	18–24	CO_2
Corynebacterium spp.[g]	BMHA	DCS	18–24 (48 h if *C. jeikeium*)	Ambient air (CO_2 if necessary)
Listeria spp.	BMHA	DCS	18–24	Ambient air
Pasteurella multocida	BMHA	DCS	18–24	Ambient air
Eikenella corrodens[g]	BMHA	DCS	18–24	CO_2

[a] This table is divided into two sections. The first part lists aerobic organisms and rapidly growing facultatively anaerobic organisms for which the FDA has cleared certain Etest strips for testing in the clinical laboratory. The second part lists organisms for which Etest studies have been performed but for which Etest strips have not yet been granted clearance by the FDA. Testing of Etest strip-organism combinations that are not yet FDA cleared should be done with caution. Always check the Etest package insert or contact the manufacturer to determine the FDA clearance status for testing an agent against a particular isolate from a patient. Not every drug is cleared for each organism group listed. Abbreviations: CMHA, chocolate, MHA; DCS, direct colony suspension standardized to a 0.5 McFarland turbidity standard; DCS/LPG, direct colony suspension or log-phase growth standardized to a 0.5 McFarland turbidity standard; GC, GC agar base with 1% defined supplement; HTM, *Haemophilus* test medium.

[b] All incubation temperatures are 34 to 35°C.

[c] Use a photometric device to adjust turbidity. This step is critical in avoiding erroneous results due to under- or overincoulation.

[d] And other penicillinase-stable penicillins.

[e] Use broth for inoculum suspension.

[f] Generally, only a beta-lactamase test is warranted for *Moraxella catarrhalis.*

[g] Use a 1.0 McFarland standard for inoculum preparation (manufacturer's recommendation).

Etest QC

Test Date	Tech	Antimicrobial	Manuf. Lot #	Date Rec'd	Exp. Date	Agar Lot #	Agar Exp. Date	QC Organism	Expected Range (µg/ml)	Result	Comments/Corrective Action

5.9 Agar Dilution MIC Test for Anaerobic Bacteria

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The anaerobe agar dilution MIC method is a semiquantitative method for the determination of in vitro activity of an antimicrobial agent against anaerobic bacterial isolates. A series of agar plates, each containing a unique concentration of an antimicrobial agent, are inoculated with up to 36 isolates. After 48 h of incubation at 35°C in an anaerobic environment, the MIC is determined by observing the lowest concentration of antimicrobial agent which will inhibit visible growth of the test isolate. MICs obtained are interpreted as susceptible, intermediate, or resistant based on criteria defined by the NCCLS (2).

Recommendations for performance of the agar dilution method for susceptibility testing of anaerobic bacteria are found in the NCCLS M11 standard (2). This method uses brucella agar supplemented with hemin, vitamin K_1, and laked sheep blood, which is satisfactory for testing most anaerobic species.

II. SPECIMEN

Prepare inoculum directly from four or five isolated colonies of similar colony morphology grown anaerobically for 24 to 48 h on an anaerobic brucella blood agar plate.

A. Use 24-h growth for rapid growers (e.g., *Bacteroides fragilis* group), if sufficient growth is available; 48-h growth is preferable for most other species.
B. Subculture stock, frozen, or lyophilized isolates two times prior to testing.
C. Do not expose agar plates containing test isolates to air for longer than 20 to 30 min.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Media and reagents
See Appendix 5.9–1 for additional information.
1. Brucella agar supplemented with (final concentrations) vitamin K_1 (1 μg/ml), hemin (5 μg/ml), and laked sheep blood (5%). Store at 2 to 8°C.
2. Broth media
   a. Brucella broth dispensed in aliquots of 5 ml. Store at 2 to 8°C.
   b. THIO supplemented with vitamin K_1, hemin, and either a marble chip or sodium bicarbonate dispensed in aliquots of 5 ml. Store at 2 to 8°C.
3. Sterile 0.9% NaCl dispensed in aliquots of 9 ml in screw-cap tubes. Store at 2 to 8°C.
4. Sterile 0.9% NaCl dispensed in aliquots of 9.9 ml in screw-cap tubes. Store at 2 to 8°C.
5. Brucella agar deeps supplemented with vitamin K_1 and hemin. Store at 2 to 8°C.

B. Supplies
1. Sterile cotton-tipped swabs
2. Sterile polystyrene screw-cap tubes (e.g., 16 by 125 mm for making antimicrobial agent dilutions and 20 by 15 mm for agar deep tubes)
3. Sterile plastic pipettes
4. 0.5 McFarland turbidity standard
5. Sterile 100-mm round petri plates or 100-mm square petri plates

III. MATERIALS *(continued)*

C. Equipment

1. Vortex mixer
2. Adjustable micropipette and sterile pipette tips
3. 35°C anaerobic atmosphere (in container large enough to hold plates) containing 5 to 10% H_2, 5 to 10% CO_2, and 80 to 90% N_2 (e.g., anaerobe chamber, anaerobe pouch, or anaerobic jar)
4. Boiling water bath, steamer, or autoclave to melt agar
5. 48 ± 2°C water bath
6. Steers replicator (autoclaved)
7. 35°C ambient-air incubator (CO_2)
8. Bunsen burner

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. QC strains

1. *B. fragilis* ATCC 25285
2. *Bacteroides thetaiotaomicron* ATCC 29741
3. *Eubacterium lentum* ATCC 43055

B. Monitoring accuracy

1. Test QC isolates by following routine procedures, and record results on a QC log sheet. Record lot numbers and expiration dates of medium and antimicrobial agents. See Appendix 5.9–2.
2. Compare with expected results listed in the most recent NCCLS standards tables (2). Note any out-of-control results and document; proceed with corrective action if necessary.

C. Include at least two of the QC strains listed above with each set of clinical isolates being tested. Ideally, the QC strains selected should provide on-scale MICs for each of the antimicrobial agents being tested.

D. Additional controls

1. Positive growth control plate (anaerobically incubated plate without antimicrobial agent) must demonstrate good growth (confluent spot) and not show any contaminating organisms. If adequate growth is not obtained on the positive growth control plate, the MICs should not be read or interpreted for that organism.
2. Negative growth control plate (aerobically incubated plate without antimicrobial agent) must be free of any growth.
3. Purity plate should demonstrate a pure culture.
4. Inoculum count verification plate should show approximately 50 to 200 colonies.

V. PROCEDURE

A. Organizational considerations

Agar dilution is a very complicated, multistep procedure performed over several days. It is important to completely plan out the entire procedure and have all supplies and work areas ready in advance in order to complete an agar dilution run in a timely manner. See Appendix 5.9–3 for work flow suggestions. This procedure can be performed by a single person; however, it is much easier and more efficient when at least two people are involved. Inoculum suspensions and the inoculation of the agar plates can be done outside of the anaerobic chamber. However, the time between isolate exposure to air and placement of the inoculated plates in an anaerobic environment should not exceed 30 min.

B. Preparation of antimicrobial agent-containing plates

1. Determine antimicrobial agents and concentrations to be tested. At a minimum, test six dilutions (two dilutions below the susceptible breakpoint; three dilutions representing the susceptible, intermediate, and resistant interpretive categories; and one dilution above the resistant breakpoint). Additional dilutions may be required to include concentrations that would represent acceptable ranges for QC organisms.

V. PROCEDURE *(continued)*

2. Label one petri plate for each concentration of each antimicrobial agent to be tested. Also label control plates equal to twice the number of antimicrobial agents plus two in which to pour agar without antimicrobial agent. Stack plates in an organized fashion.
3. Label one test tube (16 by 125 mm) with the intermediate concentration (10 times the final concentration) for each dilution of each antimicrobial agent (*see* Appendix 5.9–4).
4. Thaw (at room temperature) stock solutions of antimicrobial agents.
5. Prepare intermediate dilutions of stock solutions.
6. Label one tube of agar for each antimicrobial dilution.
7. Melt agar tubes and make certain that the agar is completely liquefied.
8. Place tubes with melted agar into the 48°C water bath and allow them to equilibrate to the water bath temperature (approximately 20 min). Then perform the following.
   a. Remove the melted agar deep; wipe off excess water.
   b. Add 1 ml of laked sheep blood for round or 1.5 ml for square petri plates (*see* Appendix 5.9–5).
   c. Add 2 ml of antimicrobial agent for round or 3 ml for square petri dishes.
   d. For growth control plates, add the appropriate amount (2 or 3 ml) of sterile water in place of antimicrobial agent.
9. Mix by gently inverting and rotating tubes three times.
10. Pour agar into prelabeled petri plates on a level surface.
    a. Make certain that the agar is evenly distributed in plate.
    b. Rotate plate to move any bubbles to the edge of the plate.
    c. If any bubbles remain on the central surface or if there are large bubbles on the edge, *quickly* pass a Bunsen burner flame over the surface to eliminate them before the agar solidifies.
11. Following solidification of the agar in the plates (5 to 10 min), the next step is drying the plates. If the plates are too moist, the inocula may run together. Suggestions for drying the plates include the following.
    a. Allow the plates to remain with lids slightly ajar in a 35°C ambient-air incubator (preferably, one that is not humidified) for 45 to 60 min.
    b. Invert plates and leave the lids ajar in a laminar-flow hood or biological safety cabinet for 30 to 45 min.
12. For routine testing, use plates as soon as they are dry or store at 2 to 8°C in a plastic bag for up to 7 days. Use plates containing imipenem, clavulanic acid, or any other antimicrobial agent of known instability on the day they are poured.

C. Inoculum preparation

1. Using a loop or swab, transfer colonies as follows.
   a. Growth method
      (1) Pick colonies from a supplemented brucella blood agar plate 24 to 48 h old to enriched THIO or supplemented brucella broth. (Prior to using THIO, prereduce by boiling for 5 min and then cool.)
      (2) Incubate at 35°C under anaerobic conditions for 4 to 6 h (but no longer than 24 h) or until the turbidity is equivalent to that of a 0.5 McFarland turbidity standard (1.5×10^8 CFU/ml). Ambient-air incubation is acceptable when using prereduced THIO. If turbidity is not reached by 24 h, use the direct colony suspension method.
   b. Direct colony suspension method
      (1) Pick colonies from a supplemented brucella blood agar plate 24 to 48 h old, and suspend in brucella broth.
      (2) Gently invert the tube or vortex to obtain a homogeneous suspension.
      (3) Adjust the turbidity to match that of a 0.5 McFarland standard, which has been vortexed.

V. PROCEDURE *(continued)*

2. Prepare inoculum suspensions no more than 15 min before inoculation of plates.

D. Inoculation

1. Allow agar dilution plates to warm to room temperature, and label them.

2. Within 15 min of preparing the inoculum, inoculate the agar dilution plates.

3. Pipette approximately 0.5 to 0.6 ml of each standardized suspension into the appropriate well of a Steers replicator reservoir block. See layout example in Appendix 5.9–5.

4. Make certain that the plates contain some indication as to the orientation of inocula (e.g., labels or markings on plates or dye in one of the seed wells).

5. Inoculate plates in the following order.

a. Two control plates without drug: label as "pre-(name of antimicrobial agent 1)."

b. Antimicrobial agent 1 plates: lowest to highest concentrations (e.g., 0.06 to 128 μg/ml).

c. Two control plates without drug: label as "between (names of antimicrobial agents 1 and 2)." These plates are necessary to show that the organism is still viable throughout the inoculation process.

d. Antimicrobial agent 2 plates: lowest to highest concentrations

e. Two control plates without drug: label as "between (names of antimicrobial agents 2 and 3)."

f. Continue this pattern until all antimicrobial agent-containing plates are inoculated.

g. After the last antimicrobial agent (*x*) plate, inoculate two control plates without drug and label them "post-(name of antimicrobial agent *x*)". No more than four antimicrobial dilution sets of plates should be inoculated using a single replicator seed tray-pin set in order to minimize the chance of antimicrobial agent carryover and contamination of the seed tray of the replicator. If more than four antimicrobial agents are tested, several replicators are needed.

h. An additional plate without drug can be inoculated and used as an inoculum control plate. Do not incubate, but store these plates at 4°C during the incubation period. Remove the plates from the refrigerator at the time the MICs are read. The inoculum control plates are often helpful when poor-growing organisms are encountered, as they allow for a comparison between poor growth and inoculum dried on the agar plate with no growth.

6. Inoculate plates by positioning inoculator prongs in reservoir block wells to pick up inocula (prongs of Steers replicator generally pick up 1 to 5 μl). Then carefully apply prongs to the agar surface (final inoculum concentration is approximately 10^5 CFU per spot). Perform this procedure slowly to avoid splashing, which can lead to cross-contamination of inocula.

7. Following inoculation, move each plate aside and allow it to dry (agar surface up) with the top slightly ajar.

E. Incubation

1. When inocula on the plates have dried (usually 5 to 10 min after inoculation), invert plates (cover down).

2. Incubate one plate from each pair of growth control plates inoculated at the beginning, between antimicrobial agents, and at the end of the procedure at 35°C in a CO_2 incubator (aerobic purity plates).

3. Incubate all antimicrobial agent-containing plates and remaining growth control and colony count plates in an anaerobic environment at 35°C in CO_2 for 42 to 48 h.

4. Incubate all plates with lid side down.

V. PROCEDURE *(continued)*

F. Reading MICs

1. Following incubation, examine purity plates first. If a mixed culture is apparent, repeat the test.
2. Examine growth control plates.
   a. Positive growth control plates should demonstrate a confluent spot indicating adequate growth. The growth pattern should be the same on all growth control plates throughout the run.
   b. Negative growth control plates should show no growth.
   c. If either type of growth control plate is unacceptable, repeat the test.
3. Read agar dilution plates in the order that they were stamped, and place each plate against a dark nonreflective background. It is helpful to line up all plates (lowest to highest concentration) of one antimicrobial agent and read one organism at a time.
4. The MIC is the lowest concentration of antimicrobial agent where a *marked* change in growth appears compared to the growth control plate. A marked change can consist of no growth, faint haze, one discrete colony, or several tiny colonies. NCCLS M11 contains photographs and is a good reference for reading appropriate endpoints (2).
5. Record results on agar dilution MIC worksheet (*see* Appendix 5.9–6).

G. Inoculum count verification plates

1. Colony counts should be performed periodically at the completion of a run to verify the inoculum concentration. Frequency of testing is up to the individual laboratory and should be based on the frequency of use of the test method. For laboratories that infrequently perform this procedure, colony counts should be done every time the test is performed. For laboratories that perform this procedure at least once a week, colony counts can be performed monthly.
2. Colony count procedure
   a. Select approximately two to five wells to test.
   b. Remove 0.01 ml from the respective replicator block well immediately after completion of the inoculation process and add this to 9.9 ml of 0.9% saline (1:1,000 dilution). Mix by at least three gentle inversions.
   c. Transfer 0.1 ml from the first tube to a second tube containing 9.9 ml (1:100 dilution). Mix by at least three gentle inversions.
   d. Plate 0.1 ml to a supplemented brucella blood agar plate.
   e. Using a loop or bent glass (“hockey stick”), thoroughly spread the inoculum over the surface of the plate by streaking in several directions.
   f. Incubate under anaerobic conditions for 48 h.
   g. The plate count should be approximately 10^2 organisms (50 to 200 colonies per plate).

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Interpretation

Interpret MIC based on criteria specified by the NCCLS (2).

B. Reporting

Report the MIC along with its categorical interpretation: susceptible (S), intermediate (I), or resistant (R).

Example: *B. fragilis*

Drug	MIC (μg/ml)	Interpretation
Ampicillin-sulbactam	8/4	S
Cefoxitin	8	S
Clindamycin	4	I
Metronidazole	1	S
Piperacillin-tazobactam	0.25	S

C. Important reporting rules

The majority of *B. fragilis* group isolates are beta-lactamase producers; therefore, these isolates do not require beta-lactamase testing and should always be reported as resistant to penicillin and ampicillin regardless of the MIC (1).

VII. PROCEDURE NOTES

A. For some fastidious isolates, it may be necessary to prereduce the plates and to perform all manipulations in an anaerobic environment.

B. CHOC can be used in place of BAP for aerobic purity plates.

C. With some anaerobic species, such as *E. lentum*, which either grow slowly or produce small colonies, multiple plates may need to be inoculated in order to obtain sufficient growth to make an inoculum suspension equivalent to a 0.5 McFarland standard.

D. Most anaerobic isolates can be stored at $\leq -60°C$ in 20% glycerol or 20% sterile skim milk.

VIII. LIMITATIONS

A. Agar dilution method is the standard reference method for susceptibility testing of anaerobic bacteria; however, it is very labor-intensive and not ideal for testing a small number of isolates.

B. As with any procedure, experience is important. Those who perform agar dilution susceptibility tests on anaerobes should have experience in anaerobic bacteriology. Laboratories with few requests for susceptibility tests should consider sending them to a referral laboratory with experience in anaerobic susceptibility testing.

C. Anaerobic organisms differ markedly in size and shape; therefore, a standardization of inocula by using a 0.5 McFarland standard may not yield 1.5×10^8 CFU/ml for all organisms.

D. Modifications of this method and of the medium composition may permit the growth of the more fastidious anaerobic organisms, but appropriate QC tests must be included and the results must conform to published values. If modifications are made, reporting of results should acknowledge changes from standard methods.

E. Because many infections involving anaerobes are polymicrobial and successful treatment often involves a combination of surgical intervention and the use of empirical broad-spectrum antimicrobial therapy, the relative importance of the susceptibility of a single organism to predict a favorable clinical outcome is difficult to determine.

VIII. LIMITATIONS *(continued)*

F. When testing anaerobic species that are known to swarm (e.g., *Clostridium difficile* or *Clostridium tetani*), empty wells should be left between the test isolates in the Steers replicator reservoir block. With *Clostridium septicum*, it is recommended that only one isolate be tested per plate.

G. Beta-lactamase testing with a chromogenic cephalosporin-based method can be performed with anaerobic species. A beta-lactamase-producing isolate should be reported as resistant to penicillin and ampicillin, regardless of the MIC. Some anaerobic species are known to be resistant to beta-lactam antimicrobial agents by mechanisms other than beta-lactamases. Therefore, a negative beta-lactamase test does not ensure susceptibility to all beta-lactam antimicrobial agents.

REFERENCES

1. **Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold.** 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual*, 6th ed. Star Publishing Co., Belmont, Calif.
2. **NCCLS.** 2001. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Anaerobically*, 5th ed. Approved standard M11-A5. NCCLS, Wayne, Pa.

SUPPLEMENTAL READING

Citron, D. M., and D. W. Hecht. 2003. Susceptibility test methods: anaerobic bacteria, p. 1141–1148. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.

Finegold, S. M., and W. L. George. 1989. *Anaerobic Infections in Humans.* Academic Press, Inc., San Diego, Calif.

Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.). 1977. *Anaerobe Laboratory Manual*, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.

APPENDIX 5.9–1

Preparation of Media and Reagents

A. Supplemented brucella blood agar (1, 2)

1. Use the following formulation of brucella agar powder.

pancreatic digest of casein	10 g
peptic digest of animal tissue	10 g
dextrose	1 g
yeast extract	2 g
sodium chloride	5 g
sodium bisulfite	0.1 g
agar	15 g

2. Combine the following in 1,000 ml of distilled water.

brucella agar powder	43 g
hemin stock solution	1 ml
vitamin K_1 working solution	1 ml

3. Boil to dissolve agar.
4. Autoclave at 121°C for 15 min.
5. Cool to 48 to 50°C.
6. Aseptically dispense molten agar into sterile screw-cap tubes (17 or 27 ml depending on the size of the petri dishes being used). See Appendix 5.9–5.
7. Cap tightly, and store at 2 to 8°C for up to 1 month.
8. Add laked sheep blood just before adding the antimicrobial dilutions (final concentration, 5%). See item V.B in procedure 5.9 for more details.

APPENDIX 5.9–1 *(continued)*

B. Enriched THIO (1, 2)
1. Use the following formulation of enriched THIO powder.

pancreatic digest of casein	17 g
papaic digest of soybean meal	3 g
dextrose	6 g
sodium chloride	2.5 g
sodium thioglycolate	0.5 g
agar	0.7 g
L-cystine	0.25 g
sodium sulfite	0.1 g

2. Combine the following in 1,000 ml of distilled water.

THIO medium powder without indicator	30 g
hemin stock solution	1 ml
vitamin K_1 working solution	1 ml

3. Boil to dissolve agar (THIO contains a small amount of agar).
4. Dispense in 5-ml amounts into screw-cap tubes (13 by 100 mm).
5. Autoclave at 121°C for 15 min.
6. Cap tightly, and store at 2 to 8°C in the dark for up to 6 months.
7. Just prior to use, boil for 5 min and cool. Add 0.25 ml of filter-sterilized sodium bicarbonate stock solution per 5 ml of THIO.
8. Alternatively, substitute three or four marble chips per 5 ml of THIO instead of sodium bicarbonate. Add before autoclaving.

C. Hemin stock solution (final concentration, 5 mg/ml)
1. Dissolve 0.1 g of hemin in 2 ml of 1.0 N sodium hydroxide (American Chemical Society certified).
2. Bring volume to 20 ml with distilled water. Hemin must be completely dissolved before the addition of water.
3. Sterilize at 121°C for 15 min.
4. Store at 2 to 8°C in a tightly closed container, protected from light, for up to 1 month.

D. Vitamin K_1 stock solution (final concentration, 10 mg/ml)
1. Add 20 ml of 95% ethanol to 0.2 ml of vitamin K_1 (3-phytylmenadione), and mix thoroughly.
2. Store at 2 to 8°C in tightly closed dark container for up to 1 year.

E. Vitamin K_1 working solution (final concentration, 1 mg/ml)
1. Add 1 ml of vitamin K_1 stock solution to 9 ml of sterile distilled water, and mix thoroughly.
2. Store at 2 to 8°C in tightly closed dark container for up to 1 month.

F. Laked sheep blood
1. Lyse blood by freezing at −20°C or below.
2. Thaw blood by either rapidly thawing in a water bath at 35 to 37°C or slowly thawing overnight at 2 to 8°C.
3. Store at −20°C for up to 6 months in 50- to 100-ml aliquots.
4. Bring to room temperature and mix thoroughly by inverting several times before use.
5. Ready-to-use laked sheep blood is commercially available.

G. Sodium bicarbonate stock solution (final concentration, 20 mg/ml)
1. Dissolve 2 g of sodium bicarbonate in 100 ml of distilled water.
2. Filter sterilize through a membrane filter (0.20- to 0.22-μm pore size) into a sterile screw-cap container.
3. Store at 2 to 8°C in a tightly closed container for up to 1 month.

References

1. **Jousimies-Somer, H. R., P. Summanen, D. M. Citron, E. J. Baron, H. M. Wexler, and S. M. Finegold.** 2002. *Wadsworth-KTL Anaerobic Bacteriology Manual*, 6th ed. Star Publishing Co., Belmont, Calif.
2. **NCCLS.** 2001. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Anaerobically*, 5th ed. Approved standard M11-A5. NCCLS, Wayne, Pa.

APPENDIX 5.9–2

Anaerobe agar dilution MIC QC table (*see* p. 5.9.11)

APPENDIX 5.9–3

Timetable for Agar Dilution Susceptibility Testing of Anaerobic Bacteria

A. 3 to 4 weeks (or more) before run
   1. Read procedure carefully.
   2. Check supplies of the following, and order what is necessary.
      a. Antimicrobial agents
      b. Solvents and diluents
      c. Supplies
      d. Ingredients necessary for preparation of agar and broth media

B. 2 weeks before run
   1. Order or prepare agar and broth media.
   2. Order sheep blood.
   3. Prepare antimicrobial stock solutions and freeze.

C. 5 to 7 days before run
   1. Subculture test isolates and QC organisms from ≤ -60°C freezer to supplemented brucella blood agar plate. Check purity of isolates.
   2. Lake sheep blood if not purchased as ready to use. Store in refrigerator or freezer.

D. 3 to 4 days before run
   1. Subculture test and QC organisms a second time onto anaerobic supplemented brucella blood agar plates.
   2. Number and list test isolates on form for recording results.
   3. Prepare supplemented brucella agar deeps.

E. 1 day before run
   1. Label antimicrobial dilution tubes and add appropriate volumes of diluent to them. Cap tubes tightly.
   2. Label petri plates and mark for correct orientation.
   3. Make certain a 48 $\pm$ 2°C water bath is available for the next day.
   4. Autoclave Steers replicators.
   5. If using the growth method, inoculate test and QC isolates to enriched THIO and incubate at 35°C for 6 to 24 h.

F. Day of run
   1. Remove antimicrobial stock solutions from freezer and thaw them at room temperature.
   2. Remove laked sheep blood from refrigerator or freezer and allow it to warm to room temperature.
   3. Melt agar tubes and place them in water bath.
   4. Prepare intermediate dilutions of antimicrobial agents from stock solutions.
   5. Disinfect area where plates are to be poured.
   6. Add laked sheep blood and antimicrobial solutions to agar deeps.
   7. Pour plates.
   8. Allow agar to solidify in plates. (*Note:* Allow approximately 30 min per antimicrobial agent per person to make dilutions and pour plates; e.g., it will take one person approximately 1.5 h to do steps F.4 through 7 for three antimicrobial agents.)
   9. Allow plates to dry with their lids ajar in an incubator.
   10. Prepare and standardize inoculum suspensions (allow 30 to 45 min for 36 isolates).
   11. Pipette inoculum suspensions into Steers replicator reservoir block.
   12. Inoculate plates.
   13. When inocula have dried, invert and place them in an anaerobe jar or anaerobic chamber. Incubate for 42 to 48 h.
   14. Incubate aerobic plates in a 5 to 7% CO_2 incubator at 35°C.
   15. Autoclave Steers replicator and rinse thoroughly with tap and distilled water. Dry.

G. After incubation
   1. Check positive growth control plates.
   2. Check negative growth (contamination) control plate.
   3. Read and record results.

APPENDIX 5.9–4

Preparation of antimicrobial dilutions from stock solutions[a]

Concn (μg/ml) of antimicrobial solution	Amt (ml) of:		Intermediate antimicrobial concn (μg/ml)[b]
	Antimicrobial solution	Diluent	
5,120	2	2	2,560
5,120	2	6	1,280
2,000	6.4	3.6	1,280
1,280	2	2	640
1,280	1	3	320
1,280	1	7	160
160	2	2	80
160	1	3	40
160	1	7	20
20	2	2	10
20	1	3	5
20	1	7	2.5
2.5	2	2	1.25
2.5	1	3	0.6
2.5	1	7	0.3

[a] Reproduced with permission from **NCCLS.** 2003. *Performance Standards for Antimicrobial Susceptibility Testing of Anaerobic Bacteria.* Thirteenth informational supplement M100-S13. NCCLS, Wayne, Pa. Copies of the current edition may be obtained from NCCLS, 940 West Valley Rd., Suite 1400, Wayne, PA 19087-1898. *See* also **NCCLS.** 2001. *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria,* 5th ed. Approved standard M11-A5. NCCLS, Wayne, Pa. The antimicrobial agent is diluted 10-fold when it is added to the agar. Therefore, the intermediate antimicrobial concentration is 10 times the final concentration of the antimicrobial agent in the agar.

[b] Begin with stock solution at a concentration of 5,120 or 2,000 μg/ml.

APPENDIX 5.9–5

Volumes of components required for preparation of agar dilution plates when using round or square petri plates

Component	Vol (ml) required	
	Round plate	Square plate
Agar	17	25.5
Blood	1	1.5
Antimicrobial dilution	2	3
Total	20	30

Patterns for arrangement of isolates in Steers replicator

Pattern for 100-mm round plate						Pattern for 100-mm-square plate					
	1	2	3	4		1	2	3	4	5	6
5	6	7	8	9	10	7	8	9	10	11	12
11	12	13	14	15	16	13	14	15	16	17	18
17	18	19	20	21	22	19	20	21	22	23	24
23	24	25	26	27	28	25	26	27	28	29	30
	29	30	31	32		31	32	33	34	35	36

APPENDIX 5.9–6

Agar dilution MIC worksheet (*see* p. 5.9.12)

Anaerobe Agar Dilution MIC QC

Antimicrobial agent: _____
Stock solution concentration (µg/ml): _____
Agar: _____

Acceptable range (µg/ml):

Bacteroides fragilis ATCC 25285 _____
Bacteroides thetaiotaomicron ATCC 29741 _____
Eubacterium lentum ATCC 43055 _____

Test Date	Tech	Antimicrobial stock solution		Agar		Results (µg/ml)		
		Prep. date or lot #	Exp. date	Prep. date or lot #	Exp. date	*B. fragilis* ATCC 25285	*B. thetaiotaomicron* ATCC 29741	*E. lentum* ATCC 43055

Agar Dilution MIC Worksheet

Antimicrobial agent: _____ Tech _____ Test Date _____

Agar lot # or prep. date: _____ Antimicrobial stock solution lot# or prep. date: _____

	Isolate	Pre*		Antimicrobial concentration (µg/ml)												Post**	
		Aer	Ana	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	Aer	Ana
1																	
2																	
3																	
4																	
5																	
6																	
7																	
8																	
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Record growth (+ or –) for each organism (each drug concentration and growth control plates).

* Pre: growth control plates inoculated immediately preceding first dilution of antimicrobial-containing plates

** Post: growth control plates inoculated immediately following last dilution of antimicrobial-containing plates

5.10.1 Minimum Bactericidal Concentration Testing

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The minimum bactericidal concentration (MBC) test can be used to assess the ability of an antimicrobial agent to kill a bacterial isolate. MBC tests are performed after consultation in very select clinical situations when it is necessary to determine the bactericidal activity of an antimicrobial agent against a bacterial isolate. These situations include those in which immune mechanisms offer little help in eradicating the infecting organisms, such as in patients with endocarditis or osteomyelitis or in immunosuppressed patients, particularly those with neutropenia. Bactericidal tests provide a rough prediction of bacterial eradication, but other factors may also impact the bacteriologic responses of patients. MBC testing is an accepted parameter in the evaluation of new antimicrobial agents and is frequently used as a research tool.

Although MIC tests have been well standardized, this is not the case for tests that assess bactericidal activity. The MBC and time-kill curve procedures described here represent the authors' recommendations.

Following performance of a conventional broth macrodilution or microdilution MIC susceptibility test (5), the tubes or wells containing concentrations of antimicrobial agent equal to and greater than the MIC are subcultured to determine whether the initial inoculum was inhibited from multiplying (static action) or was killed (bactericidal action) (4). The MBC test endpoint is defined as the lowest concentration of antimicrobial agent that kills ≥99.9% of the test inoculum. *The 99.9% cutoff is an arbitrary in vitro value with 95% confidence limits that has uncertain clinical relevance.* Generally, MBC tests are performed with one or two specific antimicrobial agents rather than the panel of antimicrobial agents commonly employed in routine MIC testing. In contrast to MIC tests, organisms in the MBC test inoculum must be in the log phase of growth, since many antimicrobial agents exert a bactericidal effect only on growing bacteria (4).

II. SPECIMEN

A. Prepare inoculum from four or five isolated colonies of similar colony morphology grown for 18 to 24 h on BAP or nonselective supplemented media if necessary.

B. Subculture stock, frozen, or lyophilized isolates three times prior to testing.

III.A. MATERIALS: MACRODILUTION METHOD

Include QC information on reagent container and in QC records.

A. Media and reagents (storage conditions)

The media listed include those necessary for testing rapidly growing aerobic bacteria. Refer to Appendix 5.10.1–1 for a complete listing of suggested media for testing fastidious aerobic bacteria.

1. Cation-adjusted Mueller-Hinton broth (CAMHB) dispensed in approximately 250-ml aliquots into flasks and in 5-ml aliquots into tubes. Store at 2 to 8°C.

2. Sterile 0.9% NaCl dispensed in 0.9-ml aliquots into capped tubes. Store at 2 to 8°C.

3. BAP. Store at 2 to 8°C.

4. Antimicrobial agent stock solutions, 1 ml of 1,280-μg/ml solution. Store at −70°C. Refer to procedure 5.14 for preparation.

5. Beaker of 95% ethanol to sterilize bent glass rods (optional)

III.A. MATERIALS: MACRODILUTION METHOD *(continued)*

6. Media for maintenance of QC organisms
   a. TSA slants. Store at 2 to 8°C.
   b. Brucella broth–15% glycerol dispensed into vials appropriate for freezing. Store at 2 to 8°C.

B. Supplies

1. Sterile acid-washed borosilicate glass test tubes (13 by 100 mm) with metal or plastic closures
2. Sterile 1-, 5-, and 10-ml glass or plastic serologic pipettes and pipette bulb
3. McFarland 0.5 turbidity standard
4. Sterile disposable polystyrene capped tubes (5 ml; 12 by 75 mm)
5. Sterile cotton-tipped swabs

C. Equipment

1. Test tube racks with 12 spaces per row
2. Micropipettes, 10- and 100-μl capacity with sterile disposable tips
3. Calibrated 0.001-ml loop
4. Vortex mixer
5. Mechanical test tube shaker (fitted in incubator)
6. −70°C freezer
7. 35 ± 2°C ambient-air incubator (CO_2 incubation needed for some organisms)
8. Bunsen burner and bent glass rod "hockey sticks" (optional)

ANALYTICAL CONSIDERATIONS

IV.A. QUALITY CONTROL: MACRODILUTION METHOD

A. QC strains

1. Choose QC strains according to individual antimicrobial agents tested. (See NCCLS standard M7 for details on choosing appropriate QC strains and expected results [5]. See procedure 5.13 for information on appropriate storage and maintenance of QC strains.)
   a. Test QC strains according to the standard procedure for testing patient isolates.
   b. Select a QC strain that will give a midrange endpoint for the antimicrobial agent tested.
2. Record MIC test results for individual agents on QC log worksheet (Appendix 5.10.1–2), and compare MICs obtained with expected results.

B. Growth control tube should show 3 to 4+ turbidity. Purity plate should show good growth and no contaminating organisms.

C. Sterility control should be free of any growth.

D. Inoculum controls should show approximately 75 to 150 colonies.

E. Test considered in control

1. Test broth supports growth of the test and control organisms.
2. MICs for QC strains are within acceptable limits for each individual agent.
3. MBCs for QC strains are within acceptable limits for each individual agent. This is an optional QC parameter (8).
4. Growth and inoculum controls show appropriate growth.
5. Purity plates are free of contaminating organisms.

F. Do not report patient results if any result is out of control (at any point during testing).

1. Notify supervisor.
2. Ascertain the reason for failure, remedy the situation, and repeat the test.
3. See troubleshooting tips in procedures 5.2 and 5.13.

V.A. PROCEDURE: MACRODILUTION METHOD

A. Final concentrations to test

1. Determine MIC for test isolate.
2. Generally, include final concentration ranges from at least eight times the expected MIC to one-half or one-fourth the expected MIC. A range from 128 to 0.06 μg/ml is usually adequate. Avoid testing high concentrations that may not be clinically achievable.

V.A. PROCEDURE: MACRODILUTION METHOD *(continued)*

3. Determine the number of tubes to prepare. Include enough tubes for QC. Each tube will contain a 1-ml volume of antimicrobial agent.

B. Antimicrobial agent working concentrations

See procedure 5.14 for antimicrobial stock solution preparation.

1. Assemble 14 sterile glass test tubes for each antimicrobial agent to be tested.
2. Label the tubes 1 through 14. Place tubes 1 through 12 in the first row and tubes 13 and 14 in the second row of a test tube rack.
   a. Tube 14 is the broth sterility control (broth only).
   b. Tube 13 is the growth control (antimicrobial agent-free broth plus organism).
   c. Tubes 1 through 12 are for antimicrobial agent dilutions.
3. Using a 10-ml pipette, dispense 1 ml of broth into tubes 2 through 14.
4. Dispense 1.8 ml of broth into tube 1.
5. Use a 1-ml pipette (or a 1,000-μl micropipette) to add 0.2 ml of 1,280-μg/ml stock solution to 1.8 ml of CAMHB in tube 1 to obtain a solution of 128 μg/ml.
6. Vortex.
7. Using a new pipette, transfer 1 ml from tube 1 into tube 2.
8. Vortex.
9. Repeat steps V.A.B.7 and 8 sequentially through tube 12.
10. Discard 1 ml from tube 12.
11. The final concentrations of antimicrobial agent will be 128 to 0.06 μg/ml when the tubes are inoculated. If the volume of inoculum exceeds 10% of the tube volume, the diluting effect of the inoculum on the antimicrobial agent must be taken into account (5).

C. Preparation of inoculum

1. Using a sterile swab, transfer organisms from four or five colonies of similar morphology to 5 ml of CAMHB to obtain a suspension equivalent to the turbidity of a McFarland 0.5 standard.
2. Transfer 0.1 ml of the standardized suspension to 5 ml of CAMHB.
   a. Fastidious organisms may require transfer of a larger volume to achieve the required turbidity in a reasonable time.
   b. Do not test fastidious organisms that do not grow well in broth.
3. Vortex for 15 to 20 s.
4. Incubate on a mechanical shaker at 35 ± 2°C until visibly turbid. Do not allow growth to produce heavy turbidity. This preparation should correspond to an early to mid-logarithmic growth phase for most rapidly growing organisms. Growth phase may be confirmed by monitoring an organism growth curve in broth for 4 to 6 h.
   a. Sample aliquots of broth and organism every 30 min.
   b. Enumerate organisms by performing serial dilution bacterial counts on agar.
   c. Plot CFU per milliliter versus time on semilog paper.
   d. Determine approximate time and approximate turbidity density required to reach mid-logarithmic growth.
5. Adjust turbidity of the suspension with sterile 0.85% NaCl to match a McFarland 0.5 turbidity standard (approximately 1.5×10^8 CFU/ml).
6. Add 0.3 ml of the standardized log-phase suspension to 9.7 ml of CAMHB (1:32 dilution) to obtain a suspension containing 4×10^6 to 5×10^6 CFU/ml. (This volume of inoculum suspension is sufficient to inoculate six sets of antimicrobial dilution tubes.)
7. Vortex.
8. Use the inoculum suspension within 15 min of preparation.

V.A. PROCEDURE: MACRODILUTION METHOD *(continued)*

D. Inoculation and incubation

1. Using a micropipette to inoculate tubes, add 0.1 ml of suspension containing 5×10^6 CFU/ml by placing the pipette tip just below the broth meniscus.
   - **a.** Use a new pipette tip for each tube, and avoid contact of the tip with the wall of the tube.
   - **b.** Mix by gently drawing solution back and forth into the pipette tip 6 to 10 times without causing air bubbles or splashes.
   - **c.** Final organism concentration is approximately 5×10^5 CFU/ml.
2. Using a 0.001-ml calibrated loop, inoculate a purity plate by subculturing 0.001 ml of the McFarland 0.5-matched suspension onto the surface of a BAP. Streak for isolation.
3. Prepare an inoculum count verification plate as follows.
   - **a.** Remove 0.1 ml from tube 13 (drug-free growth control) immediately after inoculation.
   - **b.** Add to 0.9 ml of saline (1:10). Vortex.
   - **c.** Transfer 0.1 ml of solution from tube prepared in step V.A.D.3.b into 0.9 ml of saline (1:10). Vortex.
   - **d.** Transfer 0.2 ml of solution from tube prepared in step V.A.D.3.c into 0.8 ml of saline (1:5). Vortex.
   - **e.** Plate 0.1 ml of the final dilution onto the surface of a BAP. Perform in duplicate (should contain approximately 100 CFU).
   - **f.** Allow the inoculum to dry slightly, and spread it thoroughly across the agar surface as described in Appendix 5.10.1–3.
4. Incubate the rack of tubes at 35 ± 2°C for 20 h. Note the time.
5. Invert and incubate purity and inoculum count verification plates at 35 ± 2°C.
6. After 20 h of incubation, remove the rack from the incubator.
7. Gently vortex or swirl each tube by hand. This mixing should draw any organisms from the top of the meniscus back into the broth containing antimicrobial agent.
8. Place the rack of tubes back into the incubator, and incubate for an additional 4 h (24 h total).

E. Reading and recording MICs

1. Examine corresponding purity plate. If results are mixed, repeat the test.
2. Examine growth control (tube 13) for 3 to 4+ turbidity, indicating adequate growth. Assess growth as follows.
   - **a.** ± to 1+, very light haze in tube
   - **b.** 2+, light haze in tube
   - **c.** 3+ to 4+, heavy turbidity or fine granular growth throughout tube or dense precipitate at bottom of tube, with hazy supernatant
3. Examine the uninoculated broth control (tube 14). Compare questionable growth with that in this tube when necessary.
4. Examine the inoculum count verification plate. Record results on worksheet (Appendix 5.10.1–4). If count is inadequate (<75 or >150 CFU), repeat the test. Determine the actual number of organisms tested in each tube as follows.
   - **a.** Count the colonies growing on the plate.
   - **b.** Multiply the number of colonies by the reciprocal of the volume of the aliquot plated (0.1 ml) and the dilution factor (5×10^2).
     Example: 123 CFU × 1/0.1 ml × (5×10^2) = 6.2×10^5 CFU/ml
5. Examine tubes 1 through 12. Pick each tube up, gently mix the contents, and hold the tube in transmitted light. Look for growth as evidenced by a button in the bottom of the tube, turbidity, or a combination of both compared with the growth and sterility control tubes.
6. Determine the MIC (the lowest concentration of antimicrobial agent that completely inhibits the growth of the organism).
7. Record results on worksheet (Appendix 5.10.1–4).

V.A. PROCEDURE: MACRODILUTION METHOD *(continued)*

F. Preparation and incubation of MBC subcultures

It is recommended that 100-µl aliquots be subcultured to a single agar plate; however, a procedure for this volume as well as for 10 µl is described, since some laboratories may elect to go with the smaller volume.

1. Using a 10- or 100-µl micropipette, mix the solution by aspirating it up and down 6 to 10 times, and remove an aliquot from each of the tubes showing no growth except tube 14.
2. Plate each sample onto a BAP, using one of the following methods (perform subcultures in duplicate).
   - **a.** Plate 10-µl aliquots onto one-fourth of an agar plate, and spread the aliquot over the entire quadrant.
   - **b.** Plate 10- or 100-µl aliquots onto a single agar plate.
     (1) Allow samples to air dry for 15 to 20 min.
     (2) Use a bent glass rod to spread the aliquot over the entire plate surface (*see* Appendix 5.10.1–3).
3. Invert plates, and incubate them at 35 ± 2°C.
4. Read preliminary results daily and final results (number of colonies growing on each subculture plate) at the following times.
   - **a.** 24 h for rapidly growing gram-negative rods
   - **b.** 48 h for staphylococci and enterococci
   - **c.** 72 h for all other organisms

G. Determining the MBC

1. From the initial MIC inoculum, determine the number of colonies allowable for a 99.9% MBC endpoint.
   - **a.** If a 10-µl aliquot was plated, use Appendix 5.10.1–5, and record the number of allowable colonies based on the final inoculum size.
     Example: If inoculum was 6.2×10^5 CFU/ml, then >29 CFU is defined as the rejection value.
   - **b.** Based on plating a 100-µl sample, calculate the rejection value.
     Example: initial inoculum × aliquot plated × allowable viable percent = $(6.2 \times 10^5$ CFU/ml) × 0.1 ml × 0.001 = 62 CFU. A plate showing >62 CFU does not meet the 99.9% endpoint; ≤62 colonies indicate ≥99.9% killing.
2. Examine each subculture plate, and record the number of colonies.
3. The MBC is defined as the lowest concentration showing ≥99.9% killing.
   - **a.** With 10-µl aliquot subcultures, when the sum of the colonies growing on duplicate subcultures of each dilution is equal to or less than the rejection value, that dilution is bactericidal at ≥99.9%. Below is an example of how to use Appendix 5.10.1–5 for an organism for which the MIC is 8 µg/ml and with an inoculum of 6.2×10^5 CFU/ml (TNTC, too numerous to count).

Tube no.	Amount of agent (µg/ml)	CFU/plate	Total CFU	≥99.9% Kill
1	128	2, 0	2	Yes
2	64	3, 8	11	Yes
3	32	10, 8	18	Yes
4	16	15, 11	33	No
5	8	TNTC	TNTC	No

MBC = 32 µg/ml

   - **b.** With 100-µl aliquot subcultures, when the number of colonies growing on both duplicate subcultures is equal to or less than the rejection value, that dilution is bactericidal at ≥99.9%.

PREANALYTICAL CONSIDERATIONS

III.B. MATERIALS: MICRODILUTION METHOD

Include QC information on reagent container and in QC records.

A. Media and reagents (storage conditions)

The media listed include those necessary for testing rapidly growing aerobic bacteria. Refer to Appendix 5.10.1–1 for a complete listing of suggested media for testing fastidious aerobic bacteria.

1. CAMHB dispensed in approximately 250-ml aliquots into flasks and in 5-ml aliquots into tubes. Store at 2 to 8°C.
2. Sterile 0.9% NaCl dispensed in 0.9-ml aliquots into capped tubes. Store at 2 to 8°C.
3. BAP. Store at 2 to 8°C.
4. Antimicrobial agent stock solution, 1 ml of 1,280-μg/ml solution. Store at −70°C. See procedure 5.14 for preparation.
5. Beaker of 95% ethanol to sterilize bent glass rods (optional)
6. Media for maintenance of QC organisms
   a. TSA slants. Store at 2 to 8°C.
   b. Brucella broth–15% glycerol dispensed into vials appropriate for freezing. Store at 2 to 8°C.
7. Sterile distilled water with 0.02% Tween 80 dispensed in aliquots of 25 ml in screw-cap tubes. Store at 25°C.

B. Supplies

1. Prepared broth microdilution (U-bottom) tray containing 100 μl of antimicrobial solution per well. Store at −70°C. See procedures 5.2 and 5.15.
2. Microdilution tray sealers
3. Sterile single-inoculum reservoir trays
4. Sterile disposable plastic multipronged inoculators (or other inoculating device)
5. Sterile 1-, 5-, and 10-ml glass or plastic serologic pipettes and pipette bulb
6. McFarland 0.5 turbidity standard
7. Sterile disposable polystyrene capped tubes (5 ml; 12 by 75 mm)
8. Sterile cotton-tipped swabs

C. Equipment

1. Micropipettes, 100- and 1,000-μl (optional) capacity with sterile disposable tips
2. Calibrated 0.001-ml loop
3. Microdilution tray reading device
4. Microdilution tray storage container
5. Vortex mixer
6. Mechanical test tube shaker (fitted in incubator)
7. −70°C freezer
8. 35 ± 2°C ambient-air incubator (CO_2 incubator needed for some organisms)
9. Bunsen burner and bent glass rod "hockey sticks" (optional)
10. Mechanical microdilution tray shaker-mixer (optional)

ANALYTICAL CONSIDERATIONS

IV.B. QUALITY CONTROL: MICRODILUTION METHOD

See item IV.A above.

V.B. PROCEDURE: MICRODILUTION METHOD

A. Final concentrations to test

1. Determine MIC for test isolate.
2. Generally, include final concentration ranges from at least eight times the expected MIC to one-half or one-fourth the expected MIC. A range from 128 to 0.06 μg/ml is usually adequate. Avoid testing high concentrations that may not be clinically achievable.
3. Determine the number of broth microdilution trays to prepare. Include enough trays for QC. For example, testing the organism of interest in duplicate with one QC strain singly requires three trays. The final volume of each well will be 100 μl.

V.B. PROCEDURE: MICRODILUTION METHOD *(continued)*

B. Preparation of trays

Prepare broth microdilution tray containing 100 μl of antimicrobial solution per well (see procedure 5.14 for antimicrobial stock solution preparation and procedures 5.2 and 5.15 for routine broth microdilution methods).

1. Format a 96-well microdilution tray with serial twofold dilutions (128 to 0.06 μg/ml), or use a prepared, commercially purchased tray.
2. When preparing trays, use one well as the broth sterility control and two wells for the antimicrobial agent-free growth controls. If a commercially prepared tray is used, remove contents from a well of low concentration (preferably that of a narrow-spectrum beta-lactam; do not use quinolone or aminoglycoside wells), wash the well several times with 100-μl aliquots of CAMHB, and replace the contents of the well with 100 μl of CAMHB.
3. Multiple agents can be tested on one tray.
4. Test one organism per tray.

C. Preparation of inoculum

1. Using a sterile swab, transfer organisms from four or five colonies of similar morphology to 5 ml of CAMHB to obtain a suspension with a turbidity approximately equal to that of a McFarland 0.5 standard.
2. Transfer 0.1 ml of the standardized suspension to 5 ml of CAMHB.
   - **a.** Fastidious organisms may require transfer of a larger volume to achieve the required turbidity in a reasonable time.
   - **b.** Do not test fastidious organisms that do not grow well in broth.
3. Vortex for 15 to 20 s.
4. Incubate on a mechanical shaker at 35 ± 2°C until visibly turbid. Do not allow growth to produce heavy turbidity. This preparation should correspond to an early to mid-logarithmic growth phase for most rapidly growing organisms. Growth phase may be confirmed by monitoring an organism growth curve in broth for 4 to 6 h.
   - **a.** Sample aliquots of broth and organism every 30 min.
   - **b.** Enumerate organisms by performing serial dilution bacterial counts on agar.
   - **c.** Plot CFU per milliliter versus time on semilog paper.
   - **d.** Determine approximate time and approximate turbidity density required to reach logarithmic growth.
5. Adjust turbidity with sterile 0.9% NaCl to match a McFarland 0.5 turbidity standard (approximately 1.5×10^8 CFU/ml).
6. Calculate volume of standardized suspension to be added to 25 ml of water diluent to obtain final organism concentration of approximately 5×10^5 CFU/ml in each well.

   Example: When using a disposable plastic multipronged inoculating device that delivers 0.01 ml per well

   McFarland 0.5 suspension = 1.5×10^8 CFU/ml

   Add 0.8 ml of suspension to 25 ml of water diluent (1:31 dilution) = 4×10^6 to 5×10^6 CFU/ml

   Prongs deliver 0.01 ml of suspension = 4×10^4 to 5×10^4 CFU per well (4×10^5 to 5×10^5 CFU per ml)
7. Vortex.
8. Use the inoculum suspension within 15 min of preparation.

D. Inoculation and incubation

1. Add 0.8 ml of standardized suspension to 25 ml of water-Tween 80.
2. Mix by inverting tubes five or six times (try to avoid producing air bubbles).
3. Within 15 min, inoculate the MIC tray.
   - **a.** Remove the inoculator-reservoir set from the plastic packaging.
   - **b.** Remove prongs from the reservoir tray, and pour contents of the diluted inoculum into the tray.

V.B. PROCEDURE: MICRODILUTION METHOD *(continued)*

c. Dip prongs into the inoculum suspension.
(1) Orient sterility well so that it is not inoculated.
(2) Press down on the inoculator firmly to ensure that all prongs come in contact with the organism solution.
d. Inoculate MIC tray by dipping the filled prongs carefully into the MIC tray.
e. Discard prongs.
f. If the volume of inoculum exceeds 10% of the well volume, the diluting effect of the inoculum on the antimicrobial agent must be taken into account (5).

4. Using a 0.001-ml calibrated loop, inoculate a purity plate by subculturing 0.001 ml of the McFarland 0.5-matched suspension onto the surface of a BAP. Streak for isolation.
5. Prepare an inoculum count verification plate.
a. Remove 0.1 ml from one of the agent-free growth control wells after inoculation (optional: remove only 0.05 ml).
b. Add to 0.9 ml of sterile 0.9% NaCl (1:10) (optional: add only 0.45 ml). Vortex.
c. Transfer 0.1 ml from tube prepared in step V.B.D.5.b into 0.9 ml of sterile 0.9% NaCl (1:10) to obtain a 1:100 dilution of the initial inoculum (1:10 $\times$ 1:10 = 1:100). Vortex.
d. Transfer 0.2 ml of solution from tube prepared in step V.B.D.5.c into 0.8 ml of sterile 0.9% NaCl (1:5). Vortex.
e. Plate 0.1 ml of the final dilution onto the surface of a BAP. Perform in duplicate.
f. Allow the inoculum to dry for 15 to 20 min, and spread it thoroughly across the agar surface as described in Appendix 5.10.1–3.
g. BAP plate will contain approximately 100 CFU.
6. Stack inoculated MIC trays in stacks of no more than four, and place the stacks in a microdilution tray storage container with the top loose (or into plastic bags). An empty tray can be placed on top of the stack.
7. Incubate MIC trays at 35 $\pm$ 2°C for 24 h. Note the time.
8. Invert the plates, and place purity and inoculum verification control plates at 35 $\pm$ 2°C for rapidly growing organisms.
9. After 24 h of incubation, remove the trays and plates from the incubator.

E. Reading and Recording MICs

1. Place the tray on an appropriate reading device, and read broth microdilution MICs as described in procedure 5.2.
2. Examine growth well for 3 to 4+ turbidity, indicating adequate growth. Assess growth as follows.
a. $\pm$ to 1+, very light haze in well
b. 2+, light haze in well
c. 3+ to 4+, heavy turbidity or fine granular growth throughout well or dense precipitate at bottom of well with hazy supernatant
3. Examine the uninoculated broth control. Compare questionable growth with that in this well when necessary.
4. Examine corresponding purity control plate. If results are mixed, repeat the test.
5. Examine the inoculum count verification plate. Record results on worksheet (Appendix 5.10.1–4). If the count is inadequate (<75 or >150 CFU), repeat the test.
a. Count the colonies growing on the plate.
b. Multiply the number of colonies by the reciprocal of the volume of the aliquot plated and the dilution factor.
Example: 123 CFU $\times$ (1/0.1 ml) $\times$ (5×10^2) = 6.2×10^5 CFU/ml

V.B. PROCEDURE: MICRODILUTION METHOD *(continued)*

6. Examine each well, and record growth or no growth for each well.
7. Determine the MIC (the lowest concentration of antimicrobial agent that completely inhibits the growth of the organism).
8. Record results on worksheet (Appendix 5.10.1–4).

F. Preparation and incubation of MBC subcultures

1. Mix the contents of each well to achieve a uniform suspension of organisms by using one of the following methods.
   a. Place tray on a mechanical microdilution tray shaker-mixer. Set the speed high enough to displace the well contents halfway up the internal wall. Shake for approximately 1 min.
   b. Aspirate the contents of a well in and out of a 100-µl pipette tip 6 to 10 times.
   c. Gently tap the plate against a solid object until a uniform suspension is obtained in each well.
2. Using a 100-µl micropipette, remove the entire contents of each well showing no visible growth, except sterility control well.
3. Plate each 100-µl sample onto a separate BAP.
4. Allow samples to air dry for 15 to 20 min.
5. Spread samples over the entire plate surface with a bent glass rod (*see* Appendix 5.10.1–3).
6. Invert plates, and incubate them at 35 ± 2°C.
7. Read preliminary results daily and final results (number of colonies growing on agar) at the following times.
   a. 24 h for rapidly growing gram-negative rods
   b. 48 h for staphylococci and enterococci
   c. 72 h for all other organisms

G. Determining the MBC

See item V.A above.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Interpretation

1. Record MICs for each agent tested on the worksheet (Appendix 5.10.1–4). Interpret results according to criteria in procedure 5.2.
2. Record MBCs for each agent tested on the worksheet (Appendix 5.10.1–4).
3. There are currently no established criteria for interpretation of MBCs.

B. Reporting

Report MIC (and interpretation) and MBC.

Example: *Staphylococcus aureus*

Drug	MIC (µg/ml)	Interpretation	MBC (µg/ml)
Oxacillin	≤0.5	S	≤0.5
Vancomycin	1	S	2

VII. PROCEDURE NOTES

A. Planning antimicrobial concentration ranges to test

1. The dilution scheme described here is appropriate for testing most antimicrobial agents. However, in some situations, testing of higher or lower concentrations may be warranted. A 12-tube set of antimicrobial concentrations is not mandatory; i.e., a 10-tube set may be satisfactory. Generally, use two or three concentrations below the expected MIC and five or six concentrations above the expected MIC. However, there is no standardized range to

VII. PROCEDURE NOTES
(continued)

test. Do not test extremely high concentrations that are interpreted as resistant and represent levels not achievable clinically.

a. Example: Cefazolin MIC for *Escherichia coli* = 4 μg/ml
Test concentrations ranging from 0.5 to 128 μg/ml.

b. Example: Ciprofloxacin MIC for *E. coli* = 0.125 μg/ml
Test concentrations ranging from 0.015 to 8 μg/ml.

2. Some investigators test only the MIC, two times the MIC, and four times the MIC for antimicrobial agents known to be bactericidal with organisms that do not routinely exhibit various phenomena such as the paradoxical effect, tolerance, or persistence (e.g., *E. coli* and gentamicin).

3. Commercially prepared panels can be adapted for MBC determination as described in item V.B above.

B. Procedure

1. Alternative broth macrodilution methods include using a 2- or 10-ml final tube volume. Inocula and dilutions must be adjusted accordingly.

2. When anaerobes are tested, use a final inoculum of 1×10^6 to 5×10^6 CFU/ml (6).

3. High final inoculum densities (5×10^7 CFU/ml) are sometimes tested to study inoculum effect. However, after 24 h of incubation, tubes or wells at or above the obvious MIC usually appear slightly turbid or hazy or have a small pellet at the bottom of the tube or well. An additional control is needed to differentiate between true growth turbidity and background turbidity when a final inoculum of 10^7 organisms per ml is used. Set up an additional broth tube or well containing 0.0002% merthiolate solution inoculated with the organism suspension for comparison. Prepare an additional 1:100 dilution to enumerate initial inoculum from growth control. A 99.9% MBC endpoint results in 100 times the allowable number of viable CFU, so subcultures require a 1:100 dilution before being plated onto agar media to facilitate counting bacterial colonies.

4. Slightly turbid broth hazes are sometimes observed during reading of the broth macrodilution MIC when standard inoculum densities at antimicrobial concentrations above an obvious endpoint are used. Process for MBC determination to determine if viable organisms, a contaminant, or nonviable organisms are present at the 24-h endpoint.

5. To determine MBCs, two subculture volumes (10 and 100 μl) have been used for broth macrodilution methods. The larger volume is preferred, and 100 μl should always be used for broth microdilution methods. Do not use volumes of $<$10 μl, because too few colonies may be transferred. Use of volumes of $>$100 μl may result in antimicrobial agent carryover.

a. Subculture volume depends on the following.

(1) Material costs (e.g., plate four samples per agar plate with 10 μl or one sample per agar plate with 100 μl)

(2) The nature of the organism tested and previous published references (e.g., swarming *Proteus* species, staphylococci)

(3) Contribution of drug carryover

(4) Numbers of CFU remaining in MIC tube ($<$10, 100, 500), desired lower limit of detection, and initial inoculum size (standard or high)

b. Do not use a disposable multiprong inoculator device to subculture MBCs, because the volume transferred is too variable.

6. Antimicrobial agent carryover may be a problem when high concentrations are tested, and this may prevent viable bacteria from growing into colonies on agar. Suggested ways to overcome this problem (10) are as follows.

a. Test concentrations no more than 16 times the MIC so that effects are overcome by dilution onto agar medium.

VII. PROCEDURE NOTES
(continued)

b. Use an entire agar plate, and spread the sample over a large surface area.
c. Enzymatically inactivate penicillin or ampicillin by overlaying the spread aliquot with 0.1 ml of a broad-spectrum beta-lactamase such as Penase.
d. Filter aliquots over sterile disposable 0.2-µm-pore-size filters, and resuspend the organisms in broth.
e. Chemically inactivate the antimicrobial agent.
f. Remove the antimicrobial agent by using Amberlite beads (Rohm and Hass Co.).

7. Medium described here for testing MICs and MBCs does not contain serum, which determines the contribution of protein-binding effects. A human serum broth diluent 1:1 ratio (CAMBH-human serum) could be used for antimicrobial agents that are highly protein bound (4).
8. Do not test known bacteriostatic antimicrobial agents for MBCs. Consult published references for the agent and organism of interest.
9. Organisms not in logarithmic growth phase may be tested with novel agents to determine differences in bactericidal activity between the two phases. However, most antimicrobial agents exert a bactericidal effect only on actively growing bacteria.
10. Plates used for subculturing organisms should not be freshly prepared, because the excess moisture on the agar surface will not evaporate quickly and developing bacterial colonies may coalesce. Freshly prepared or purchased commercial media should be allowed to sit out overnight without plastic packaging at room temperature to allow excess moisture to evaporate. Subculture aliquots will then quickly wick into the agar, dispersing residual antimicrobial agent and reducing the activity against viable organisms.
11. Take appropriate precautions when working with 95% ethanol. It is highly flammable.
12. Do not use calibrated loops for subculturing aliquots to determine MBCs. The loops are too variable for subculturing potentially low numbers of bacteria that might be significant in these tests.
13. Insufficient contact between the bacteria and the antimicrobial agent may occur when bacteria adhere to the tube or well in which the test is performed and escape the antibacterial action of the agent during incubation. Mixing the contents of the tube prior to subculturing for MBC testing resuspends the organisms. A falsely elevated MBC may result. Acid-washed glass tubes and inoculation of antimicrobial dilutions by using a subsurface inoculation technique minimize this event. It has not been established if this phenomenon occurs in microdilution wells.
14. CO_2 incubation alters pH and affects the activities of some antimicrobial agents. It should therefore be avoided.

C. Interpretation

1. In general, bactericidal antimicrobial agents produce MBCs within 2 twofold dilutions of the MIC.
2. Numerous interpretative problems that may be related to technical or biological issues can occur with MBC test results. These have been described by various investigators (1, 2, 3, 7, 9, 10) and for various organisms such as *S. aureus*, usually with beta-lactams.

VII. PROCEDURE NOTES
(continued)

a. The paradoxical effect, or Eagle effect, occurs when increasing numbers of bacteria survive at progressively higher concentrations of antimicrobial agent even though several lower concentrations meet the 99.9% MBC endpoint. This effect may be observed with various bacterial species and antimicrobial agents, particularly beta-lactam agents.

b. Persisters are small numbers of bacteria that survive the bactericidal effect of an antimicrobial agent so that in the MBC test, they would appear as colonies numbering close to the 0.1% cutoff. A few concentrations may meet the 99.9% MBC definition, but several concentrations will exhibit growth just in excess of the rejection value. Retesting the MICs for persisters results in MICs identical to those for the original organism culture.

c. Tolerance occurs when a significant percentage of cells survive the apparent bactericidal effect of an antimicrobial agent without an accompanying MIC increase. With the MBC test, this can be observed when a series of dilutions grow organisms 10 to 100 times the allowable rejection value. The MBC/MIC ratio is $\geq$32 after 24 h of incubation of the MIC test.

d. If a paradoxical effect, persisters, or tolerance seems to be occurring, try the following.

(1) Reconfirm the MICs for surviving organisms to determine if resistant subpopulations have been selected.

(2) Repeat MIC and MBC tests with a fresh subculture of the original isolate to confirm if the effect is repeatable when the same method is used.

(3) Determine if organisms were in mid-logarithmic phase when tested.

(4) Perform a time-kill assay to determine the validity of the phenomenon.

VIII. LIMITATIONS

A. Although parts of the MBC procedures described above have been published in NCCLS documents (4), they are not considered standardized methods. This and other references describe multiple alternatives and comparisons for several steps in the MBC procedure (7, 9).

B. The clinical value and interpretation of the MBC are limited because of various technical issues and unknown correlation with clinical outcome. However, clinicians may be able to assess patient response to an antimicrobial agent regimen.

C. Broth macrodilution and microdilution results may not produce the same MBC endpoint results because of technical differences in the methods.

REFERENCES

1. **Eagle, H., and A. D. Musselman.** 1948. The rate of bacterial action of penicillin *in vitro* as a function of its concentration, and its paradoxically reduced activity at high concentrations against certain organisms. *J. Exp. Med.* **88:**99–131.
2. **Gunnison, J. B., M. A. Fraher, and E. Jawetz.** 1963. Persistence of *Staphylococcus aureus* in penicillin *in vitro*. *J. Gen. Microbiol.* **34:**335–349.
3. **Handwerger, S., and A. Tomasz.** 1985. Antibiotic tolerance among clinical isolates of bacteria. *Rev. Infect. Dis.* **7:**368–386.
4. **NCCLS.** 1999. *Methods for Determining Bactericidal Activity of Antimicrobial Agents.* Approved guideline M26-A. NCCLS, Wayne, Pa.
5. **NCCLS.** 2003. *Methods for Dilution Susceptibility Tests for Bacteria That Grow Aerobically*, 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.
6. **NCCLS.** 2001. *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria*, 5th ed. Approved standard M11-A5. NCCLS, Wayne, Pa.

REFERENCES *(continued)*

7. **Pearson, R. D., R. T. Steigbigel, H. T. Davis, and S. W. Chapman.** 1980. Method for reliable determination of minimal lethal antibiotic concentrations. *Antimicrob. Agents Chemother.* **18:**699–708.
8. **Reimer, L. G., C. W. Stratton, and L. B. Reller.** 1981. Minimum inhibitory and bactericidal concentrations of 44 antimicrobial agents against three standard control strains in broth with and without human serum. *Antimicrob. Agents Chemother.* **19:**1050–1055.
9. **Shanholtzer, C. J., L. R. Peterson, M. L. Mohn, J. A. Moody, and D. N. Gerding.** 1984. MBCs for *Staphylococcus aureus* as determined by macrodilution and microdilution techniques. *Antimicrob. Agents Chemother.* **26:**214–219.
10. **Woolfrey, B. F., R. T. Lally, and M. N. Edberer.** 1985. Evaluation of oxacillin tolerance in *Staphylococcus aureus* isolates by a novel method. *Antimicrob. Agents Chemother.* **28:**381–388.

SUPPLEMENTAL READING

Gerding, D. N., J. Foxworth, C. E. Hughes, D. M. Bamberger, and T. A. Larson. 1996. Extravascular antimicrobial distribution and the respective blood concentrations in humans, p. 835–899. *In* V. Lorian (ed.), *Antibiotics in Laboratory Medicine*, 4th ed. The Williams & Wilkins Co., Baltimore, Md.

Taylor, P. C., F. D. Schoenknecht, J. C. Sherris, and E. C. Linner. 1983. Determination of minimal bactericidal concentrations of oxacillin for *Staphylococcus aureus:* influence and significance of technical factors. *Antimicrob. Agents Chemother.* **23:**142–150.

APPENDIX 5.10.1–1

MBC testing conditions for various bacteria[a]

Organism or group	Broth diluent for MIC[b]	MBC		
			Incubation[c]	
		Subculture agar	Length (h)	Atmosphere
Members of the family *Enterobacteriaceae* and non-glucose-fermenting gram-negative rods	CAMHB	BAP[a]	24	Ambient air
Enterococcus spp.	CAMHB	BAP	48	Ambient air
Haemophilus spp.	HTM	CHOC	72	Ambient air (CO_2 if needed)
Staphylococcus spp.	CAMHB[d]	BAP	48	Ambient air
Streptococcus pneumoniae	CAMHB with 2–5% LHB or THB	BAP	72	Ambient air (CO_2 if needed)
Streptococcus spp.	CAMHB with 2–5% LHB or THB	BAP	72	Ambient air (CO_2 if needed)
Nutritionally variant *Streptococcus* spp.	CAMHB or THB with 0.001% pyridoxal HCl	CHOC with 0.001% pyridoxal HCl	72	CO_2

[a] Inhibitory portion of test is to be incubated for 24 h. This table applies to broth macrodilution and microdilution.

[b] Specified broth appropriate for growing inoculum to log phase and for time-kill assays. HTM, *Haemophilus* test medium; LHB, lysed horse blood; THB, Todd-Hewitt broth.

[c] All incubations were at 35°C.

[d] Add 2% NaCl for penicillinase-resistant penicillins (oxacillin, methicillin, nafcillin, cloxacillin, dicloxacillin, and flucloxacillin).

APPENDIX 5.10.1–2

Worksheet for MIC and MBC QC (*see* p. 5.10.1.16)

APPENDIX 5.10.1–3

Technique for Using Bent Glass Rod "Hockey Sticks" for Colony Counts

A. Allow broth aliquot transferred onto agar to dry slightly (15 to 20 min). Agar plates should be free of excess moisture to prevent the development of coalescing bacterial colonies.
B. Using one sterile rod, spread the aliquot evenly across the entire surface in two or three different directions to create a bacterial lawn.
C. To sterilize the glass rod, *use extreme caution* when performing the following procedure, because 95% ethanol is very flammable. Acetone or methanol may be used in place of 95% ethanol.
   1. Place ethanol in a glass beaker large enough to accommodate the bent length of the rod. The ethanol should be deep enough to cover the bent end of the rod. Restore the ethanol volume as needed. Place beaker in a metal or glass pan or bowl to confine accidental spills. Keep a large nonburnable container (e.g., metal waste can or bucket) nearby to place over beaker and pan to extinguish accidental fires.
   2. Place incinerator with flame well away from the ethanol, and/or contain it in a flame-resistant box.
   3. Submerge bent portion of rod into 95% ethanol for at least 1 min.
   4. Remove the rod from the ethanol, and allow excess liquid to drain into beaker.
   5. Place rod containing residual alcohol in the burner flame to ignite the alcohol and sterilize the rod.
   6. Hold rod parallel to the bench top until the flames have subsided.
   7. Lay rod parallel to the bench top on a test tube rack to cool.
   8. Reuse once rod has cooled.
D. Clean rods in detergent and water after use.

APPENDIX 5.10.1–4

Worksheet for MBCs (*see* p. 5.10.1.17)

APPENDIX 5.10.1–5

Rejection value and calculated sensitivity and specificity for each initial concentration based on duplicate 0.01-ml samples[a]

Final inoculum (CFU/ml)[b]	Rejection value[c]	Sensitivity[d] (%)	Specificity[d] (%)
1×10^5	4	77	97
2×10^5	8	89	99
3×10^5	15	99	99
4×10^5	20	99	99
5×10^5	25	99	99
6×10^5	29	99	99
7×10^5	33	99	99
8×10^5	38	99	99
9×10^5	42	99	99
1×10^6	47	99	99
2×10^6	91	99	99
3×10^6	136	99	99
4×10^6	182	99	99
5×10^6	227	99	99
6×10^6	273	99	99
7×10^6	318	99	99
8×10^6	364	99	99
9×10^6	409	99	99
1×10^7	455	99	99

[a] When the sum of colonies from duplicate samples was equal to or less than the rejection value, the antimicrobial agent was declared lethal (a 0.999 or greater reduction in the final inoculum). (Adapted with permission from **R. D. Pearson, R. T. Steigbigel, H. T. Davis, and S. W. Chapman.** 1980. Method for reliable determination of minimal lethal antibiotic concentrations. *Antimicrob. Agents Chemother.* **18:**699–708.) A 5% error (pipette error plus full sampling error for determination of inoculum) was used. Error was based on duplicate samples for determination of the final inoculum size.

[b] As determined from colony count plate.

[c] Number of colonies.

[d] Sensitivity and specificity were calculated for each specific final inoculum concentration and rejection value.

MIC/MBC QC

Year _____

Antimicrobial _____

Expected Range (µg/ml)

QC Organism _____ MIC _____ MBC _____
QC Organism _____ MIC _____ MBC _____
QC Organism _____ MIC _____ MBC _____

			Tube Dilution Set						Results																				
Date Test	Tech	Date Prep.	Tech	Broth Type	Broth Lot #	Lot # Stock AMIC	Exp. Date	QC Org	MIC (µg/ml)	MBC (µg/ml)	Purity Cont.	Inoc. Cont	MBC Cutoff	Tech	Record number of CFU from sub of respective concentration (µg/ml) for MBC														
																512	256	128	64	32	16	8	4	2	1	.5	.25	.12	.06	.03
															24 h														
															48 h														
															24 h														
															48 h														
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															48 h														

MBC Worksheet

	ANTIMICROBIAL LOT #		Date Tech											MIC µg/ml		MBC µg/ml		INOC. COUNT MBC cutoff:		
		µg/ml	512	256	128	64	32	16	8	4	2	1	0.5	0.25	0.12	0.06	0.03	CONTROLS		
	MIC																	+	−	PUR
	MBC	24 hrs																		MIC
		48 hrs																		
		72 hrs																		MBC

	ANTIMICROBIAL LOT #		Date Tech											MIC µg/ml		MBC µg/ml		INOC. COUNT MBC cutoff:		
		µg/ml	512	256	128	64	32	16	8	4	2	1	0.5	0.25	0.12	0.06	0.03	CONTROLS		
	MIC																	+	−	PUR
	MBC	24 hrs																		MIC
		48 hrs																		
		72 hrs																		MBC

	ANTIMICROBIAL LOT #		Date Tech											MIC µg/ml		MBC µg/ml		INOC. COUNT MBC cutoff:		
		µg/ml	512	256	128	64	32	16	8	4	2	1	0.5	0.25	0.12	0.06	0.03	CONTROLS		
	MIC																	+	−	PUR
	MBC	24 hrs																		MIC
		48 hrs																		
		72 hrs																		MBC

	ANTIMICROBIAL LOT #		Date Tech											MIC µg/ml		MBC µg/ml		INOC. COUNT MBC cutoff:		
		µg/ml	512	256	128	64	32	16	8	4	2	1	0.5	0.25	0.12	0.06	0.03	CONTROLS		
	MIC																	+	−	PUR
	MBC	24 hrs																		MIC
		48 hrs																		
		72 hrs																		MBC

	ANTIMICROBIAL LOT #		Date Tech											MIC µg/ml		MBC µg/ml		INOC. COUNT MBC cutoff:		
		µg/ml	512	256	128	64	32	16	8	4	2	1	0.5	0.25	0.12	0.06	0.03	CONTROLS		
	MIC																	+	−	PUR
	MBC	24 hrs																		MIC
		48 hrs																		
		72 hrs																		MBC

5.10.2 Time-Kill Assay

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The time-kill assay is used for examining the rate at which concentrations of an antimicrobial agent(s) kill a bacterial isolate. Time-kill assays can be used to study both concentration-dependent and time-dependent bactericidal activities of antimicrobial agents. Time-kill assays are used to evaluate new antimicrobial agents and combinations of agents (*see* procedure 5.10.3); confirm the paradoxical effect, persisters, or tolerance; and help explain treatment failures in clinical situations in which bactericidal activity may be crucial to patient outcome.

Although the time-kill assay has been described in an NCCLS protocol (1), the assay, like the minimal bactericidal concentration (MBC) method, and the sample times for bacterial enumeration have not been standardized.

A standardized inoculum is added to broth containing various concentrations of an antimicrobial agent and to broth without agent (control). At the initial time of inoculation and at various times thereafter, the numbers of viable CFU remaining are determined by performing serial dilution bacterial colony counts on an aliquot from each test vial. Results for the control and each agent concentration are plotted versus time. Generally, a 3-$\log_{10}$ CFU/ml decrease in bacterial counts in antimicrobial solution compared with counts for the growth control indicates an adequate bactericidal response.

Prior to performance of the time-kill assay, several parameters must be defined. Determine the MIC of the specific antimicrobial agent for the organism of interest by a standard MIC procedure, preferably a broth macrodilution method. From the MIC, determine other concentrations of antimicrobial agent to test, usually the MIC, two times the MIC, and four times the MIC. Depending on the mechanism of action (e.g., cell wall-active agent) and growth rate of the organism, determine sample time points (usually 4, 8, 24, and sometimes 48 h after inoculation). For rapidly bactericidal agents such as quinolones or aminoglycosides, shorter sampling periods may be suitable (e.g., 1, 2, 4, 6, and 24 h).

II. SPECIMEN

See procedure 5.10.1, item II.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Media and reagents (storage conditions)

The media listed include those necessary for testing rapidly growing aerobic bacteria. Refer to Appendix 5.10.2–1 for a complete listing of suggested media for testing fastidious aerobic bacteria.

1. Cation-adjusted Mueller-Hinton broth (CAMHB) dispensed in approximately 250-ml aliquots into flasks and in 5-ml aliquots into tubes. Store at 2 to 8°C.
2. Sterile 0.9% NaCl dispensed in 0.9-ml aliquots into capped disposable polystyrene tubes (12 by 75 mm). Store at 25°C.
3. Sterile 0.9% NaCl dispensed in 9.9-ml aliquots into capped disposable polystyrene capped tubes (17 by 100 mm) (optional). Store at 25°C.
4. BAP. Store at 2 to 8°C.
5. Antimicrobial agent stock solution, 1 ml of 1,280-μg/ml solution. Store at −70°C. See procedure 5.14 for preparation.
6. Beaker of 95% ethanol to sterilize bent glass rods (optional)
7. Media for maintenance of QC organisms
   a. TSA slants. Store at 2 to 8°C.
   b. Brucella broth–15% glycerol dispensed into vials appropriate for freezing. Store at 2 to 8°C.

III. MATERIALS *(continued)*

B. Supplies

1. Sterile acid-washed borosilicate glass test tubes (16 by 150 mm) with metal or plastic closures
2. Sterile disposable screw-cap tubes (50 ml; 30 by 115 mm)
3. Sterile disposable polystyrene capped tubes (5 ml; 12 by 75 mm)
4. Sterile 1-, 5-, and 10-ml glass or plastic serologic pipettes and pipette bulb
5. Sterile cotton-tipped swabs
6. McFarland 0.5 and 1.0 turbidity standards
7. Semilog graph paper, 10 cycle (or 10 by 10 graph paper if counts are converted to $\log_{10}$ units before being graphed)

C. Equipment

1. Test tube racks with 12 spaces per row
2. Test tube rack to accommodate 50-ml tubes
3. Micropipette, 100-µl capacity with sterile disposable tips
4. Automatic single-channel micropipette to deliver 500 µl (optional)
5. Calibrated 0.001-ml loop
6. Vortex mixer
7. Mechanical test tube shaker (fitted in incubator)
8. −70°C freezer
9. 35 ± 2°C ambient-air incubator (CO_2 incubation needed for some organisms)
10. Bunsen burner and bent glass rod "hockey sticks" (optional)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

There are no standard QC recommendations for time-kill assays, but see the MBC procedure in procedure 5.10.1. Additionally, the following parameters should be controlled.

A. Test media

1. Growth control tube should support growth of the test strain.
2. Sterility control tube should show no growth.

B. Inoculum

1. Purity plate should show good growth and no contaminating colonies.
2. Colony count at time zero in growth control tube should be between 10^5 and 10^6 CFU/ml of final inoculum.

C. Antimicrobial agent concentration in reaction tubes

1. Aliquots of each dilution should be tested by broth macrodilution or microdilution methods with appropriate QC strains (refer to appropriate previous procedures).
2. Results must be within acceptable MIC ranges for test to be in control.

V. PROCEDURE

A. Final concentrations to test

1. Determine MIC for test isolate by broth macrodilution.
2. Test these final concentration ranges: MIC, two times the MIC, and four times the MIC (and one-fourth and one-half the MIC if desired).
   - **a. Example:** Vancomycin MIC for *Staphylococcus aureus* = 0.25 µg/ml. Test vancomycin concentrations of 0.25, 0.5, and 1 µg/ml (and 0.06 and 0.125 µg/ml if desired) in 10 ml of CAMHB each.
   - **b.** Test separate control tubes, one containing 10 ml of CAMHB plus inoculum (growth control) and one containing 10 ml of CAMHB only (sterility control).
3. Determine number of tubes and volume of antimicrobial agent to prepare, including enough tubes for QC.

V. PROCEDURE *(continued)*

B. Preparation of antimicrobial agent working concentrations

See procedure 5.14 for antimicrobial stock solution preparation.

1. Prepare intermediate dilutions of the stock antimicrobial agent.

a. Dilute stock solution in sterile water (or other appropriate diluent) so that the concentration is 100 times the highest concentration that will be tested in the time-kill test. For example, if the highest concentration tested is 1 μg/ml, dilute 1,280-μg/ml stock 1:12 (0.25 ml into 2.75 ml) to obtain 100 μg/ml.

b. In tubes (12 by 75 mm), perform 2 (or 4 if one-half and one-fourth MICs will be tested) serial twofold dilutions to obtain solutions of 50.0 and 25.0 μg/ml (and 12.0 and 6.0 μg/ml if desired). For example, dilute 0.5 ml of 100-μg/ml stock into 0.5 ml of sterile water to obtain 50 μg/ml. Then dilute 0.5 ml of 50-μg/ml stock into 0.5 ml of sterile water to obtain 25 μg/ml and so forth.

2. Prepare final working concentrations of antimicrobial agents for time-kill assay.

a. Assemble one 50-ml tube (30 by 115 mm) for each concentration (procedure below describes preparation of 20-ml volumes of each drug concentration, which is sufficient for one kill curve and QC or two kill curves).

(1) Use 10 ml for each drug concentration in the time-kill assay.

(2) Use 1 ml for MIC for QC strain.

b. Dispense 19.8 ml of CAMHB into each tube.

c. Add 0.2 ml of 100-fold-concentrated antimicrobial agent to obtain a 1:100 dilution. For example, dilute 0.2 ml of 100-μg/ml stock into 19.8 ml of CAMHB to obtain 1 μg/ml. Then dilute 0.2 ml of 50-μg/ml stock into 19.8 ml of CAMHB to obtain 0.5 μg/ml.

d. Vortex.

3. Dispense 10 ml of each final concentration into sterile glass tubes (16 by 150 mm) for time-kill assay.

4. Dispense 10 ml of CAMHB into each control and sterility tube.

5. Dispense appropriate volumes of antimicrobial agent concentrations and growth control broth for QC MIC (e.g., 1 ml each for broth macrodilution MIC; follow the protocol for performing MIC QC in procedure 5.2).

C. Inoculum preparation

See Appendix 5.10.2–2 also.

1. Using a sterile swab, transfer organisms from four or five colonies of similar morphology to 5 ml of CAMHB to obtain a suspension equivalent to the turbidity of a McFarland 0.5 standard.

2. Transfer 0.1 ml of the standardized suspension to 5 ml of CAMHB.

a. Fastidious organisms may require transfer of a larger volume to achieve the required turbidity in a reasonable time.

b. Do not test fastidious organisms that do not grow well in broth.

3. Vortex for 15 to 20 s.

4. Incubate on a mechanical shaker at 35 ± 2°C until visibly turbid. Do not allow growth to produce heavy turbidity. This preparation should correspond to an early to mid-logarithmic growth phase for most rapidly growing organisms. Growth phase may be confirmed by monitoring an organism growth curve in broth for 4 to 6 h.

a. Sample aliquots of broth and organism every 30 min.

b. Enumerate organisms by performing serial dilution bacterial counts on agar.

c. Plot CFU per milliliter versus time on semilog paper.

d. Determine approximate time and approximate turbidity density required to reach mid-logarithmic growth.

V. PROCEDURE *(continued)*

5. Adjust turbidity of suspension with sterile 0.9% NaCl to match a McFarland 1.0 turbidity standard (approximately 3.0×10^8 CFU/ml).
6. Dilute standardized suspension 1:5 by adding 1 ml of suspension to 4 ml of CAMHB (approximately 6×10^7 CFU/ml).

D. Inoculation and incubation

1. Add 0.1 ml of inoculum containing 6×10^7 CFU/ml to each tube containing 10 ml except sterility control tube.
   - **a.** Use a new pipette tip for each tube.
   - **b.** Vortex gently.
   - **c.** Final organism concentration is approximately 6×10^5 CFU/ml.
2. Using a 0.001-ml calibrated loop, inoculate a purity plate by subculturing 0.001 ml of the McFarland 1.0 standard suspension onto the surface of a BAP. Streak for isolation.
3. Immediately after inoculation, remove 0.1 ml from each reaction tube to perform serial dilution bacterial plate counts for time zero. Refer to Appendix 5.10.2–3.
4. Incubate tubes and purity plate at 35 ± 2°C.
5. At the next sample time (e.g., 4 h), remove tubes from the incubator.
   - **a.** Vortex gently.
   - **b.** Carefully remove a 0.1-ml aliquot from each tube (except sterility control) to perform serial dilution bacterial plate counts.
   - **c.** Remove aliquots from all tubes within 5 to 10 min so that the growth cycle is not disrupted by temperature changes.
6. Incubate tubes at 35 ± 2°C immediately after sampling.
7. Repeat steps V.D.5 and 6 for other time points as necessary.

E. Reading and recording results

1. At 24 h, visually inspect all reaction tubes and note the MIC in the time-kill assay tubes. Also perform serial dilution bacterial plate counts for all tubes, including the growth control.

   Example: At 24 h, the vancomycin MIC = 0.25 μg/ml for test isolate *S. aureus.*
2. After overnight incubation, confirm that test is in control (e.g., QC MIC, purity control). Final colony counts for some slower-growing organisms will require 48 or 72 h of incubation (*see* Appendix 5.10.2–1).
3. For each agent tested, record the MIC observed in the time-kill assay tubes on the worksheet (Appendix 5.10.2–4).
4. Determine the initial inoculum size at time zero to confirm that the appropriate initial bacterial density was tested (approximately 6×10^5 CFU/ml). Record data on the worksheet (Appendix 5.10.2–4).
5. Count the colonies on each of the serial dilution bacterial count plates. Record data on the worksheet (Appendix 5.10.2–4).
   - **a.** Average the counts from plates showing 30 to 300 CFU.
   - **b.** Do not use plates with <30 or >300 CFU in data tabulation unless these are the only counts available for the time point.
   - **c.** Convert to actual CFU per milliliter by multiplying average of raw counts by dilution factors (*see* Appendix 5.10.2–3).
   - **d.** Convert CFU per milliliter to $\log_{10}$ data (optional).
6. Plot CFU per milliliter (*y* axis) versus time (*x* axis) as shown in Appendix 5.10.2–5. Use semilog paper for actual numbers (e.g., 8.4×10^7 CFU/ml) or graph paper (10 by 10) for numbers converted to $\log_{10}$ values (e.g., 7.9 $\log_{10}$ CFU/ml).

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Interpretation

1. Interpret MICs according to criteria in procedure 5.2.

2. Determine which antimicrobial agent concentrations result in a 3-$\log_{10}$ CFU/ml decrease compared with the growth control.

3. Determine the time required to obtain a 3-$\log_{10}$ CFU/ml decrease compared with the growth control for all agent concentrations meeting this endpoint.

B. Reporting

1. Report MICs, and state whether bactericidal activity occurred at a particular concentration.

Example: For *S. aureus*, vancomycin MIC = 0.25 μg/ml. Vancomycin was bactericidal at concentrations of 0.25 μg/ml at 8 h and 0.5 μg/ml at 4 h.

2. Report results graphically as shown in Appendix 5.10.2–5.

VII. PROCEDURE NOTES

A. Procedure

1. Various alternatives for several of the steps in the time-kill assay have been suggested.

a. Perform the test in larger volumes of media (e.g., 30 to 100 ml or more instead of 10 ml) so that there are more challenge bacteria.

b. Perform the test in smaller volumes of media (e.g., 1 or 2 ml) so that an MBC can be determined by using methods in procedure 5.10.1. Technical problems associated with large volumes could thus be avoided.

c. Incubate with agitation when volumes of >10 ml are tested.

d. Sample more frequently for quinolones and aminoglycosides.

2. Testing high concentrations of antimicrobial agents (e.g., a beta-lactam at 128 μg/ml) will require preparing more than 1 ml of 1,280-μg/ml stock or preparing 1 ml of 12,800-μg/ml stock reagent.

3. Serial dilution bacterial plate counts

a. Saline volumes (0.9 ml) for performing dilutions in preparation for colony counts should be dispensed aseptically after autoclaving to minimize volume inconsistencies that may occur during autoclaving of small volumes. Cap tubes tightly for storage to prevent evaporation. These tubes can be stored at 25°C for a few days, but 2 to 8°C is needed for longer storage.

b. When plating suspensions containing high numbers of bacteria (e.g., 10^7 CFU/ml), one 9.9-ml dilution tube of saline can be used (to prepare a 1:100 dilution) in place of two 0.9-ml dilution tubes of saline, thereby minimizing dilution steps and number of plates.

c. The most concentrated sample tested, a 0.1-ml aliquot from the primary reaction tube, represents a 10^{-1} dilution when CFU per milliliter are calculated. Therefore, if this aliquot is plated directly onto an agar plate, the lower limit of detection is 10 CFU/ml.

d. Bacterial colonies can be counted after 24 h of incubation in most situations, but an additional day of incubation usually results in larger colonies that are easier to count. Some fastidious organisms need >24 h of incubation (*see* Appendix 5.10.2–1).

e. Replicate counts from the same dilution should agree within 10%.

f. Antimicrobial agent carryover may be a problem when higher antimicrobial concentrations are tested (e.g., >128 μg/ml) or when a 0.1-ml aliquot from the primary reaction tube is plated onto agar. Enzymatic or chemical inactivators (such as beta-lactamase or a slightly acidic pH for aminoglycosides) can be incorporated into the subculture media (2). Spread the sample over a larger surface area to dilute the antimicrobial agent.

VII. PROCEDURE NOTES *(continued)*

g. Use a latex finger cot, latex glove, or bandage on the thumb of the hand used to snap caps off saline dilution tubes to prevent blistering.

4. Higher initial starting bacterial densities (e.g., 6×10^7 CFU/ml) may be tested. Adjust dilutions accordingly.

5. Since the observed MIC may vary 1 twofold dilution between runs and between macro- and microdilution methods, test several concentrations above and below the expected MIC.

6. Inactivation of antimicrobial agents by organisms during the time-kill assay may result in organism regrowth after an initial decrease in viable organism counts.

B. Strategic planning of bacterial plate count dilutions

1. Growth control kinetics

a. Most rapidly growing organisms will multiply 2 to 3 $\log_{10}$ CFU/ml in the first 4 h from an initial 5-$\log_{10}$ CFU/ml inoculum.

b. Growth control tubes should be slightly turbid at 6 or 8 h after inoculation. Barely visible turbidity approximates 10^7 CFU/ml. McFarland turbidity standards can be used to determine approximate numbers of bacteria in reaction tubes. Use turbidity as a guide for planning dilutions. Nonviable cells sometimes contribute to turbidity.

c. Most organisms exhaust medium nutrients and do not exceed 10 $\log_{10}$ CFU/ml at 24 h.

2. Agents exhibiting time-dependent killing (e.g., beta-lactams)

a. At concentrations equal to or higher than the MIC, organisms are killed slowly. The result is usually a decrease of approximately 1 $\log_{10}$ CFU/ml in the first 4 h, 2 $\log_{10}$ CFU/ml in 8 h, and $\geq$3 $\log_{10}$ CFU/ml at 24 h.

b. At concentrations lower than the MIC, organisms are not killed and should mimic the growth control counts. A short lag in growth may be encountered at 4 h with the one-half MIC and will result in a 1- to 2-$\log_{10}$ CFU/ml increase in growth from the initial inoculum.

3. Agents exhibiting concentration-dependent killing (e.g., glycopeptides, aminoglycosides, quinolones)

a. Organisms are killed faster with increasing concentrations, so for concentrations higher than the MIC, the kill curves will be greater than the curve for the observed MIC. Organisms are typically killed at approximately 1 $\log_{10}$ CFU/ml/h at the MIC. At two and four times the MIC, organisms may decrease 2 and 3 $\log_{10}$ CFU/ml, respectively, in the first hour.

b. At concentrations lower than the MIC (e.g., one-fourth and one-half the MIC), some subpopulations of organisms are killed, but other populations remain viable, resulting in curves that are higher than that for the MIC but lower than that for the growth control.

4. When initiating a study in which bactericidal action against the study organism is unknown, perform an extensive dilution series at each time point to accommodate a wide range of organism densities (e.g., 6 to 8 serial 10-fold dilutions at 12 h).

5. Enumerating bacterial colonies

a. If the only agar plates with viable bacteria show $<$30 or $>$300 colonies, count the colonies and record.

b. If the lowest dilution (0.1 ml from primary reaction plated on agar) results in no colonies, the lower limit of detection is $\leq$10 CFU/ml.

c. If the highest dilution of the series results in $>$1,000 colonies, record and graph as an absolute number (e.g., 10^6 CFU/ml) for an approximate curve. Footnote the graph, and note in procedure that the dilution series should be extended upon retesting.

VIII. LIMITATIONS

A. See limitations under MBC testing procedure (procedure 5.10.1).

B. If the time-kill assay runs for >48 h, the antimicrobial agent could be inactivated. If the time-kill assay runs for 48 h, an increase in bacterial counts after a notable decrease could occur because of selection of resistant mutants.

REFERENCES

1. **NCCLS.** 1999. *Methods for Determining Bactericidal Activity of Antimicrobial Agents.* Approved guideline M26-A. NCCLS, Wayne, Pa.
2. **Woolfrey, B. F., R. T. Lally, and M. N. Ederer.** 1985. Evaluation of oxacillin tolerance in *Staphylococcus aureus* isolates by a novel method. *Antimicrob. Agents Chemother.* **28:**381–388.

APPENDIX 5.10.2–1

MBC testing conditions for various bacteria[a]

Organism or group	Broth diluent for MIC[b]	MBC		
			Incubation[c]	
		Subculture agar	Length (h)	Atmosphere
Members of the family *Enterobacteriaceae* and non-glucose-fermenting gram-negative rods	CAMHB	BAP[a]	24	Ambient air
Enterococcus spp.	CAMHB	BAP	48	Ambient air
Haemophilus spp.	HTM	CHOC	72	Ambient air (CO_2 if needed)
Staphylococcus spp.	CAMHB[d]	BAP	48	Ambient air
Streptococcus pneumoniae	CAMHB with 2–5% LHB or THB	BAP	72	Ambient air (CO_2 if needed)
Streptococcus spp.	CAMHB with 2–5% LHB or THB	BAP	72	Ambient air (CO_2 if needed)
Nutritionally variant *Streptococcus* spp.	CAMHB or THB with 0.001% pyridoxal HCl	CHOC with 0.001% pyridoxal HCl	72	CO_2

[a] Inhibitory portion of test is to be incubated for 24 h. This table applies to broth macrodilution and microdilution.

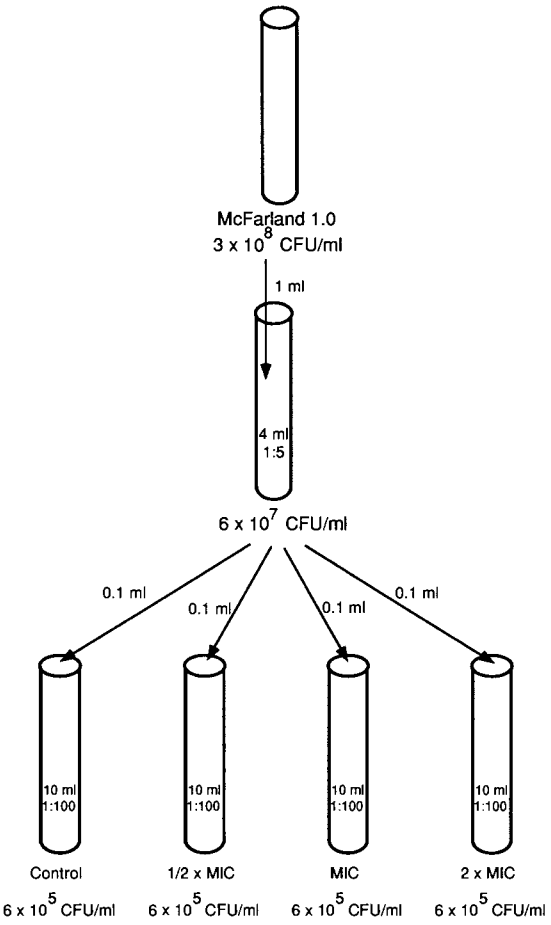
[b] Specified broth appropriate for growing inoculum to log phase and for time-kill assays. HTM, *Haemophilus* test medium; LHB, lysed horse blood; THB, Todd-Hewitt broth.

[c] All incubations were at 35°C.

[d] Add 2% NaCl for penicillinase-resistant penicillins (oxacillin, methicillin, nafcillin, cloxacillin, dicloxacillin, and flucloxacillin).

APPENDIX 5.10.2–2

Preparation of inoculum for reaction tubes in time-kill assay.



APPENDIX 5.10.2–3

Determining Colony Counts from Control and Antimicrobial Tubes for Time-Kill Assay

A. Primary method

1. Perform serial dilution plate counts from the inoculated reaction tubes (growth control tube and antimicrobial tubes) in duplicate. The number of organisms in the control tube will increase with time, whereas the number of organisms in the antimicrobial agent-containing tubes will either increase or decrease depending on whether the antimicrobial agent kills the organism.
2. At time zero, which is the organism inoculation step, prepare 3 serial 10-fold dilutions (through 10^{-4}) in 0.9-ml aliquots of sterile 0.9% NaCl (as shown in Fig. 5.10.2–A1). Prepare dilutions by adding 0.1 ml of each higher concentration to 0.9 ml of 0.9% NaCl. Use a new pipette tip, and vortex well between dilutions.
3. Return primary reaction tubes to the incubator as quickly as possible.
4. Assuming that reaction tubes at time zero contain approximately 6×10^5 CFU/ml, plate 0.1 ml from the 10^{-2}, 10^{-3}, and 10^{-4} dilutions onto two separate agar plates as shown in Fig. 5.10.2–A1. Process as described in Appendix 5.10.1–3.

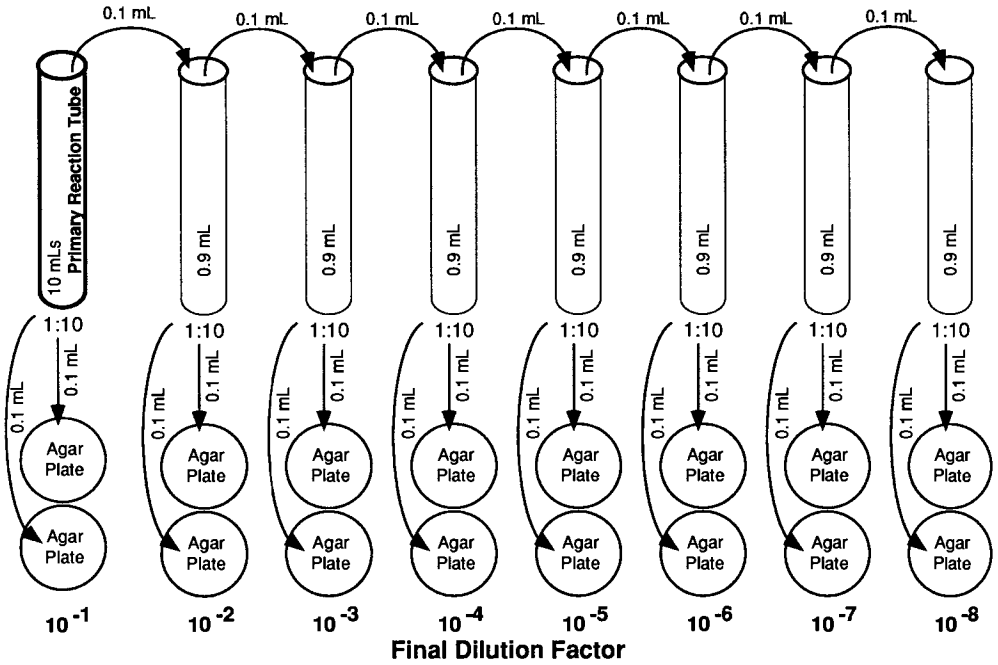


Figure 5.10.2–A1 Dilution scheme for bacterial colony counts.

APPENDIX 5.10.2–3 *(continued)*

5. At the next sample time following a defined interval of incubation (e.g., 4 h), prepare 4 10-fold dilutions (through 10^{-5}) from clear tubes and 6 10-fold dilutions (through 10^{-7}) from reaction tubes that demonstrate any evidence of turbidity. Dilute very turbid tubes to 10^{-8}. Plate 0.1-ml aliquots of each dilution as described above.
   a. Depending on the time point and previous experience with the drug-organism combination, it may not be necessary to prepare and plate all dilutions from clear tubes.
   b. Depending on the time point, degree of turbidity, and previous experience with the drug-organism combination, it may not be necessary to plate all dilutions from turbid tubes.
   c. Plate 0.1 ml from each reaction tube onto two separate agar plates if they are needed for detecting low numbers of viable organisms. *Note:* One 9.9-ml saline tube can be used in place of two 0.9-ml saline tubes for a 1:100 dilution.
6. Following incubation of subculture plates for the times specified in Appendix 5.10.2–1, count the colonies on those plates that show between 30 and 300 colonies.
7. Determine the number of colonies in each reaction tube at each time by averaging the counts obtained.
   Example: At 4 h in tube containing 1 μg of vancomycin per ml

Dilution	No. of CFU		Average no. of CFU
10^{-2}	>300	>300	>300
10^{-3}	282	264	273*
10^{-4}	30	28	29*

*within acceptable counting range

Average (actual) CFU/ml

$$\left.\begin{array}{l} 273 \times 1/10^{-3} = 2.7 \times 10^5 \text{ CFU/ml} \\ 29 \times 1/10^{-4} = 2.9 \times 10^5 \text{ CFU/ml} \end{array}\right] \text{ average } = 2.8 \times 10^5 \text{ CFU/ml}$$

B. Alternative method (1)
1. Proceed with preparation of serial 10-fold dilutions as described above.
2. Instead of subculturing 0.1-ml aliquots to an entire agar plate, use a micropipette to subculture 0.025-ml aliquots (1:40 dilution) of each dilution in spots around the periphery of an agar plate as shown in Fig. 5.10.2–A2.
3. Incubate as described above, and count the colonies within each spot for those dilutions demonstrating a countable number of colonies.

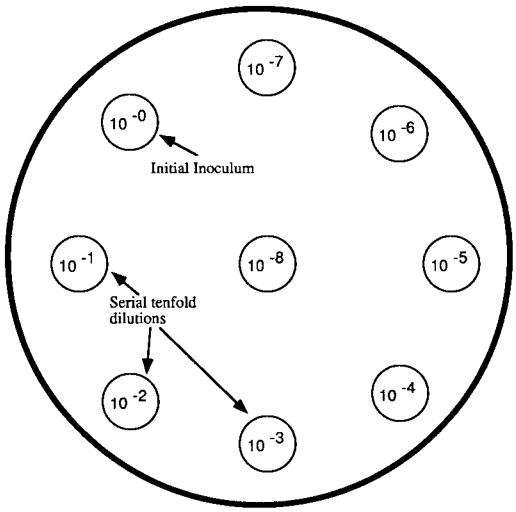


Figure 5.10.2–A2 Plate configuration for inoculating 0.25 ml of each dilution from reaction tube to agar plate for colony counts. (Adapted and reprinted with permission from **G. M. Eliopoulos and R. C. Moellering.** 1996. Antimicrobial combinations, p. 330–396, Fig. 9.5 [p. 339]. *In* V. Lorian [ed.], *Antibiotics in Laboratory Medicine*, 4th ed. The Williams & Wilkins Co., Baltimore, Md.)

APPENDIX 5.10.2–3 *(continued)*

4. Determine the number of colonies in the reaction tube by averaging the numbers of counts obtained. Multiply the count by 40 to obtain the CFU per milliliter in reaction tube. An example follows:

Dilution	No. of CFU		Average no. of CFU
10^{-1}	Confluent	Confluent	Confluent
10^{-2}	52	Confluent	52*
10^{-3}	5	7	6*
10^{-4}	0	0	0
10^{-5}	0	0	0
10^{-6}	0	0	0
10^{-7}	0	0	0
10^{-8}	0	0	0

*within acceptable counting range

Average CFU $= (5.6 \times 10^3) \times 40 = 224 \times 10^3 = 2.2 \times 10^5$

Reference

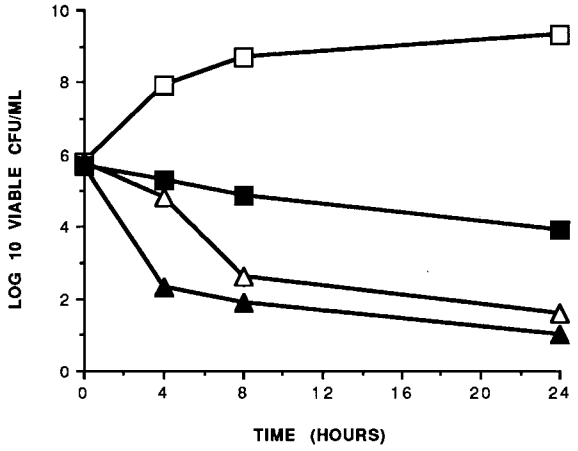
1. **Eliopoulos, G. M., and C. T. Eliopoulos.** 1988. Antibiotic combinations: should they be tested? *Clin. Microbiol. Rev.* **1:**139–156.

APPENDIX 5.10.2–4

Time-kill assay worksheet (*see* p. 5.10.2.12)

APPENDIX 5.10.2–5

Sample graph for time-kill assay of *S. aureus* with vancomycin. Symbols: □, growth control; ■, vancomycin at 0.25 μg/ml; △, vancomycin at 0.5 μg/ml; ▲, vancomycin at 1 μg/ml.



Time-Kill Assay Worksheet

Date / Tech _____
Patient _____
Antimicrobial _____
Broth _____ Lot # _____
MIC = _____ µg/ml

Organism _____
Specimen # _____
Lot # stock solution _____
Agar _____ Lot # _____

Antimicrobial concentration tested (µg/ml)	Time (h)	Actual CFU count at respective dilution (0.1-ml volumes plated in duplicate)								Actual CFU*		Log_{10} CFU*	
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	Average CFU/ml	ΔCFU/ml (control – drug)	Average CFU/ml	ΔCFU/ml (control – drug)
+ control	0												
+ control	4												
+ control	8												
+ control	24												
1× MIC µg/ml	0												
1× MIC µg/ml	4												
1× MIC µg/ml	8												
1× MIC µg/ml	24												
2× MIC µg/ml	0												
2× MIC µg/ml	4												
2× MIC µg/ml	8												
2× MIC µg/ml	24												
4× MIC µg/ml	0												
4× MIC µg/ml	4												
4× MIC µg/ml	8												
4× MIC µg/ml	24												

* Plot actual or log_{10} CFU

5.10.3 Time-Kill Assay for Determining Synergy

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The time-kill assay can be used to study the dynamics of synergism or antagonism for a combination of antimicrobial agents by determining the number of viable bacteria remaining over time after exposure to each individual agent and various combinations. Therefore, time-kill assay data can assess both the rate and extent of killing. This method is used primarily to study organisms from patients with meningitis or endocarditis for whom clinical treatment failed or as a research tool to study novel antimicrobial agents.

The procedure described here is not a standardized procedure and represents recommendations. Additionally, the steps in this procedure that are the same as those in the MBC and time-kill procedures have been cross-referenced to those in procedures 5.10.1 and 5.10.2.

Prior to performance of the time-kill assay, concentrations of individual agents and combinations most likely to result in synergism are planned from data resulting from a checkerboard synergism test (*see* procedure 5.12). The time-kill assay for synergism uses minimal bactericidal concentration (MBC) broth macrodilution and time-kill procedures described in procedures 5.10.1 and 5.10.2. However, only a limited number of antimicrobial concentrations can be studied at one time because of practical limitations. Synergism in the time-kill assay is usually defined as a $\geq$2-$\log_{10}$ CFU/ml decrease by the combination compared with the most active single agent (1, 2). Antagonism is usually defined as a $\geq$2-$\log_{10}$ CFU/ml increase by the combination compared with the most active single agent; however, smaller $\log_{10}$ CFU per milliliter increases may suggest antagonistic interactions (1, 2). The outcome can be represented graphically as in Appendix 5.10.3–2.

II. SPECIMEN

Refer to procedure 5.10.1.

III. MATERIALS

Refer to procedure 5.10.2.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

There are no standard recommendations for time-kill synergy assays. However, refer to the MBC procedure in procedure 5.10.1, the time-kill procedure in procedure 5.10.2, and procedure 5.12. Additionally, the following parameters should be controlled.

A. Test aliquots of each dilution of antimicrobial agent in reaction tubes by broth macrodilution or microdilution methods with appropriate QC strains (*see* procedure 5.10.1).

B. Results must be within acceptable MIC ranges for test to be in control.

V. PROCEDURE

A. Planning concentrations to test

1. Determine the MIC and MBC of each agent for the organism. Test the MIC and one-half, one-fourth, and two times the MIC *for each agent.*

a. Example (*see* procedure 5.12 for synergy examples)**:** *Pseudomonas aeruginosa,* piperacillin MIC = 128 μg/ml; amikacin MIC = 32 μg/ml

b. Test piperacillin at 32, 64, 128, and 256 μg/ml and amikacin at 8, 16, 32, and 64 μg/ml.

2. Determine combination interaction by using broth microdilution checkerboard methods (*see* procedure 5.12).

Example: The combination of piperacillin and amikacin resulted in synergism at multiple combinations.

3. Choose combination concentrations most likely to result in synergism (or antagonism).

a. When synergism is expected, test combinations at one-fourth and one-eighth the MIC of each agent.

Example: Piperacillin MIC = 128 μg/ml; amikacin MIC = 32 μg/ml. Test the combinations of piperacillin, 32 μg/ml, and amikacin, 8 μg/ml; and piperacillin, 16 μg/ml, and amikacin, 4 μg/ml.

b. When antagonism is expected, test combinations at two times the MIC of agent A and at two times the MIC of agent B.

B. Preparation of antimicrobial agent working concentrations

See procedure 5.14 for antimicrobial stock solution preparation.

1. Prepare intermediate dilutions of the stock antimicrobial agent. Dilute stock solution in sterile water (or other appropriate diluent) so that the concentration is 100 times the final concentration that will be tested in the time-kill test (*see* procedure 5.10.2).

2. Prepare final working concentrations of antimicrobial agents for the time-kill assay.

a. For individual agents, dispense the following into sterile glass tubes (16 by 150 mm).

(1) 9.9 ml of cation-adjusted Mueller-Hinton broth (CAMHB)

(2) 0.1 ml of 100-fold-concentrated antimicrobial agent solution

b. For combination agents, dispense the following into sterile glass tubes (16 by 150 mm).

(1) 9.8 ml of CAMHB

(2) 0.1 ml of 100-fold-concentrated antimicrobial agent A solution

(3) 0.1 ml of 100-fold-concentrated antimicrobial agent B solution

C. Inoculum preparation

See procedure 5.10.2.

D. Inoculation and incubation

See procedure 5.10.2.

E. Reading and recording results

See procedure 5.10.2, and modify the worksheet in Appendix 5.10.3–1 to record results for both individual agents and agent combinations at various times.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Interpretation

1. Interpret individual agent MICs according to criteria in procedure 5.2.

2. Determine for individual agents the $\log_{10}$ CFU per milliliter decrease compared with the growth control at sample times.

3. Determine the difference in $\log_{10}$ CFU per milliliter between the most active individual agent at one-fourth the MIC and the combination of agents A and B at one-fourth or one-eighth the MIC.

VI. REPORTING RESULTS *(continued)*

4. Determine whether the definition of synergism or antagonism is met (1, 2).
   - **a.** Synergism is usually defined as a $\geq$2-$\log_{10}$ CFU/ml decrease by the combination compared with the most active single agent.
   - **b.** Antagonism is usually defined as a $\geq$2-$\log_{10}$ CFU/ml increase by the combination compared with the most active single agent.
5. Determine the shortest period necessary to obtain synergism or antagonism.
6. Retest the MICs for organisms remaining after 24 h of antimicrobial action to confirm that a resistant subpopulation has not been selected.

B. Reporting

1. Report MIC results, and state whether bactericidal activity occurred at a particular concentration for individual agents.
2. Report results by stating whether synergism (or antagonism) occurred with a particular combination of agents.

   Example: For *P. aeruginosa*, piperacillin MIC = 128 μg/ml, amikacin MIC = 32 μg/ml, and the combination of piperacillin at 32 μg/ml and amikacin at 8 μg/ml exhibited synergism at 4 h.
3. Report results graphically. Plot growth control, one-fourth MIC for agent A, one-fourth MIC for agent B, and one-fourth MIC for agents A and B combined. Refer to example in Appendix 5.10.3–3.

VII. PROCEDURE NOTES

See procedure 5.10.2

A. When evaluating organisms from clinical treatment failures, actual (measured) or expected achievable antimicrobial agent concentrations can be used for individual and combination concentrations in place of MIC data.

B. Ideally, concentrations of individual agents tested should not show significant killing when compared with the growth control.

VIII. LIMITATIONS

See procedure 5.10.2. The number of combination concentrations and sample times are limited to a few tests.

REFERENCES

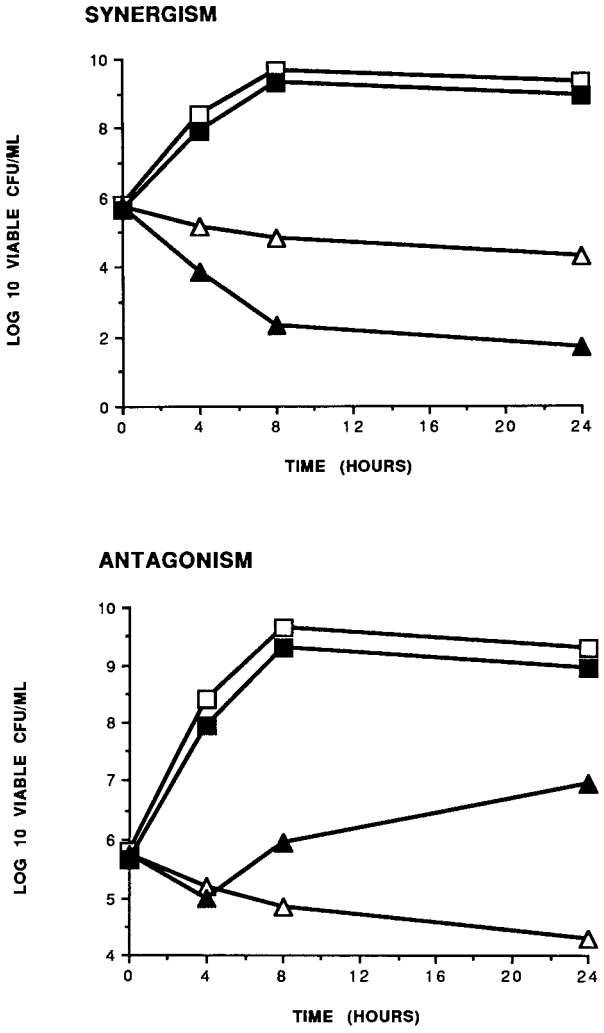
1. **Eliopoulos, G. M., and C. T. Eliopoulos.** 1988. Antibiotic combinations: should they be tested? *Clin. Microbiol. Rev.* **1:**139–156.
2. **Eliopoulos, G. M., and R. C. Moellering, Jr.** 1996. Antimicrobial combinations, p. 330–396. *In* V. Lorian (ed.), *Antibiotics in Laboratory Medicine,* 4th ed. The Williams & Wilkins Co., Baltimore, Md.
3. **NCCLS.** 1999. *Methods for Determining Bactericidal Activity of Antimicrobial Agents.* Approved guideline M26-A. NCCLS, Wayne, Pa.

APPENDIX 5.10.3–1

Time-kill assay worksheet (*see* p. 5.10.3.6)

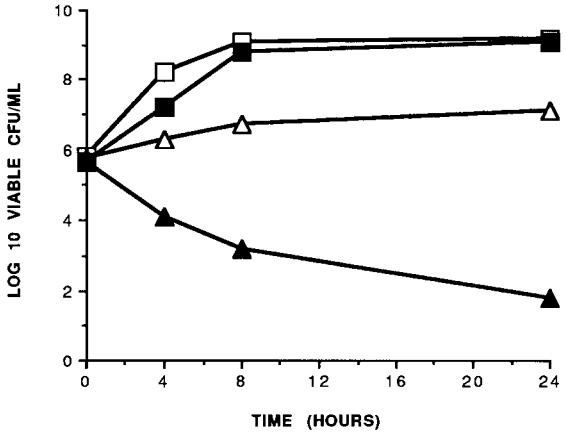
APPENDIX 5.10.3–2

Graphic representation of time-kill assay showing synergism and antagonism. Symbols: □, growth control; ■, agent A; △, agent B; ▲, agents A and B. (Adapted and reprinted with permission from **G. M. Eliopoulos and R. C. Moellering, Jr.** 1996. Antimicrobial combinations, p. 330–396, Fig. 9.6 [p. 340]. *In* V. Lorian [ed.], *Antibiotics in Laboratory Medicine*, 4th ed. The Williams & Wilkins Co., Baltimore, Md.)



APPENDIX 5.10.3–3

Sample graph of time-kill assay of *P. aeruginosa* with piperacillin and amikacin. Symbols: □, growth control; ■, piperacillin (32 μg/ml); △, amikacin (8 μg/ml); ▲, piperacillin (32 μg/ml) and amikacin (8 μg/ml).



Time-Kill Assay Worksheet

Date / Tech _____ Organism _____
Patient _____ Secimen # _____
Antimicrobial (A) _____ Lot # stock solution (A) _____
Antimicrobial (B) _____ Lot # stock solution (B) _____
Broth _____ Lot # _____ Agar _____ Lot # _____
MIC (A) = _____ µg/ml_____ MIC (B) = _____ µg/ml _____

Antimicrobial concentration tested (µg/ml)	Time (h)	Actual CFU count at respective dilution (0.1-ml volumes plated in duplicate)								Actual CFU*		$\log_{10}$ CFU*	
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	Average CFU/ml	ΔCFU/ml (control – drug)	Average CFU/ml	ΔCFU/ml (control – drug)

* Plot actual or $\log_{10}$ CFU

5.11 Serum Inhibitory and Bactericidal Titers

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Schlichter and MacLean first used serum inhibitory titers (SIT) to assess the effectiveness of penicillin in the treatment of subacute bacterial endocarditis (2). Other recognized clinical indications for obtaining serum bactericidal titers (SBT) to monitor effective therapy are osteomyelitis, closed-space infections such as meningitis or joint infection, and the use of oral antimicrobial agents after intravenous therapy. Controversy surrounds and will continue to surround the methodology, and there are few clinical situations in which the test is indicated.

There is currently no single standardized method for determining SIT and SBT. However, the NCCLS has described several methods in an approved guideline which contains substantial information about the use of this test in addition to methods (1).

The single most contested issue is the choice of diluent for the test, and the second issue is the use of patient serum versus the use of an ultrafiltrate of patient serum (*see* Appendix 5.11–1). It is the author's preference to use broth without added serum and patient serum rather than ultrafiltrate.

The SIT and SBT tests are designed to determine the bacteriostatic and bactericidal activities of an antimicrobial agent(s) in a patient's serum against the patient's own infecting organism. The test integrates the pharmacokinetic and pharmacodynamic properties of the antimicrobial agent(s) that the patient is receiving. The serum is diluted geometrically, and a standard inoculum of the patient's organism is added to each tube or well and then incubated for 24 h at 35°C. The SIT is the highest dilution of the serum that inhibits visible growth. A sample from each tube showing no growth is then plated onto agar without any antimicrobial agent and incubated for 24 to 72 h. The SBT is the highest dilution of the serum that reduces (kills) the concentration of the patient's organism by 99.9% (1).

II. SPECIMEN

Observe standard precautions.

A. Patient isolate

An 18- to 48-h agar plate (noninhibitory agar) containing a pure culture of the patient's infecting organism, streaked for isolation (minimum, 20 to 30 colonies)

B. Serum

1. Timing

a. Trough: 0 to 30 min prior to the next dose

b. Peak

(1) 60 min after a 30-min intravenous infusion of drug

(2) 60 min after an intramuscular dose of drug

(3) 90 min after an oral dose of drug

(4) If two or more antimicrobial agents are being administered concomitantly, collect the peak sample 60 min after the second drug is given. If one drug is given more frequently than the other, collect the sample following the less frequently given drug.

c. Trough and peak should ideally be collected around the same dose.

II. SPECIMEN *(continued)*

2. Collection and processing
- **a.** Collect the following aseptically in a red-stopper Vacutainer tube.
  - (1) 2 to 5 ml of blood (1 to 2.5 ml of serum) for the microdilution procedure
  - (2) 5 to 7 ml of blood (2.5 to 3.5 ml of serum) for the macrodilution procedure
- **b.** Place tube on ice, and deliver to laboratory immediately.
- **c.** Centrifuge (before removing the stopper) long enough to obtain cell-free serum.
- **d.** Using aseptic technique (for this step and all steps of the procedure), remove the serum to a sterile vial or small test tube.
- **e.** Specimens can be kept at 2 to 8°C for up to 2 h.
- **f.** Freeze serum (not blood) for up to 2 months at −20°C or lower if testing will be delayed (−70°C or lower is preferred and is necessary when patient has received very temperature-labile drugs such as imipenem or combinations containing clavulanic acid).
- **g.** Never refreeze a thawed specimen prior to testing, as this could lead to significant antimicrobial inactivation.

C. Body fluids: CSF, joint fluid, peritoneal fluid, etc.
1. Collect in a sterile tube.
2. Filter sterilize by using a 0.45-μm-pore-size filter if there is any suspicion that the fluid contains viable organisms.
3. Volumes required are the same as those for serum.
4. Follow handling and storage instructions for serum.

III.A. MATERIALS: MICRODILUTION PROCEDURE

Include QC information on reagent container and in QC records.

A. Media and reagents (storage conditions)

The media listed include those necessary for testing rapidly growing and some other aerobic bacteria. Refer to Appendix 5.11–1 for information on serum diluents and use of ultrafiltrates. Refer to Appendix 5.11–2 for a complete list of suggested media for testing fastidious aerobic bacteria.
1. Cation-adjusted Mueller-Hinton broth (CAMHB). Store at 2 to 8°C.
2. Plated media
   - **a.** BAP. Store at 2 to 8°C.
   - **b.** CHOC. Store at 2 to 8°C.
3. Sterile 0.9% NaCl. Store at 25°C.

B. Supplies
1. Red-stopper Vacutainer tubes (2- to 7-ml capacity)
2. Sterile glass or plastic vial (2- to 5-ml capacity)
3. Sterile glass test tubes (16 by 150 mm) with metal or plastic closures
4. Sterile 1-, 5-, and 10-ml serological pipettes and pipette bulb
5. McFarland 0.5 turbidity standard
6. Sterile polystyrene 96-well U-bottom microdilution trays
7. Sterile 0.05-ml pipette droppers (optional)
8. Empty microdilution trays or tray-sealing tape
9. Microdilution tray storage container or plastic bags
10. Sterile V-shaped reservoir tray or petri plate (100 mm)

C. Equipment
1. Vortex mixer
2. Micropipettes, 10-, 25-, 50-, and 100-μl capacity with sterile disposable tips
3. 0.001-ml calibrated loops
4. Eight-channel micropipette (optional), 50-μl capacity with sterile disposable tips
5. Microdilution tray reading device (mirror, light box, etc.)
6. Microdilution tray shaker-mixer (optional)
7. 33 to 35°C ambient-air incubator (CO_2 incubation needed for some organisms)

ANALYTICAL CONSIDERATIONS

IV.A. QUALITY CONTROL: MICRODILUTION PROCEDURE

A. Broth

Assess the performance and chemical characteristics of each batch or lot of broth medium (*see* procedures 5.2 and 5.14).

1. Check the growth capability of the broth by using an ATCC strain similar to the patient's isolate. The broth must support growth.

2. Perform a dilution susceptibility test with the antimicrobial agent(s) being administered to the patient. Use an ATCC QC strain that has an on-scale endpoint for the antimicrobial agent(s) in question. The MIC must fall within the acceptable range (*see* procedure 5.2).

B. Controls

1. The positive control must show heavy (3 to 4+) turbidity.

2. The broth sterility well must show no growth.

3. The serum (body fluid) sterility well must show no growth.

4. The broth-plus-serum sterility well must show no growth.

5. The purity and inoculum count verification plates must have no contaminating organisms.

6. The SBT subcultures must have no contaminating organisms.

C. The final inoculum must contain between 10^5 and 10^6 CFU/ml (20 to 200 colonies on inoculum count verification plate).

V.A. PROCEDURE: MICRODILUTION PROCEDURE

See Appendix 5.11–2, which gives testing conditions and media for various organisms.

A. Setup

1. Use a sterile 96-well U-bottom microdilution tray for each patient.

2. Label and mark in the following manner (*see* Appendix 5.11–3).

a. Use horizontal rows A and C for trough serum sample (run in duplicate).

b. Use horizontal rows E and G for peak serum sample (run in duplicate).

c. Use D12 and H12 for broth sterility controls (broth only).

d. Use B11 for trough serum sterility control (serum only).

e. Use F11 for peak serum sterility control (serum only).

f. Use B12 and F12 for broth-plus-serum comparison wells (to help ascertain growth versus protein precipitate).

g. Use wells A12, C12, E12, and G12 for growth control wells (broth plus organism).

3. The final volume of each well will be 0.1 ml. Each serum sample is run in duplicate.

B. Dilutions

1. Using a pipette dropper or other micropipette, add 0.05 ml of test broth to wells 2 to 12 in rows A, C, E, and G and to wells B12, D12, F12, and H12 (for an eight-channel pipetter, put a tip on every other channel for filling wells in rows A, C, E, and G).

2. Vortex patient serum.

3. Add 0.05 ml of patient serum to wells 1 and 2 of appropriate rows with a micropipette.

4. With a new pipette tip, mix serum and broth in well 2 by aspirating them in and out of the tip 6 to 10 times (an eight-channel pipetter with alternating tips could be used).

5. Use a new tip to withdraw 0.05 ml of this dilution, and add it to well 3.

6. Repeat this procedure through well 11, using a new tip for each dilution.

7. Discard 0.05 ml from well 11.

V.A. PROCEDURE: MICRODILUTION PROCEDURE *(continued)*

8. Add 0.1 ml of trough serum to well B11 and 0.05 ml of trough serum to well B12.
9. Add 0.1 ml of peak serum to well F11 and 0.05 ml of peak serum to well F12.

C. Inoculum preparation

1. Using a sterile loop, touch 5 to 10 colonies of the patient's organism, and inoculate them to 5 ml of CAMHB.
2. Incubate for 3 to 4 h at 35°C until the suspension is visibly turbid (logarithmic phase). Optimally place tube on a shaker.
3. Adjust the suspension to obtain a turbidity comparable to that of a McFarland 0.5 turbidity standard.
4. Add 0.05 ml of this suspension to 10 ml of CAMHB, and vortex (concentration = approximately 7.5×10^5 CFU/ml).
5. Use the inoculum suspension within 15 min.

D. Inoculation and incubation

1. Using a pipette dropper, micropipette, or eight-channel pipetter, add 0.05 ml of inoculum to each well in horizontal rows A, C, E, and G. (Do not touch liquid in wells.)
2. Seal tray with tray-sealing tape or an empty sterile microdilution tray, or place the tray in a plastic bag or other storage container to prevent evaporation during incubation.
3. Prepare a purity plate by thoroughly spreading 0.001 ml of the McFarland 0.5 suspension onto the surface of a 100-mm agar plate.
4. Prepare an inoculum count verification plate as follows.
   a. Prepare a 1:100 dilution of the inoculum (0.1 ml of inoculum plus 9.9 ml of sterile 0.9% NaCl).
   b. Vortex.
   c. Use a 10-µl (0.01-ml) micropipette or calibrated loop to transfer and thoroughly spread 0.01 ml of this dilution onto the surface of a BAP by streaking in several directions with a loop or bent glass rod ("hockey stick") (perform in duplicate).
5. Incubate trays at 35°C in an ambient-air incubator for 24 h (CO_2 may be required for some organisms; *see* Appendix 5.11–2).
6. After 20 h, remove trays from the incubator.
7. Mix the contents of each well to achieve a uniform suspension of organisms by one of the following methods.
   a. Place the tray on a mechanical microdilution tray shaker-mixer, and set the speed high enough to displace the well contents halfway up the internal wall. Shake for approximately 1 min.
   b. Gently tap the plate against a solid object until a uniform suspension is obtained in each well.
8. Then reincubate for another 4 h (total, 24 h).
9. Incubate the purity and inoculum count verification plates in an ambient-air incubator at 35°C (some fastidious organisms may require CO_2 or more than 24 h of incubation before colonies can be counted).

E. Reading and recording inhibitory titers

1. Place tray on reading device.
2. Examine growth control wells for 3 to 4+ turbidity, indicating adequate growth. Assess growth as follows.
   a. ± to 1+, very light haze in well
   b. 2+, light haze in well
   c. 3+ to 4+, heavy turbidity or fine granular growth throughout well or dense precipitate at bottom of well

V.A. PROCEDURE: MICRODILUTION PROCEDURE *(continued)*

d. During incubation, serum proteins may precipitate and interfere with SIT interpretation. It may be impossible to report the SIT if growth cannot be distinguished from serum protein precipitation.

3. Examine uninoculated controls. Compare questionable growth with these when necessary.

4. Examine inoculum count verification plate. If count is inadequate ($<$20 or $>$200), repeat the test.

5. The SIT is the highest dilution of patient serum resulting in no visible growth after 24 h of incubation.

6. Record the results on a worksheet (Appendix 5.11–4) (examples follow).

a. If there is no growth in wells 1 to 5 of a horizontal row, the titer is 1:32.

b. If all 11 dilutions of patient serum show growth, the titer is $<$1:2.

c. If all 11 dilutions of patient serum show no growth, the titer is $\geq$1:2,048.

7. In preparation for determining the SBT, calculate the inoculum colony count (final inoculum or actual number of organisms tested).

a. Count the colonies growing on each inoculum count verification plate, and average the two counts.

b. Multiply the average of the two counts by 10^4 (inoculum was diluted 1:100 for inoculum count verification, and you must multiply by 100 to convert to 1 ml).

c. Divide by 2 (inoculum was diluted 1:2 in the test).

F. Plating for bactericidal titers

1. After 24 h of incubation without additional shaking, use a micropipette to remove a 0.01-ml aliquot from the following.

a. Each well showing no growth

b. The well representing the highest dilution showing growth

c. Each control well

2. Plate each sample onto a quarter section of a BAP (do for each patient row), and spread the sample over the entire quadrant. Prepare these subcultures in duplicate. If a micropipette is used, use a new tip for each subculture.

3. Allow inocula to dry prior to incubation.

G. Incubation

1. Invert the plates, and incubate them at 35°C in an ambient-air incubator (CO_2 may be needed for some organisms; *see* Appendix 5.11–2).

2. Read preliminary results (number of colonies growing in each quadrant or spot) after 1 day of incubation.

3. Read the final results at the following times.

a. 24 h for rapidly growing gram-negative rods

b. 48 h for staphylococci and enterococci

c. 72 h for all other organisms

H. Determining bactericidal titers

1. Count the colonies growing in each quadrant or on each spot, and record on the worksheet.

2. Determine if a particular dilution is bactericidal.

a. Add the number of colonies growing in each quadrant from platings of duplicate wells (e.g., A1 + C1, A2 + C2, etc.).

b. Use Appendix 5.11–5 to determine whether this number of colonies is above or below the rejection value for the final inoculum that was calculated previously.

c. When the sum of colonies growing from subcultures of duplicate dilutions is equal to or less than the rejection value, that dilution of serum is declared bactericidal (indicating killing of 99.9% or more of the organisms tested).

V.A. PROCEDURE: MICRODILUTION PROCEDURE *(continued)*

3. Given here is an example of the use of Appendix 5.11–5 for a specimen with a trough SIT of 1:32, where all wells showing no growth and the lowest dilution showing growth (1:64) were subcultured (TNTC, too numerous to count).
- **a.** Number of colonies on colony count plate = 100 CFU
- **b.** Final calculated inoculum in each well: $(100 \times 10^4)/2 = 5 \times 10^5$ CFU/ml
- **c.** Duplicate subculture rejection value = 25 CFU

Duplicate subcultures from wells	No. of CFU/quadrant	Total CFU	≥99.9% Killing
A1 + C1 (1:2)	2, 0	2	Yes
A2 + C2 (1:4)	3, 8	11	Yes
A3 + C2 (1:8)	10, 8	18	Yes
A4 + C4 (1:16)	15, 11	26	No
A5 + C5 (1:32)	TNTC	TNTC	No
A6 + C6 (1:64)	TNTC	TNTC	No

4. The SBT is the highest dilution of patient serum that results in killing of 99.9% or more of the organisms tested. The SBT is 1:8 in the example above.

PREANALYTICAL CONSIDERATIONS

III.B. MATERIALS: MACRODILUTION METHOD

Include QC information on reagent container and in QC records.

A. Media and reagents (storage conditions)
The media listed include those necessary for testing rapidly growing and some other aerobic bacteria. Refer to Appendix 5.11–1 for information on serum diluents and use of ultrafiltrates. Refer to Appendix 5.11–2 for a complete listing of suggested media for testing fastidious aerobic bacteria.
- **1.** CAMHB. Store at 2 to 8°C.
- **2.** Plated media
  - **a.** BAP. Store at 2 to 8°C.
  - **b.** CHOC. Store at 2 to 8°C.
- **3.** Sterile 0.9% NaCl. Store at 25°C.

B. Supplies
- **1.** Red-stopper Vacutainer tubes (2- to 7-ml capacity)
- **2.** Sterile glass or plastic vial (2- to 5-ml capacity)
- **3.** Sterile glass test tubes (16 by 150 mm) with metal or plastic closures
- **4.** Sterile 1-, 5-, and 10-ml serological pipettes and pipette bulb
- **5.** McFarland 0.5 turbidity standard
- **6.** Sterile acid-washed borosilicate glass tubes (13 by 100 mm) with metal or plastic closures

C. Equipment
- **1.** Vortex mixer
- **2.** Micropipettes, 10-, 25-, 50-, and 100-µl capacity with sterile disposable tips
- **3.** 0.001- and 0.01-ml calibrated loops
- **4.** 33 to 35°C ambient-air incubator (CO_2 incubation is needed for some organisms)

ANALYTICAL CONSIDERATIONS

IV.B. QUALITY CONTROL: MACRODILUTION PROCEDURE

A. Broth
Assess the performance and chemical characteristics of each batch or lot of broth medium (*see* procedures 5.2 and 5.14).
- **1.** Check the growth capability of the broth by using an ATCC strain similar to the patient's isolate. The broth must support growth.
- **2.** Perform a dilution susceptibility test with the antimicrobial agent(s) being administered to the patient. Use an ATCC QC strain that has an on-scale endpoint for the antimicrobial agent(s) in question. The MIC must fall within the acceptable range (*see* procedure 5.2).

IV.B. QUALITY CONTROL: MACRODILUTION PROCEDURE *(continued)*

B. Controls
1. The positive control must show heavy (3 to 4+) turbidity.
2. The broth sterility tube must show no growth.
3. The broth-plus-serum sterility tube must show no growth.
4. The purity and colony count verification plates must have no contaminating organisms.
5. The SBT subcultures must have no contaminating organisms.

C. The final inoculum must contain between 10^5 and 10^6 CFU/ml (20 to 200 colonies on inoculum count verification plate).

V.B. PROCEDURE: MACRODILUTION PROCEDURE

See Appendix 5.11–2, which gives testing conditions and media for various organisms.

A. Setup
1. Assemble 14 sterile test tubes (13 by 100 mm) for each patient sample.
2. Label tubes 1 through 12, and place them in one row.
   - **a.** Tubes 1 through 11 are for serial dilutions of patient sera (1:2 to 1:2,048).
   - **b.** Tube 12 is for the growth control (broth plus organism).
   - **c.** Tube 13 is for the broth sterility control (broth only).
   - **d.** Tube 14 is for the serum sterility control (broth plus serum).
3. The final volume of each tube will be 1.0 ml. Each serum sample is run singly.

B. Dilutions
1. Use a sterile 5-ml pipette to add 1.0 ml of broth to tubes 2 through 14.
2. Vortex patient serum.
3. Use a sterile 2-ml pipette to add 1.0 ml of patient serum to tubes 1, 2, and 14.
4. Vortex tube 2.
5. Use a new sterile 2-ml pipette to transfer 1.0 ml of broth-serum mixture from tube 2 to tube 3. Vortex tube 3.
6. Repeat this procedure through tube 11, using a new pipette for each tube.
7. Discard 1.0 ml of mixture removed from tube 11.

C. Inoculum preparation
1. Using a sterile loop, touch 5 to 10 colonies of the patient's organism, and inoculate them to 5 ml of CAMHB.
2. Incubate for 3 to 4 h at 35°C until the suspension is visibly turbid (logarithmic phase). Optimally place tube on a shaker.
3. Adjust the suspension to obtain a turbidity comparable to that of a McFarland 0.5 turbidity standard.
4. Add 0.3 ml of this suspension to 9.7 ml of CAMHB (1:32 dilution), and vortex (concentration = approximately 4×10^6 to 5×10^6 CFU/ml).
5. Use the inoculum suspension within 15 min.

D. Inoculation and incubation
1. Using a micropipette to inoculate tubes, add 0.1 ml of inoculum to tubes 1 through 12 by placing the tip just under the meniscus of the liquid (final organism concentration, approximately 5×10^5 CFU/ml).
2. Mix by drawing the solution into the tip two or three times without causing air bubbles or splashing.
3. Use a new tip for each tube, and avoid contact of the tip with the wall of the tube.

V.B. PROCEDURE: MACRODILUTION PROCEDURE *(continued)*

4. Prepare a purity plate by subculturing 0.001 ml of the McFarland 0.5 suspension onto the surface of a 100-mm agar plate.
5. Prepare an inoculum count verification plate as follows.
   a. Prepare a 1:100 dilution of the inoculum (0.1 ml of inoculum plus 9.9 ml of sterile 0.9% NaCl).
   b. Vortex.
   c. Use a 10-µl (0.01-ml) micropipette or calibrated loop to transfer and thoroughly spread 0.01 ml of this dilution onto the surface of a BAP by streaking in several directions with a loop or bent glass rod ("hockey stick") (perform in duplicate).
6. Incubate tubes at 35°C in an ambient-air incubator for 20 h (CO_2 may be required for some organisms; *see* Appendix 5.11–2).
7. Incubate the purity and inoculum count verification plates in an ambient-air incubator at 35°C (some fastidious organisms may require CO_2 or more than 24 h of incubation before colonies can be counted).
8. Remove the rack from the incubator.
9. Gently swirl each tube by hand until a uniform turbidity is achieved (the object is to draw organisms from the top of the meniscus back into the broth antimicrobial mixture).
10. Place the rack back into the incubator, and incubate for another 4 h (24 h total).

E. Reading and recording inhibitory titers (after 24 h of incubation)

1. Examine growth control tubes for 3 to 4+ turbidity, indicating adequate growth. Assess growth as follows.
   a. ± to 1+, very light haze in tube
   b. 2+, light haze in tube
   c. 3+ to 4+, heavy turbidity or fine granular growth throughout tube or dense precipitate at bottom of tube
   d. During incubation, serum proteins may precipitate and interfere with SIT interpretation. It may be impossible to report SIT if growth cannot be distinguished from serum protein precipitation.
2. Examine uninoculated controls. Compare questionable growth with these when necessary.
3. Examine inoculum count verification plate. If count is inadequate (<20 or >200), repeat the test.
4. The SIT is the highest dilution of patient serum resulting in no visible growth after 24 h of incubation.
5. Record the results on a worksheet (Appendix 5.11–4) (examples follow).
   a. If there is no growth in tubes 1 through 5 of a row, the titer is 1:32.
   b. If all 11 dilutions of patient serum show growth, the titer is <1:2.
   c. If all 11 dilutions of patient serum show no growth, the titer is ≥1:2,048.
6. In preparation for determining the SBT, calculate the inoculum colony count (final inoculum or actual number of organisms tested).
   a. Count the colonies growing on each inoculum count verification plate, and average the two counts.
   b. Multiply the average of the two counts by 10^4 (inoculum was diluted 1:100 for inoculum count verification, and you must multiply by 100 to convert to 1 ml).
   c. Divide by 2 (inoculum was diluted 1:2 in the test).

F. Plating for bactericidal titers

1. Use a micropipette to remove 0.01 ml of vortexed broth from the following tubes.
   a. All tubes showing no growth
   b. The tube representing the highest dilution showing growth
   c. Each control tube (tubes 12, 13, and 14)

V.B. PROCEDURE: MACRODILUTION PROCEDURE *(continued)*

2. Plate each sample onto a quarter segment of a BAP, and spread the sample over the entire quadrant. Prepare these subcultures in duplicate. Use a new tip for each subculture.
3. Allow inocula to dry prior to incubation.

G. Incubation

1. Invert the plates, and incubate them at 35°C in an ambient-air incubator (CO_2 may be needed for some organisms; *see* Appendix 5.11–2).
2. Count the colonies growing in each quadrant after 1 day of incubation.
3. Read final results (number of colonies growing in each quadrant) at the following times.
a. 24 h for rapidly growing gram-negative rods
b. 48 h for staphylococci and enterococci
c. 72 h for all other organisms

H. Determining bactericidal endpoints

1. Count the colonies growing in each quadrant, and record on the worksheet.
2. Determine if a particular dilution is bactericidal.
a. Add the number of colonies growing in each quadrant from duplicate platings of each dilution.
b. Use Appendix 5.11–5 to determine whether this number of colonies is above or below the rejection value for the final inoculum that was calculated previously.
c. When the sum of colonies growing on duplicate subcultures of each dilution is equal to or less than the rejection value, that dilution of serum is declared bactericidal (indicating killing of 99.9% or more of the organisms tested).
3. An example of the use of Appendix 5.11–5 for a specimen with an SIT of 1:32 for which all tubes showing no growth and the lowest dilution showing growth (1:64) were subcultured is illustrated above in the microdilution procedure.

POSTANALYTICAL CONSIDERATIONS

VI. RESULTS

A. Interpretation

The NCCLS provides the following "approximate" guidelines when using the microdilution method (1).

1. Bactericidal titers of 1:16 and 1:32 are frequently associated with favorable clinical outcomes in patients with endocarditis.
2. Bactericidal results of 1:2 or greater are frequently associated with favorable clinical outcomes in patients with skeletal infections.
3. There is much controversy surrounding interpretation beyond that described in items VI.A.1 and 2. It is suggested that the medical literature be followed for other applications and validations (1).

B. Providing QC is acceptable, report according to laboratory policy and format. The following is an example.

The trough serum collected 10-15-90 at 08:00 when tested against *Enterococcus* spp. was bacteriostatic at a dilution of 1:32 and bactericidal at a dilution of 1:8.

VII. PROCEDURE NOTES

A. Specimen

1. Observe standard precautions when handling *all* patient specimens.
2. The decision to test peak or trough serum or both should be made by the physician based on knowledge of the clinical situation of the patient. These factors include but are not limited to the type(s) of antimicrobial agent(s) that the patient is receiving, the patient's immune status, and the patient's clinical isolate.
3. If the patient's isolate is unavailable, the test *cannot* be performed. It is inappropriate to substitute another isolate (such as an ATCC QC strain) for the patient's isolate (1).

B. Materials

1. The broth medium must permit growth of the organism and not inactivate the antimicrobial agents through pH variation, chelation, or other mechanisms.
2. Some prefer subculturing organisms for SBT to TSA plates rather than BAP. Either is acceptable, providing adequate growth is obtained. Contaminating bacteria are often more readily apparent on BAP.

C. Procedure

1. The outcome of this test is influenced by many technical factors and the fact that two different entities (serum and organism) are tested; thus, QC is difficult, and the protocol must be strictly adhered to in order to minimize artifacts and ambiguous results.
2. Testing must be performed on organisms in the log phase of growth, because the most effective killing of organisms and hence the greatest expression of antibacterial effectiveness occurs at the mid-logarithmic growth phase. A mechanical shaker may be used to accelerate growth. For slow-growing organisms, overnight incubation may be necessary.
3. Organisms can travel up and cling to the sides of non-acid-washed or plastic test tubes. These organisms will not be subjected to the lethal effects of the antimicrobial agent(s) being tested, and less bactericidal activity may be observed; i.e., a lower SBT may result. It has not been established if this phenomenon occurs in microdilution wells.
4. In the microdilution procedure, it is imperative that organisms not travel from one well to another and that no liquid be allowed to evaporate from the wells. Do not stack trays more than four trays high, so that the incubation temperature will be the same for all plates.
5. When subculturing for bactericidal endpoints, position the initial streak about 1 cm from the edge of the plate. Streak in four or five directions to spread the inoculum thoroughly so that individual colonies can be counted. This helps dilute the antimicrobial agent(s) so that it does not continue to inhibit growth of organisms.
6. Whether volumes greater than 0.01 ml should be subcultured for bactericidal determinations is a matter of controversy.
7. Some slow-growing organisms may require more than 24 h of incubation before the colony count can be determined (*see* Appendix 5.11–2).
8. The NCCLS describes an alternative macrodilution procedure where the final volume is 2.0 ml. However, the 1.0-ml volume is more frequently used (1).

D. Results

The clinical relevance of this test and the interpretation of the results are controversial. The NCCLS guidelines contain a thorough summary of the literature and an exhaustive list of references (1).

VII. PROCEDURE NOTES
(continued)

E. Other

1. Inhibitory titers for the microdilution method may tend to be a dilution higher than in the macrodilution method because of difficulty in observing small amounts of precipitate or turbidity in the wells (suspensions cannot be shaken or swirled as easily as with tubes). However, microdilution bactericidal titers should read the same as macrodilution titers.
2. Occasionally, an organism (particularly viridans group streptococci) does not grow satisfactorily in the microdilution system but produces satisfactory results with the macrodilution method.
3. Several problems are associated with the use of human serum in the clinical laboratory. These include but are not limited to transmission of infectious diseases, availability, cost, batch-to-batch variation, and difficulties in reading test results because of precipitation of serum proteins during incubation.
4. SBT activity relates to concentration of antimicrobial agent in patient serum. Other tests that complement this assay and are often performed instead of or in conjunction with it include the actual measurement of drug in patient serum. This can be done by using chemical or immunoassay techniques, and these methods are more standardized than SIT and SBT tests.
5. The lack of a standard test method has limited comparisons or pooling of results from one institution to another.

VIII. LIMITATIONS

Interpretation of the results of this test remains controversial, and the test should not be performed without consulting the director of the microbiology laboratory or an infectious disease physician. This test is designed primarily for aerobic bacteria that grow well after overnight incubation in CAMHB.

REFERENCES

1. **NCCLS.** 1999. *Methodology for the Serum Bactericidal Test.* Approved guideline M21-A. NCCLS, Wayne, Pa.
2. **Schlichter, J. G., and H. MacLean.** 1947. A method for determining the effective therapeutic level in the treatment of subacute bacterial endocarditis with penicillin: a preliminary report. *Am. Heart J.* **34:**209–211.

SUPPLEMENTAL READING

Amsterdam, D. 1996. Susceptibility testing of antimicrobials in liquid media, p. 103–105. *In* V. Lorian (ed.), *Antibiotics in Laboratory Medicine,* 4th ed. The Williams & Wilkins Co., Baltimore, Md.

Leggett, J. E., S. A. Wolz, and W. A. Craig. 1989. Use of serum ultrafiltrate in the serum bactericidal test. *J. Infect. Dis.* **160:**616–623.

Stratton, C. W. 1988. Serum bactericidal test. *Clin. Microbiol. Rev.* **1:**19–26.

Taylor, P. C., F. D. Schoenknecht, J. C. Sherris, and E. C. Linner. 1983. Determination of minimal bactericidal concentration of oxacillin for *Staphylococcus aureus:* influence and significance of technical factors. *Antimicrob. Agents Chemother.* **23:**142–150.

Weinstein, M. P., C. W. Stratton, A. Ackley, H. B. Hawley, B. D. Fisher, D. V. Alcid, D. S. Stephens, and L. B. Reller. 1985. Multicenter collaborative evaluation of a standardized serum bactericidal test as a prognostic indicator in infective endocarditis. *Am. J. Med.* **78:**262–269.

Weinstein, M. P., C. W. Stratton, H. B. Hawley, A. Ackley, and L. B. Reller. 1987. Multicenter collaborative evaluation of a standardized serum bactericidal test as a predictor of therapeutic osteomyelitis. *Am. J. Med.* **83:**218–222.

APPENDIX 5.11–1

Alternative Protocols for Variations in Sample or Diluent

If the antimicrobial agent in the patient's serum is appreciably protein bound, either an ultrafiltrate of the patient's serum or pooled human serum plus broth diluent should be used.

I. USE OF POOLED HUMAN SERUM AS DILUENT

A. Principle

Heat-inactivated normal human serum (NHS) can be added to broth for use as a diluent in order to minimize the effects of protein binding of the antimicrobial agent. Since some antimicrobial agents (e.g., some beta-lactams) are highly protein bound and since only unbound drug exerts antibacterial activity, use of a serum-broth diluent may more closely simulate antibacterial activity in vivo (1). Generally, titers will be lower when broth-serum is used, particularly when the patient is receiving a beta-lactam. Most studies using serum-broth diluents have included CAMHB as the broth, and projected activity with other broths can only be speculated.

B. Supplemental materials required

1. Media and reagents (storage conditions)
   a. NHS, pooled or single donor. Store at −20°C.
   b. Antibiotic medium 5 or nutrient agar. Store at 25°C.
   c. 1 N HCl. Store at 25°C.
   d. 1 N NaOH. Store at 25°C.
   e. Materials needed to perform an acidimetric beta-lactamase test (*see* procedure 5.3)
2. Supplies
   a. Sterile 100-mm petri plates
   b. Sterile 6-mm filter paper disks
   c. Filtration units with 5.0-, 0.8-, and 0.45-μm-pore-size filters
   d. Sterile containers to hold approximately 100 ml of filtered serum
3. Equipment
   a. 56°C water bath
   b. pH meter
4. *Bacillus subtilis* ATCC 6633

C. QC

Serum must be free of beta-lactamase and antibacterial activity.

1. Test each batch or lot of NHS to make certain that it does not contain beta-lactamases.
   a. Perform a beta-lactamase test on an aliquot of the NHS (*see* procedure 5.3).
   b. Use the acidimetric method (other methods, including chromogenic cephalosporin methods, may give false-positive results).
2. Using a bioassay method, test each batch or lot of NHS to make certain that it does not contain nonspecific antibacterial activity.
   a. Grow *B. subtilis* ATCC 6633 overnight at 35°C in CAMHB (turbidity should be at least that of a McFarland 4 standard).
   b. Prepare 100 ml of antibiotic medium 5 according to the manufacturer's directions (only 5 ml is required for this test, but it is best to make agar in 100-ml volumes).
   c. After autoclaving, place molten agar in a 56°C water bath, and allow it to equilibrate to 56°C.
   d. Add 0.1 to 0.2 ml of the overnight suspension of *B. subtilis* (containing approximately 10^9 to 10^{10} CFU/ml) to the 100 ml of cooled agar.
   e. Mix well.
   f. Dispense a 5.0-ml aliquot into a 100-mm petri plate, and allow it to solidify.
   g. Dispense a 20-μl aliquot of NHS onto a sterile 6-mm filter paper disk (perform in duplicate).
   h. Place disks on surface of solidified agar.
   i. Incubate plates overnight at 35°C.
   j. Examine for any evidence of antibacterial activity (zone of inhibition) around disk.

APPENDIX 5.11–1 *(continued)*

k. Results
(1) No zone of inhibition around the disk means that no antibacterial activity is present. The serum is acceptable for use as a diluent.
(2) Any zone of inhibition around the disk indicates antibacterial activity. The serum is not acceptable for use as a diluent.

D. Preparation of NHS (pooled or single donor)
1. Determine the pH of each bottle of NHS before use, and filter the NHS (process approximately 100 ml at a time).
a. Adjust to pH 7.2 to 7.4 with 1 N HCl (approximately 0.1 ml of 1 N HCl changes the pH 0.1 U) or 1 N NaOH.
b. Filter the pH-adjusted 100 ml of NHS through a 5-μm-pore-size filter (two filters will be needed, as filters clog after approximately 50 ml is filtered).
c. Filter again through a 0.8-μm-pore-size filter (use two).
d. Finally, filter through a 0.45-μm-pore-size filter.
2. Aliquot 8-ml portions of filtered serum into sterile plastic tubes, and freeze at −20°C.
a. Microdilution: one 8-ml serum aliquot is needed for each microdilution tray (i.e., peak and trough samples run in duplicate).
b. Macrodilution: one 8-ml serum aliquot is needed for each patient sample (16 ml needed for trough and peak samples).

E. Method
1. Thaw appropriate number of serum tubes.
2. Heat inactivate serum at 56°C for 30 min.
3. Dilute with 8 ml of CAMHB for a working solution.
4. Follow the microdilution or macrodilution procedure, substituting 50:50 CAMHB-NHS for CAMHB when making the patient serum dilutions and preparing the inoculum.

II. USE OF ULTRAFILTRATE OF PATIENT'S SERUM

A. Principle

Ultrafiltrate of patient serum can be used instead of patient serum in order to minimize the effects of protein binding of the antimicrobial agent. Since some antimicrobial agents are highly protein bound and since only unbound drug exerts antibacterial activity, use of an ultrafiltrate of patient serum may more closely simulate antibacterial activity in vivo. Serum proteins and protein-bound antimicrobial agent are removed from patient serum during preparation of the ultrafiltrate. This ultrafiltrate contains only free antimicrobial agent and no serum proteins. Generally, titers obtained with ultrafiltrate will be 1 to 3 dilutions lower than those obtained with unfiltered patient serum diluted in CAMHB.

B. Supplemental materials needed
1. Centrifree micropartition system YMT membrane (Amicon Corp., Danvers, Mass.)
2. Millipore filter, 0.45-μm pore size

C. Method
1. Prepare the ultrafiltrate by centrifuging patient serum for 30 min at 1,000 × *g* through a Centrifree micropartition system YMT membrane.
2. Sterilize the ultrafiltrate by passage through a 0.45-μm-pore-size Millipore filter.
3. Use this ultrafiltrate in place of the patient's serum for the macrodilution or microdilution test.
4. Ultrafiltrate can be frozen at −20°C or lower for up to 2 months for future testing.

Reference

1. **NCCLS.** 1999. *Methodology for the Serum Bactericidal Test.* Approved guideline M21-A. NCCLS, Wayne, Pa.

APPENDIX 5.11–2

SIT and SBT test conditions and media for various bacteria[a]

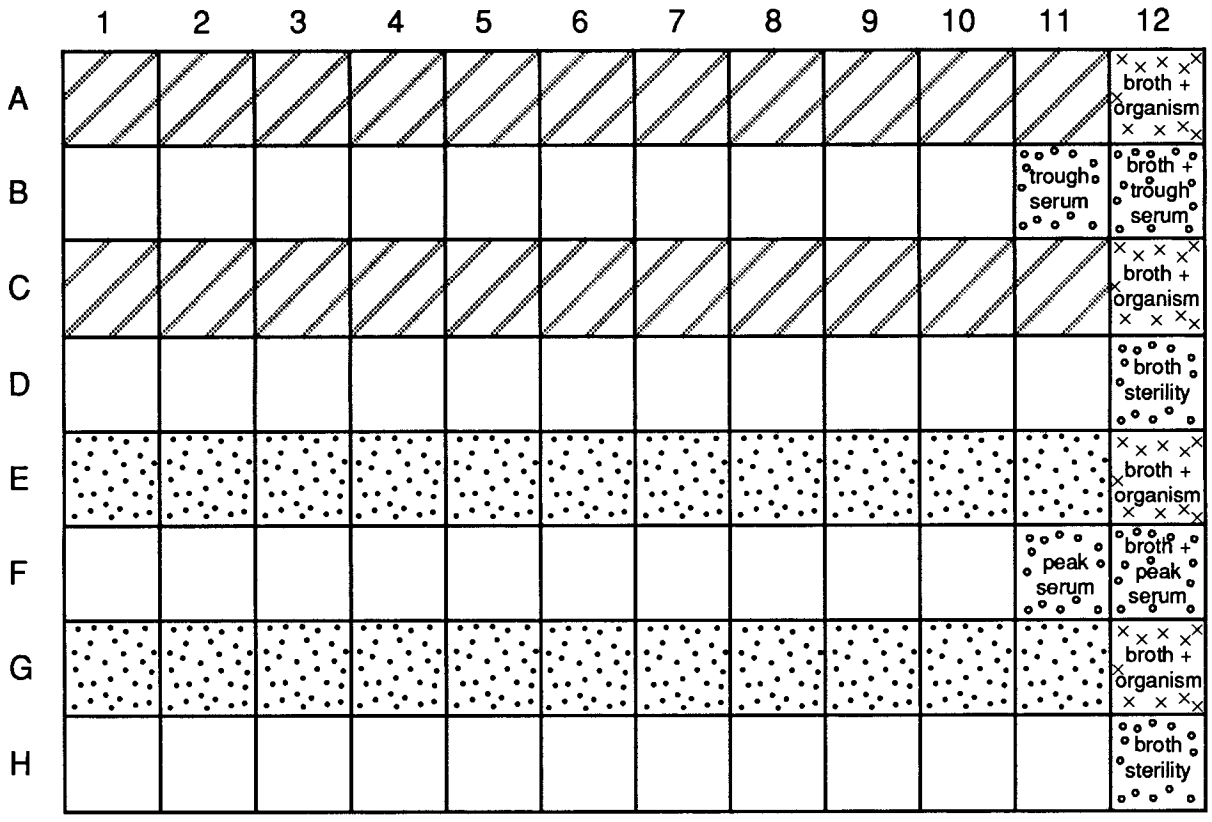
Organism or group	Broth diluent for patient serum[b]	SBT		
			Incubation[c]	
		Subculture agar	Time (h)	Atmosphere
Members of the family *Enterobacteriaceae* and non-glucose-fermenting gram-negative rods	CAMHB	BAP	24	Ambient air
Enterococcus spp.	CAMHB	BAP	48	Ambient air
Haemophilus spp.	HTM	CHOC	72	CO_2
Staphylococcus spp.	CAMHB	BAP	48	Ambient air
Streptococcus pneumoniae	CAMHB with 2–5% LHB	BAP	72	CO_2
Streptococcus spp.	CAMHB with 2–5% LHB	BAP	72	CO_2
Nutritionally variant *Streptococcus* spp.	CAMHB or THB with 0.001% pyridoxal HCl	CHOC with 0.001% pyridoxal HCl	72	CO_2

[a] Inhibitory portion of test (SIT) is incubated for 24 h. The information here applies to microdilution and macrodilution methods.

[b] Specified broth is appropriate for growing inoculum to log phase also. Broth supplemented with NHS may be used (*see* Appendix 5.11–1). HTM, *Haemophilus* test medium; LHB, lysed horse blood; THB, Todd-Hewitt broth.

[c] All incubations are at 35°C.

APPENDIX 5.11–3 Configuration of Microdilution Plate for SIT and SBT



Use one plate for each patient, and test each sample in duplicate. Row: a horizontal line of 12 wells, designated by a letter. Column: a vertical line of 8 wells, designated by a number. Well: each well has a specific letter and number corresponding to row and column location. Symbols: ◪, trough dilution rows; ⊡, peak dilution rows; ⊠, no-growth control wells; ⊠, growth control wells; □, empty wells.

APPENDIX 5.11–4

Worksheet for SIT and SBT tests (*see* p. 5.11.16)

APPENDIX 5.11–5

Rejection value and calculated sensitivity and specificity for each initial concentration based on duplicate 0.01-ml samples[a]

Final inoculum (CFU/ml)[b]	Rejection value[c]	Sensitivity (%)[d]	Specificity (%)[d]
1×10^5	4	77	97
2×10^5	8	89	99
3×10^5	15	99	99
4×10^5	20	99	99
5×10^5	25	99	99
6×10^5	29	99	99
7×10^5	33	99	99
8×10^5	38	99	99
9×10^5	42	99	99
1×10^6	47	99	99
2×10^6	91	99	99
3×10^6	136	99	99
4×10^6	182	99	99
5×10^6	227	99	99
6×10^6	273	99	99
7×10^6	318	99	99
8×10^6	364	99	99
9×10^6	409	99	99
1×10^7	455	99	99

[a] When the sum of colonies from duplicate samples was equal to or less than the rejection value, the antimicrobial agent was declared lethal (a 0.999 or greater reduction in the final inoculum). (Adapted from **R. D. Pearson, R. T. Steigbigel, H. T. Davis, and S. W. Chapman.** 1980. Method for reliable determination of minimal lethal antibiotic concentrations. *Antimicrob. Agents Chemother.* **18:**699–708.) A 5% error (pipette error plus full sampling error for determination of final inoculum) was used. Error was based on duplicate samples for determination of the final inoculum size.

[b] As determined from colony count plate.

[c] Number of colonies.

[d] Sensitivity and specificity were calculated for each specific final inoculum concentration and rejection value.

Serum Inhibitory and Bactericidal Titer Worksheet

Patient Name _____ Organism _____
Hospital ID Number _____ Site _____
Trough Sample # _____ Peak Sample # _____
Date of Sample _____ Date of Sample _____
Time of Sample _____ Time of Sample _____
Antimicrobials patient received in past 48 hours:

Drug _____ Time Last Dose _____ Drug _____ Time Last Dose _____
Drug _____ Time Last Dose _____ Drug _____ Time Last Dose _____

Test Date _____ Test Broth _____

TROUGH	Date		Date		Date		Date		Date		
Sample #	Tech		Tech		Tech		Tech		Tech		
Tube/Well #	1	2	3	4	5	6	7	8	9	10	11
Dilution 1:	2	4	8	16	32	64	128	256	512	1024	2048
Serum Inhibitory Titer (+ = growth) — [b] Row A											
Row C											
Serum Bactericidal Titer (record # of colonies) — Row A, 24 h											
Row C, 24 h											
Row A, 48 h											
Row C, 48 h											
Row A, 72 h											
Row C, 72 h											

SIT: _____ SBT:_____
[a] cc = _____
Reject number of colonies for 99.9% killing _____

Growth controls OK?:
Broth + Org_____
Sterility Controls OK?:
Broth _____
Serum _____
Broth + Serum _____
Comments:

PEAK	Date		Date		Date		Date		Date		
Sample #	Tech		Tech		Tech		Tech		Tech		
Tube/Well #	1	2	3	4	5	6	7	8	9	10	11
Dilution 1:	2	4	8	16	32	64	128	256	512	1024	2048
Serum Inhibitory Titer (+ = growth) — Row E											
Row G											
Serum Bactericidal Titer (record # of colonies) — Row E, 24 h											
Row G, 24 h											
Row E, 48 h											
Row G, 48 h											
Row E, 72 h											
Row G, 72 h											

SIT: _____ SBT:_____
[a] cc = _____
Reject number of colonies for 99.9% killing _____

Growth controls OK?:
Broth + Org_____
Sterility Controls OK?:
Broth _____
Serum _____
Broth + Serum _____
Comments:

[a] cc = colony count determined from inoculum count verification plates
[b] "Row" relates to microdilution tray (duplicate testing)

5.12 Synergism Testing: Broth Microdilution Checkerboard and Broth Macrodilution Methods

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Microbiologists commonly use a two-dimensional, two-agent broth microdilution checkerboard to evaluate combinations of antimicrobial agents against microorganisms. A broth macrodilution limited series, agar dilution method, or disk diffusion method can also be used. Test methods are based on NCCLS broth dilution susceptibility methods (5) for evaluating the inhibitory or bactericidal activity (4) of specific concentrations in combination at a fixed time. In vitro interactions are calculated algebraically and interpreted as synergistic, indifferent, or antagonistic depending on whether the antibacterial activity of the combination is greater than, equivalent to, or less than, respectively, the activities of the individual agents. In vitro interactions can also be represented geometrically with isobolograms (1).

Combination methods evaluating agent interaction are not routine. Situations in which to consider such testing include those in which the predictability of synergism is unknown, as with a new antimicrobial agent, or when the predictability is unreliable because of the development of bacterial resistance factors or treatment failures. Appendix 5.12–1 lists reported synergistic and antagonistic combinations in vivo for various organisms (2, 3, 6). Methods for determining synergistic activity have not been standardized, and controversy over the value of these tests in the clinical setting continues.

II. SPECIMEN

A. Isolated colonies (minimum of three to five) of similar colony morphology grown overnight at 35°C on BAP (or nonselective supplemented medium if necessary)

B. Isolates from frozen, lyophilized, or other stock conditions require three consecutive subcultures prior to testing.

III.A. MATERIALS: BROTH MICRODILUTION METHOD

Include QC information on reagent container and in QC records.

See procedure 5.2 for additional information, including testing materials and methods for testing fastidious organisms.

A. Media and reagents (storage conditions)

1. Cation-adjusted Mueller-Hinton broth (CAMHB) dispensed in approximately 250-ml aliquots into flasks and in 5-ml aliquots into capped tubes. Store at 2 to 8°C.
2. Diluents for antimicrobial agent stock solutions. Store at 2 to 8°C.
3. Sterile distilled reagent-grade water with 0.02% Tween 80 dispensed in 25-ml aliquots into screw-cap tubes. Store at 25°C.
4. Prepared microdilution synergy trays containing 100 µl of antimicrobial solution per well. Store at −70°C. See item V.A below.
5. BAP. Store at 2 to 8°C.
6. Sterile 0.9% NaCl

B. Supplies

1. Sterile 96-well polystyrene U-bottom microdilution trays
2. Microdilution tray lids, empty microdilution trays, or tray sealers
3. Sterile single-inoculum reservoir trays

III.A. MATERIALS: BROTH MICRODILUTION METHOD *(continued)*

4. Sterile disposable plastic multipronged inoculators (or other inoculating device)
5. Sterile polystyrene capped tubes (12 ml; 17 by 100 mm) or screw-cap tubes (50 ml; 30 by 115 mm)
6. Sterile reservoir for antimicrobial dilutions (V-shaped tray or sterile petri plate)
7. Sterile 1-, 5-, and 10-ml serologic pipettes and pipette bulb
8. Sterile cotton-tipped swabs
9. McFarland 0.5 turbidity standard
10. Microdilution tray storage container or plastic bags

C. Equipment

1. Automatic multichannel pipetter to deliver 50 µl and sterile disposable tips
   ◩ **NOTE:** If only a few panels are being prepared, a single-channel pipette can be used.
2. 0.001-ml calibrated loop
3. Microdilution tray reading device
4. Vortex mixer
5. −70°C freezer
6. 35 ± 2°C ambient-air incubator (CO_2 incubation needed for some organisms)

ANALYTICAL CONSIDERATIONS

IV.A. QUALITY CONTROL: BROTH MICRODILUTION METHOD

A. QC strains

Choose QC strains according to individual antimicrobial agents tested. (*See* procedure 5.2 for additional QC recommendations.)

1. **Example:** For piperacillin and amikacin, use *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853.
2. Test each QC organism on a separate panel according to the standard protocol for testing patient isolates.
3. Record MIC results of individual agents, and compare MICs obtained with MICs expected.

B. Growth controls

Growth control should show heavy turbidity. Purity plate should show good growth and be free of contaminating organisms.

C. Sterility control should be free of any growth.

D. Inoculum controls

Inoculum count verification plate should show approximately 75 to 150 colonies.

E. If any QC result is out of control, inform supervisor, and proceed with corrective action. Document accordingly.

V.A. PROCEDURE: BROTH MICRODILUTION METHOD

A. Preparing checkerboard microdilution panels

1. Plan final concentrations of user-defined panel configuration (example in Appendix 5.12–2).
   a. Determine single-agent MICs for test isolate, or use commonly tested therapeutic range.
      (1) **Example:** *P. aeruginosa* isolated from a blood culture was associated with a piperacillin MIC of 128 µg/ml and an amikacin MIC of 32 µg/ml.
      (2) **Example:** To test several isolates of *P. aeruginosa*, use known MIC range (i.e., piperacillin MIC of 0.5 to 256 µg/ml and amikacin MIC of 0.5 to 32 µg/ml).
   b. Include concentrations ranging from four to eight times the expected MIC to at least 1/8 to 1/16 times the expected MIC in the final panel in order to observe the occurrence and magnitude of synergism or antagonism.
   c. Determine the number of panels to prepare (one for each test isolate and one for each QC strain).

V.A. PROCEDURE: BROTH MICRODILUTION METHOD *(continued)*

2. Prepare intermediate antimicrobial agent concentrations (*see* procedure 5.14 for antimicrobial stock solution preparation).
- **a.** In separate tubes, prepare intermediate concentrations of each of the two antimicrobial agents as shown in Appendix 5.12–3. The intermediate concentrations are two times the final panel concentrations because equal volumes of each agent concentration are added to a well.
- **b.** Prepare sufficient intermediate concentration volumes to dispense respective *x*- and *y*-axis concentrations as listed in Appendix 5.12–2.
  - (1) Examples of per-panel requirements
    - (a) Piperacillin (512 μg/ml): 8 wells × 0.05 ml per well = 0.4 ml of piperacillin at 1,024 μg/ml
    - (b) Piperacillin (256 μg/ml): 8 wells × 0.05 ml per well = 0.4 ml of piperacillin at 512 μg/ml
    - (c) Amikacin (64 μg/ml): 12 wells × 0.05 ml per well = 0.6 ml of amikacin at 128 μg/ml
    - (d) Continue accordingly.
  - (2) Include sufficient volumes for serial dilution and residual minimum volumes for reservoir (e.g., 2 to 5 ml of excess volume is needed).

3. Dispense antimicrobial agents into combination panel (0.1-ml final volume per well).
- **a.** Follow the user-defined schematic in Appendix 5.12–2.

  Example: Well D10 will contain 0.05 ml of piperacillin at 256 μg/ml and 0.05 ml of amikacin at 8 μg/ml, resulting in a 0.1-ml volume of piperacillin at 128 μg/ml and amikacin at 4 μg/ml per well.
- **b.** Pour each working concentration (Appendix 5.12–3) into individual reservoirs.
- **c.** Dispense drug A (piperacillin) with the eight-channel pipetter as follows.

  ◩ **NOTE:** If an eight-channel pipetter is not used, fill each well individually.
  - (1) Add 0.05 ml of drug A (piperacillin at 1 μg/ml) to each well in column 2: A2, B2, C2, D2, E2, F2, G2, and H2.
  - (2) Add 0.05 ml of drug A (piperacillin at 2 μg/ml) to each well in column 3: A3 through H3.
  - (3) Continue to dispense the next-higher concentration of drug A into each well in the next column through column 12.
  - (4) Do not add drug to well H12 (sterility control).
- **d.** Dispense drug B (amikacin) with the eight-channel pipetter as follows.
  - (1) Add 0.05 ml of drug B (amikacin at 2 μg/ml) to each well in row B: B1 through B12.
  - (2) Add 0.05 ml of drug B (amikacin at 4 μg/ml) to each well in row C: C1 through C12.
  - (3) Continue to dispense the next-higher concentration of drug B to each well in the next row through row H.
  - (4) Do not add drug to well H12 (sterility control).
- **e.** Add 0.05 ml of CAMHB to wells A2 through A12.
- **f.** Add 0.05 ml of CAMHB to wells B1, C1, D1, E1, F1, G1, and H1.
- **g.** Add 0.1 ml of CAMHB to wells A1 and H12.
- **h.** Use panels or seal with tape, place in tightly sealed plastic bags, and store at −70°C for up to 6 months.

B. Preparing inoculum

1. Using a sterile swab, transfer organisms from four or five colonies of similar colony morphology to 5 ml of CAMHB.

2. Incubate at 35°C until turbidity matches that of a McFarland 0.5 turbidity standard (approximately 1.5×10^8 CFU/ml).

V.A. PROCEDURE: BROTH MICRODILUTION METHOD *(continued)*

3. For preparation of the intermediate inoculum dilution, calculate volume of standardized suspension to add to 25 ml of water diluent to obtain a final organism concentration of 3×10^5 to 5×10^5 CFU/ml in each well. For example, when using a disposable plastic multipronged inoculating device that delivers 0.01 ml per well, proceed as follows.
   a. McFarland 0.5 suspension = 1.5×10^8 CFU/ml
   b. Add 0.8 ml of suspension to 25 ml of water diluent (1:31 dilution) to achieve 4×10^6 to 5×10^6 CFU/ml.
   c. Prongs deliver 0.01 ml, or 4×10^4 to 5×10^4 CFU/well (4×10^5 to 5×10^5 CFU/ml).

C. Inoculation and incubation

1. Add 0.8 ml of standardized suspension to 25 ml of water-Tween 80.
2. Mix by inverting tube five or six times (try to avoid producing air bubbles).
3. Within 1 h (immediately if testing fastidious organisms), inoculate the MIC tray.
   a. Remove the inoculator-reservoir set from the plastic packaging.
   b. Remove prongs from the reservoir tray, and pour contents of the diluted inoculum into the tray.
   c. Dip prongs into the inoculum suspension.
      (1) Orient sterility well (H12) so that it is not inoculated.
      (2) Press down on the inoculator firmly to ensure that all prongs come in contact with the organism solution.
   d. Inoculate MIC tray by dipping the filled prongs carefully into the MIC tray.
   e. Discard prongs as biohazardous waste.
   f. Using a 0.001-ml calibrated loop, inoculate a purity plate by subculturing 0.001 ml of inoculum from the reservoir onto BAP. Streak for isolation.
   g. Periodically prepare an inoculum count verification plate to validate inoculum size.
      (1) Immediately after inoculation, transfer 0.01 ml from the growth control well to 10.0 ml of sterile 0.9% NaCl, and vortex well.
      (2) Plate 0.1 ml to BAP. Thoroughly spread over the surface of the BAP by streaking in several directions with a loop.
4. Stack inoculated MIC trays no more than four high, and place them in a microdilution tray storage container with the top loose.
5. Incubate MIC trays for 16 to 20 h at 35 ± 2°C. CO_2 incubation alters the pH and affects the activity of some antimicrobial agents and should be avoided.
6. Incubate purity and inoculum verification control plates in CO_2.

D. Reading MICs

1. Examine purity control plate. If results are mixed, repeat the test. When available, examine the inoculum count verification plate (should show 30 to 70 colonies).
2. Place tray on appropriate reading device.
3. Examine growth control well (A1) for heavy turbidity, indicating adequate growth.
4. Examine the uninoculated broth control. Compare questionable growth with that in this well when necessary.
5. The MIC is the lowest concentration of antimicrobial agent(s) causing complete inhibition of growth.
6. Examine drugs A and B as single agents, and record the MICs.
7. Examine each combination well, and record growth or no growth for each well.
8. Refer to Appendixes 5.12–4 and 5.12–5 for examples of outcome and how to record them.

VI.A. CALCULATIONS: BROTH MICRODILUTION METHOD

A. For each combination interaction, calculate the fractional inhibitory concentration (FIC) of each agent as follows.

1. $\text{FIC of agent A} = \dfrac{\text{MIC of agent A in combination}}{\text{MIC of agent A alone}}$

a. Example, Appendix 5.12–4, well E5:

$$\frac{4\ \mu g/ml}{128\ \mu g/ml} = 0.03 = \text{FIC of piperacillin}$$

b. Example, Appendix 5.12–5, well C12:

$$\frac{512\ \mu g/ml}{128\ \mu g/ml} = 4 = \text{FIC of piperacillin}$$

2. $\text{FIC of agent B} = \dfrac{\text{MIC of agent B in combination}}{\text{MIC of agent B alone}}$

a. Example, Appendix 5.12–4, well E5:

$$\frac{8\ \mu g/ml}{32\ \mu g/ml} = 0.25 = \text{FIC of amikacin}$$

b. Example, Appendix 5.12–5, well C12:

$$\frac{2\ \mu g/ml}{32\ \mu g/ml} = 0.06 = \text{FIC of amikacin}$$

3. The FIC may be not determinable (ND) for some combinations of concentrations if MICs of the single agents occur at test range extremes (i.e., greater than the highest or less than or equal to the lowest concentration tested) and if combination MICs are not at least fourfold different.

a. Example: MIC of piperacillin alone is >512 μg/ml, and MIC in combination is 512 μg/ml. Therefore, piperacillin FIC is ND.

b. Example: MIC of amikacin alone and in combination is ≤1 μg/ml. Therefore, amikacin FIC is ND.

4. If an FIC is ND, record, calculate other combination indices, and refer to item VIII.C.4 for instructions on analyzing ND results.

B. Calculate the summation of FIC (ΣFIC) index for each combination as follows.

1. ΣFIC = FIC of agent A + FIC of agent B

a. Example, Appendix 5.12–4, well E5: 0.03 + 0.25 = 0.28

b. Example, Appendix 5.12–5, well C12: 4 + 0.06 = 4.06

2. *Optional:* Record maximum (ΣFIC_{max}) and minimum (ΣFIC_{min}) values for the combination when testing an individual organism (*see* item VIII.C below).

a. Example, Appendix 5.12–4: $\Sigma FIC_{max} = 1.03$ (B10) and $\Sigma FIC_{min} = 0.25$ (D7)

b. Example, Appendix 5.12–5: $\Sigma FIC_{max} = 4.06$ (C12) and $\Sigma FIC_{min} = 0.625$ (F7)

POSTANALYTICAL CONSIDERATIONS

VII.A. REPORTING RESULTS: BROTH MICRODILUTION METHOD

A. Providing QC is acceptable, interpret and record each summation (ΣFIC).

1. Interpretation of summation

a. Synergism, $x \leq 0.5$

b. Indifference, $0.5 < x \leq 4$

c. Antagonism, $x > 4$

d. Examples: ΣFIC = 0.28 = synergism
ΣFIC = 4.06 = antagonism

2. Definitions of synergism, indifference, and antagonism differ among investigators. Check journal instructions, study references, or other sources, such as reference 3.

VII.A. REPORTING RESULTS: BROTH MICRODILUTION METHOD *(continued)*

B. Report organism tested, single antimicrobial agent MICs, and combination interaction.

1. Report each single-agent MIC, and give susceptibility interpretation (*see* procedure 5.2).

Example: *P. aeruginosa*
Piperacillin MIC = 128 μg/ml = resistant
Amikacin MIC = 32 μg/ml = intermediate

2. Report antimicrobial combination and interaction using one of the following formats.

a. For a single isolate tested, report an interaction.

(1) **Example:** The combination of piperacillin and amikacin resulted in synergism.

(2) Even if synergism or antagonism occurs at only one ΣFIC within the combination, report the occurrence.

(3) If both synergism (ΣFIC_{min}) and antagonism (ΣFIC_{max}) occur for one organism within the same combination and if only one interpretation is reported, report antagonism.

b. For a single isolate tested, report concentrations at which synergism occurs.

Example: The combination of piperacillin and amikacin resulted in synergism with piperacillin at 32 or 16 μg/ml in combination with amikacin at 4 μg/ml and with piperacillin at 4 or 8 μg/ml in combination with amikacin at 8 μg/ml.

PREANALYTICAL CONSIDERATIONS

III.B. MATERIALS: BROTH MACRODILUTION METHOD

Include QC information on reagent container and in QC records.

See procedure 5.10.1 for additional information, including testing materials and methods for testing fastidious organisms.

A. Media and reagents (storage conditions)

1. CAMHB dispensed in approximately 250-ml aliquots into flasks and in 5-ml aliquots into capped tubes. Store at 2 to 8°C.

2. Diluents for antimicrobial agent stock solutions. Store at 2 to 8°C.

3. Broth macrodilution tubes containing 1-ml volumes of broth and antimicrobial agents. Store at −70°C. See item V.B below.

4. BAP. Store at 2 to 8°C.

5. Sterile 0.9% NaCl. Store at 25°C.

B. Supplies

1. Sterile polystyrene capped tubes (5 ml; 12 by 75 mm)

2. Sterile polystyrene capped tubes (12 ml; 17 by 100 mm) or screw-cap tubes (50 ml; 30 by 115 mm)

3. Sterile 1-, 5-, and 10-ml serologic pipettes and pipette bulb

4. Sterile cotton-tipped swabs

5. McFarland 0.5 turbidity standard

C. Equipment

1. 0.001-ml calibrated loop

2. Vortex mixer

3. −70°C freezer

4. 35 ± 2°C ambient-air incubator (CO_2 incubator needed for some organisms)

5. Optional equipment for dispensing antimicrobial agents: 500-μl single or repetitive dispensing pipetter with sterile disposable tips

ANALYTICAL CONSIDERATIONS

IV.B. QUALITY CONTROL: BROTH MACRODILUTION METHOD

A. QC strains

Choose QC strains according to individual antimicrobial agents tested (*see* procedure 5.2 for additional QC recommendations).

1. **Example:** For piperacillin and amikacin, use *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853.
2. Test each QC organism in a separate tube set according to the standard protocol for testing patient isolates.
3. Record MICs of individual agents, and compare MICs obtained with MICs expected.

B. Growth controls

Growth control should show heavy turbidity. Purity plate should show good growth and be free of contaminating organisms.

C. Sterility control should be free of any growth.

D. Inoculum controls

Inoculum count verification plate should show approximately 75 to 150 colonies.

E. If any QC result is out of control, inform supervisor, and proceed with corrective action. Document accordingly.

V.B. PROCEDURE: BROTH MACRODILUTION METHOD

A. Preparing broth macrodilution limited checkerboard

1. Plan user-defined configuration as shown in Appendix 5.12–6.
   - **a.** Determine single-agent MICs by broth macrodilution.
     Example: Piperacillin MIC = 4 μg/ml and amikacin MIC = 8 μg/ml
   - **b.** Test single-agent concentrations at one-half the MIC, the MIC, and two times the MIC, and test combination concentrations to detect synergism and antagonism as shown in Appendix 5.12–6.
   - **c.** Determine number of sets of tubes needed (one set for each test isolate and one set for each QC strain).
2. Prepare intermediate antimicrobial concentrations (*see* procedure 5.14 for antimicrobial stock solution preparation).
   - **a.** In separate tubes, prepare intermediate concentration solutions of each of the two antimicrobial agents as shown in Appendix 5.12–7. The intermediate concentrations are four times the final tube concentrations shown in Appendix 5.12–6.
   - **b.** Prepare sufficient volumes needed for each organism (0.5 ml of each drug concentration [intermediate concentration is four times the final concentration] required for each tube).
     (1) Example of per-tube requirements
     (a) Piperacillin (2 μg/ml) is used for three tubes (PP 2, PP 2 + AK 4, and PP 2 + AK 16): 0.5 ml × 3 tubes = 1.5 ml of piperacillin at 8 μg/ml
     (b) Piperacillin (8 μg/ml) is used for five tubes (PP 8, PP 8 + AK 1, PP 8 + AK 2, PP 8 + AK 4, and PP 8 + AK 8): 0.5 ml × 5 tubes = 2.5 ml of piperacillin at 32 μg/ml
     (2) Include sufficient volumes for serial dilutions, and allow extra volumes for pipetting (1 to 2 ml of excess volume is needed).
3. Dispense antimicrobial agents into macrodilution tubes (0.5-ml volumes of each of the two antimicrobial agents).
   - **a.** Follow the user-defined schematic in Appendix 5.12–6, and use intermediate antimicrobial agent concentrations from Appendix 5.12–7 to ob-

V.B. PROCEDURE: BROTH MACRODILUTION METHOD *(continued)*

tain solutions at twice the desired final concentration. For example, the tubes designated piperacillin (8 μg/ml) and amikacin (4 μg/ml) in Appendix 5.12–6 will contain 0.5 ml of piperacillin (32 μg/ml) and 0.5 ml of amikacin (16 μg/ml).

b. Add 0.5 ml of drug A (four times the working solution) to appropriate tubes.

c. Add 0.5 ml of drug B (four times the working solution) to appropriate tubes.

d. Tubes now contain drug A at two times the actual test concentration and drug B at two times the actual test concentration.

e. Dispense 1 ml of CAMHB to the growth control and medium sterility tubes.

f. Store capped tubes at −70°C for up to 6 months.

B. Preparing inoculum

1. Using a sterile swab, transfer organisms from four or five colonies of similar colony morphology to 5 ml of CAMHB.

2. Incubate at 35°C until turbidity matches that of a McFarland 0.5 turbidity standard (approximately 1.5×10^8 CFU/ml).

C. Inoculation and incubation

1. Add 0.25 ml of standardized suspension to 25 ml of CAMHB (resulting suspension = 1.5×10^6 CFU/ml).

2. Mix by inverting tubes five or six times.

3. Use suspension within 1 h (immediately if testing fastidious organisms).

4. Using a sterile serologic pipette, add 1 ml of organism suspension to each tube. Inoculate organisms below the fluid meniscus (subsurface inoculation). Avoid touching the tube sides with the pipette. Use a separate sterile pipette for each inoculation.

a. Resulting suspension = 7.5×10^5 CFU/ml

b. Resulting antimicrobial agent concentration is diluted 1:2 to final desired concentration.

5. Using a 0.001-ml calibrated loop, inoculate a purity plate by subculturing 0.001 ml of inoculum from inoculum suspension (containing 1.5×10^6 CFU/ml) onto BAP. Streak for isolation.

6. Periodically, prepare an inoculum count verification plate to determine inoculum size.

a. Immediately after inoculation, transfer 0.01 ml from the growth control tube to 10.0 ml of sterile 0.9% NaCl, and vortex well.

b. Plate 0.1 ml to BAP. Thoroughly spread over the surface of a BAP by streaking in several directions with a loop.

7. Incubate tubes for 16 to 20 h at 35 ± 2°C. CO_2 incubation alters the pH and affects the activity of some antimicrobial agents and should be avoided.

8. Incubate purity and inoculum count verification plates in CO_2.

D. Reading MICs

1. Examine the corresponding purity control plate. If results are mixed, repeat the test. When available, examine the inoculum count verification plate (should show 75 to 150 colonies).

2. Examine growth control tube for heavy turbidity, indicating adequate growth.

3. Examine the uninoculated broth control tube. Compare questionable growth with this tube when necessary.

V.B. PROCEDURE: BROTH MACRODILUTION METHOD *(continued)*

4. The MIC is the lowest concentration of antimicrobial agent(s) demonstrating complete inhibition of growth.
5. Examine drugs A and B as single agents, and record the MICs.
6. Examine each combination tube, and record growth or no growth for each tube.
7. See Appendixes 5.12–8 and 5.12–9 for examples for outcome and recording.

VI.B. CALCULATIONS: BROTH MACRODILUTION METHOD

A. For each combination, calculate the FIC index for each agent.

1. $\text{FIC of agent A} = \dfrac{\text{MIC of agent A in combination}}{\text{MIC of agent A alone}}$

a. Example, Appendix 5.12–8:

$$\frac{1\ \mu g/ml}{4\ \mu g/ml} = 0.25 = \text{FIC of piperacillin}$$

b. Example, Appendix 5.12–9:

$$\frac{>8\ \mu g/ml}{4\ \mu g/ml} \geq 4 = \text{FIC of piperacillin}$$

2. $\text{FIC of agent B} = \dfrac{\text{MIC of agent B in combination}}{\text{MIC of agent B alone}}$

a. Example, Appendix 5.12–8:

$$\frac{2\ \mu g/ml}{8\ \mu g/ml} = 0.25 = \text{FIC of amikacin}$$

b. Example, Appendix 5.12–9:

$$\frac{1\ \mu g/ml}{8\ \mu g/ml} = 0.125 = \text{FIC of amikacin}$$

3. The FIC may be ND for some combination concentrations if MICs of the single agents occur at test range extremes (i.e., greater than the highest or less than or equal to the lowest concentration tested) and if combination MICs are not at least fourfold different.

a. Example: MIC of piperacillin alone and in combination is >8 μg/ml. Therefore, piperacillin FIC is ND.

b. Example: MIC of amikacin alone and in combination is <1 μg/ml. Therefore, amikacin FIC is ND.

4. If an FIC is ND, record, calculate other combination indices, and refer to item VIII.C.4 for instructions on analyzing ND results.

B. Calculate the ΣFIC for each combination.

1. ΣFIC = FIC of agent A + FIC of agent B

a. Example, Appendix 5.12–8: 0.25 + 0.25 = 0.5

b. Example, Appendix 5.12–9: ≥4 + 0.125 = ≥4.125

2. *Optional:* Record ΣFIC_{max} and ΣFIC_{min} for the combination when testing an individual organism (*see* item VIII.C below).

POSTANALYTICAL CONSIDERATIONS

VII.B. REPORTING RESULTS: BROTH MACRODILUTION METHOD

A. Providing QC is acceptable, interpret and record each ΣFIC.

1. Interpretation of summation

a. Synergism, $x \leq 0.5$

b. Indifference, $0.5 < x \leq 4$

c. Antagonism, $x > 4$

d. Examples: ΣFIC = 0.5 = synergism
ΣFIC = ≥4.125 = antagonism

VII.B. REPORTING RESULTS: BROTH MACRODILUTION METHOD *(continued)*

2. Definitions of synergism, indifference, and antagonism differ among investigators. Check journal instructions, study references, or other sources, such as reference 3.

B. Report organism tested, single antimicrobial agent MICs, and combination interaction.

1. Report each single-agent MIC, and give susceptibility interpretation (*see* procedure 5.2).

Example: *P. aeruginosa*
Piperacillin MIC = 4 μg/ml = susceptible
Amikacin MIC = 8 μg/ml = susceptible

2. Report antimicrobial combination and interaction using one of the following formats.

a. For a single isolate tested, report an interaction.

(1) **Example:** The combination of piperacillin and amikacin resulted in synergism.

(2) Even if synergism or antagonism occurs at only one ΣFIC within the combination, report the occurrence.

(3) If both synergism (ΣFIC_{min}) and antagonism (ΣFIC_{max}) occur for one organism within the same combination and if only one interpretation is reported, report antagonism.

b. For a single isolate tested, report concentrations at which synergism occurs.

Example: The combination of piperacillin and amikacin resulted in synergism with piperacillin at 1 μg/ml in combination with amikacin at 2 μg/ml.

VIII. PROCEDURE NOTES

A. Principle

1. The use of combinations of antimicrobial agents or concomitant agents accompanying one another is common practice, particularly in the treatment of seriously ill or immunocompromised patients. Combinations are usually empirically based on the antimicrobial spectrum and the causative bacterium, if known.

2. Potential benefits of antimicrobial combinations are provision of broad-spectrum antimicrobial coverage, minimization of resistance development, and production of a synergistic interaction to enhance bactericidal treatment efficacy while minimizing toxicity of certain frequently used agents. Disadvantages of concomitant therapy include a potential antagonistic interaction that could delay or impede treatment efficacy. Combinations may be composed of individual agents such as a beta-lactam and an aminoglycoside or as a fixed ratio such as with beta-lactam–beta-lactamase inhibitors or trimethoprim-sulfamethoxazole.

3. Fixed-ratio combinations are routinely tested as single agents in vitro by clinical laboratories using dilution or disk diffusion methods.

4. Theoretically, more than two agents may be tested. However, methods and mathematics become impractical and complex, and such testing is not recommended.

B. Methods

1. Microdilution checkerboard panels can be prepared manually (up to 20 panels) or with an automated 96-channel dispenser for larger batches (see procedure 5.15 for details on preparation methods).

2. A complete macrodilution checkerboard is too cumbersome to prepare and is not recommended.

VIII. PROCEDURE NOTES *(continued)*

3. Alternative inoculum preparation methods may be used (log-phase suspensions must be used if bactericidal activity is to be determined) (*see* procedures 5.2 and 5.10).

C. Interpretation

1. The Instructions to Authors in the January issue of *Antimicrobial Agents and Chemotherapy* defines antagonism as $\Sigma FIC > 4$. This is in part because of the 1 twofold dilution variation allowable within the test system. If each agent varies by 1 dilution within the test system, it is possible to have ΣFIC (4) = FICa (2) + FICb (2). Dilution ranges for testing, especially for a limited macrodilution series, must include a concentration above and below the expected MIC.

2. The concept of ΣFIC_{min} and ΣFIC_{max} (7) reflects interaction dynamics. Several combination concentrations are tested along the checkerboard, and multiple interaction indices are therefore calculated. This reporting method allows accounting for both synergism and antagonism if they are present within the same test panel against one isolate. Few investigators report ΣFIC_{min} and ΣFIC_{max}; most workers report only the presence of antagonism or synergism, even if exhibited only once within the checkerboard.

3. Partial synergism is defined as $\Sigma FIC = 0.5 < x < 1.0$. This designation determines drug interactions in which MICs are decreased 1 twofold dilution but not 2 twofold dilutions. Partial synergism is differentiated from indifference. This designation of partial synergism is controversial.

4. ND interactions are not problematic if other interactions are present for the combination and provide useful information within clinically therapeutic parameters. However, if the MIC interaction ranges do not provide useful information, the test may need to be repeated on a newly designed panel with appropriate ranges.

a. Example: Piperacillin MICs are >512 μg/ml, and combination interactions result in piperacillin MICs of 512 μg/ml. Repeat testing would be not useful, because piperacillin at 512 μg/ml indicates resistance.

b. Example: Amikacin MICs are 2 μg/ml, and combination interactions result in amikacin MICs of ≤1 μg/ml. Repeat testing with a panel reconfigured with amikacin at 0.25 to 16 μg/ml would be useful.

5. Checkerboards may also be graphically represented as isobolograms with interpretations (1). Checkerboard panels and isobolograms may not be meaningful to clinicians or easily reported in patient records (*see* Appendix 5.12–10).

D. Results

Bactericidal activities of the single agents and combinations can be determined when broth dilution and time-kill assay methods are used. Pay strict attention to technical details of the method described for single agents (e.g., use of log-phase inoculum suspension, use of subsurface inoculation technique to macrodilution tube inoculation, etc.; *see* procedure 5.10.1).

A. Kill curve assays can also be used to assess bactericidal activities of combinations of agents (*see* procedure 5.10.3).

B. Alternative bactericidal determination from broth microdilution method

1. Although this method is not the same as that described in procedure 5.10, it has been used successfully in the author's laboratory for several years.

2. Subculture entire well contents (0.1 ml) from wells for the MIC, two times the MIC, and four times the MIC of the individual agents (for example, Appendix 5.12–4, wells A10, A11, A12, G1, and H1) onto separate BAP.

VIII. PROCEDURE NOTES
(continued)

3. Subculture entire well contents (0.1 ml) for each combination interaction at the MIC, two times the MIC, and four times the MIC (for example, Appendix 5.12–4, wells B10, B11, B12, C9, C10, C11, D7, D8, D9, E5, E6, E7, F2, F3, F4, G2, and G3) onto separate BAP.
4. Streak aliquot in three directions to obtain isolated colonies. Incubate at 35°C overnight. Count colonies. Define MBC with a ≥99.9% cutoff based on initial starting inoculum.
5. Calculate bactericidal indices by substituting MBC and fraction bactericidal concentration for MIC and FIC.

IX. LIMITATIONS

A. Synergy testing methods are not standardized as to method reproducibility and interpretation. Synergy testing is often considered a clinical research procedure.

B. QC parameters are undefined for antimicrobial agents in combination. Only individual agents have standard QC parameters. Internal records could note and establish QC strain combination results.

C. Checkerboard testing may be considered imprecise because only 1-$\log_2$ differences are tested, results are taken at only one time point, and testing is based on a linear dose-response curve unlike the exponential dose-response curves observed in vivo.

D. Checkerboard combination interaction methods may not produce results that agree with time-kill methods, because each method measures different parameters.

REFERENCES

1. **Berenbaum, M. C.** 1978. A method for testing synergy with any number of agents. *J. Infect. Dis.* **137:**122–130.
2. **Eliopoulos, G. M., and C. T. Eliopoulos.** 1988. Antibiotic combinations: should they be tested? *Clin. Microbiol. Rev.* **1:**139–156.
3. **Eliopoulos, G. M., and R. C. Moellering, Jr.** 1996. Antimicrobial combinations, p. 330–396. *In* V. Lorian (ed.), *Antibiotics in Laboratory Medicine*, 4th ed. The Williams & Wilkins Co., Baltimore, Md.
4. **NCCLS.** 1999. *Methods for Determining Bactericidal Activity of Antimicrobial Agents.* Approved guideline M26-A. NCCLS, Wayne, Pa.
5. **NCCLS.** 2003. *Methods for Dilution Susceptibility Tests for Bacteria That Grow Aerobically*, 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.
6. **Rahal, J. J., Jr.** 1978. Antibiotic combinations: the clinical relevance of synergy and antagonism. *Medicine* **57:**179–195.
7. **Weinstein, A. J.** 1977. Principles of concomitant antibiotic therapy. *Med. J. Aust. Spec. Suppl.* **2:**19–22.

APPENDIX 5.12–1

Examples of reported combination interactions

Antimicrobial agent combination[a]	Microorganism(s)
Synergism	
Cell wall-active agent + aminoglycoside	
Penicillin, ampicillin, or vancomycin + gentamicin	*Enterococcus faecalis, Enterococcus faecium*
Penicillin, ampicillin, or vancomycin + streptomycin	*Enterococcus faecalis, Enterococcus faecium*
Penicillin + gentamicin or streptomycin	Viridans group streptococci
Penicillin or ampicillin + gentamicin or streptomycin	Group A and B streptococci
Oxacillin or vancomycin + gentamicin	Staphylococci
Beta-lactam + gentamicin, tobramycin, or amikacin	*Enterobacteriaceae*
Antipseudomonal beta-lactam + gentamicin, tobramycin, or amikacin	*Pseudomonas aeruginosa, Enterobacteriaceae*
Other	
Piperacillin + ciprofloxacin	*Pseudomonas aeruginosa*
Oxacillin or vancomycin + rifampin	Staphylococci
Antagonism	
Beta-lactam + cefoxitin	*Enterobacteriaceae*
Cefoxitin + cefamandole	*Enterobacter cloacae*
Ampicillin + chloramphenicol	Streptococci, *Klebsiella pneumoniae*

[a] In vitro reports outnumber in vivo documentation. Documentation is found in the following references: **G. M. Eliopoulos and C. T. Eliopoulos.** 1988. Antibiotic combinations: should they be tested? *Clin. Microbiol. Rev.* **1:**139–156; **G. M. Eliopoulos and R. C. Moellering, Jr.** 1996. Antimicrobial combinations, p. 330–396. *In* V. Lorian (ed.), *Antibiotics in Laboratory Medicine*, 4th ed. The Williams & Wilkins Co., Baltimore, Md.; and **J. J. Rahal, Jr.** 1978. Antibiotic combinations: the clinical relevance of synergy and antagonism. *Medicine* **57:**179–195.

APPENDIX 5.12–2

Example of format of broth microdilution checkerboard panel[a]

A1 No drug	A2 PP 0.5	A3 PP 1	A4 PP 2	A5 PP 4	A6 PP 8	A7 PP 16	A8 PP 32	A9 PP 64	A10 PP 128	A11 PP 256	A12 PP 512
B1 AK 1	B2 AK 1 + PP 0.5	B3 AK 1 + PP 1	B4 AK 1 + PP 2	B5 AK 1 + PP 4	B6 AK 1 + PP 8	B7 AK 1 + PP 16	B8 AK 1 + PP 32	B9 AK 1 + PP 64	B10 AK 1 + PP 128	B11 AK 1 + PP 256	B12 AK 1 + PP 512
C1 AK 2	C2 AK 2 + PP 0.5	C3 AK 2 + PP 1	C4 AK 2 + PP 2	C5 AK 2 + PP 4	C6 AK 2 + PP 8	C7 AK 2 + PP 16	C8 AK 2 + PP 32	C9 AK 2 + PP 64	C10 AK 2 + PP 128	C11 AK 2 + PP 256	C12 AK 2 + PP 512
D1 AK 4	D2 AK 4 + PP 0.5	D3 AK 4 + PP 1	D4 AK 4 + PP 2	D5 AK 4 + PP 4	D6 AK 4 + PP 8	D7 AK 4 + PP 16	D8 AK 4 + PP 32	D9 AK 4 + PP 64	D10 AK 4 + PP 128	D11 AK 4 + PP 256	D12 AK 4 + PP 512
E1 AK 8	E2 AK 8 + PP 0.5	E3 AK 8 + PP 1	E4 AK 8 + PP 2	E5 AK 8 + PP 4	E6 AK 8 + PP 8	E7 AK 8 + PP 16	E8 AK 8 + PP 32	E9 AK 8 + PP 64	E10 AK 8 + PP 128	E11 AK 8 + PP 256	E12 AK 8 + PP 512
F1 AK 16	F2 AK 16 + PP 0.5	F3 AK 16 + PP 1	F4 AK 16 + PP 2	F5 AK 16 + PP 4	F6 AK 16 + PP 8	F7 AK 16 + PP 16	F8 AK 16 + PP 32	F9 AK 16 + PP 64	F10 AK 16 + PP 128	F11 AK 16 + PP 256	F12 AK 16 + PP 512
G1 AK 32	G2 AK 32 + PP 0.5	G3 AK 32 + PP 1	G4 AK 32 + PP 2	G5 AK 32 + PP 4	G6 AK 32 + PP 8	G7 AK 32 + PP 16	G8 AK 32 + PP 32	G9 AK 32 + PP 64	G10 AK 32 + PP 128	G11 AK 32 + PP 256	G12 AK 32 + PP 512
H1 AK 64	H2 AK 64 + PP 0.5	H3 AK 64 + PP 1	H4 AK 64 + PP 2	H5 AK 64 + PP 4	H6 AK 64 + PP 8	H7 AK 64 + PP 16	H8 AK 64 + PP 32	H9 AK 64 + PP 64	H10 AK 64 + PP 128	H11 AK 64 + PP 256	H12 No drug

[a] Well A1, drug-free growth control; well H12, sterility control. Abbreviations: PP 0.5, 0.5 μg of piperacillin per ml; AK 1, 1 μg of amikacin per ml; etc.

APPENDIX 5.12–3

Dilution schematics for two-agent broth microdilution checkerboard

Drug and step no.	Concn (μg/ml)	Source	Diluent (ml)[a]	Intermediate working concn (μg/ml)[b]
Drug A				
1	10,240	Stock	9	1,024
2	1,024	Step 1	1	512
3	1,024	Step 1	3	256
4	1,024	Step 1	7	128
5	128	Step 4	1	64
6	128	Step 4	3	32
7	128	Step 4	7	16
8	16	Step 7	1	8
9	16	Step 7	3	4
10	16	Step 7	7	2
11	2	Step 10	1	1
Drug B				
1	1,280	Stock	9	128
2	128	Step 1	1	64
3	128	Step 1	3	32
4	128	Step 1	7	16
5	16	Step 4	1	8
6	16	Step 4	3	4
7	16	Step 4	7	2

[a] Diluent is usually CAMHB. It is combined with 1 ml of source. These values can be multiplied to obtain sufficient final working volumes.

[b] Intermediate working concentration is prepared at twice the concentration stated on panel configuration.

APPENDIX 5.12–4

Example of Broth Microdilution Showing Synergism, Partial Synergism, and Indifference

Drug A was piperacillin (PP); drug B was amikacin (AK). The test organism was *P. aeruginosa.* The inoculum colony count was 90 CFU, and the final inoculum was 4.5×10^5 CFU/ml. Shaded squares indicate wells showing growth. Abbreviations: SYN, synergism; PSYN, partial synergism; IND, indifference.

PIPERACILLIN (μg/ml)

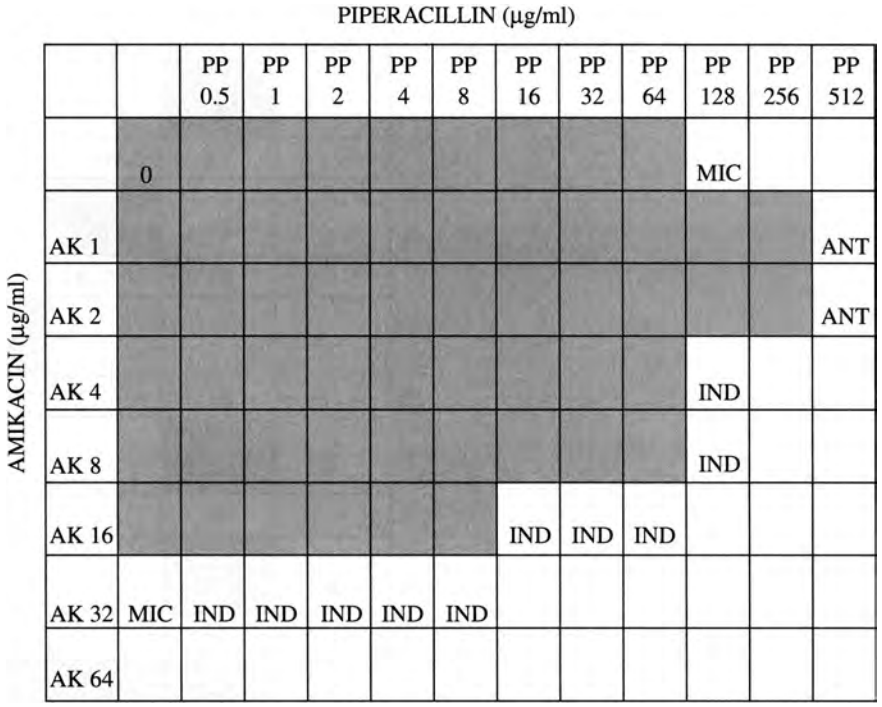
AMIKACIN (μg/ml)		PP 0.5	PP 1	PP 2	PP 4	PP 8	PP 16	PP 32	PP 64	PP 128	PP 256	PP 512
	0									MIC		
AK 1										IND		
AK 2									PSYN			
AK 4							SYN	SYN				
AK 8					SYN	SYN						
AK 16		PSYN	PSYN	PSYN								
AK 32	MIC											
AK 64												

Clear well	Piperacillin		Amikacin		ΣFIC (FIC of A + FIC of B)	Interpretation
	Concn (μg/ml)	FIC of drug A	Concn (μg/ml)	FIC of drug B		
A10	128	MIC of A	0	MIC of A	MIC of A	MIC of A
B10	128	1	1	0.03	1.03	IND
C9	64	0.5	2	0.06	0.56	PSYN or IND
D8	32	0.25	4	0.125	0.375	SYN
D7	16	0.125	4	0.125	0.25	SYN
E6	8	0.06	8	0.25	0.31	SYN
E5	4	0.03	8	0.25	0.28	SYN
F4	2	0.016	16	0.5	0.516	PSYN or IND
F3	1	0.008	16	0.5	0.508	PSYN or IND
F2	0.5	0.004	16	0.5	0.504	PSYN or IND
G1	0	MIC of B	32	MIC of B	MIC of B	MIC of B

APPENDIX 5.12–5

Example of Broth Microdilution Showing Indifference and Antagonism

Drug A was piperacillin (PP); drug B was amikacin (AK). The test organism was *P. aeruginosa.* The inoculum colony count was 90 CFU, and the final inoculum was 4.5×10^5 CFU/ml. Shaded squares indicate wells showing growth. Abbreviations: IND, indifference; ANT, antagonism.



Clear well	Piperacillin		Amikacin		ΣFIC (FIC of A + FIC of B)	Interpretation
	Concn (μg/ml)	FIC of drug A	Concn (μg/ml)	FIC of drug B		
A10	128	MIC of A	0	MIC of A	MIC of A	MIC of A
B12	512	4	1	0.03	4.03	ANT
C12	512	4	2	0.06	4.06	ANT
D10	128	1	4	0.125	1.125	IND
E10	128	1	8	0.25	1.25	IND
F9	64	0.5	16	0.5	1	IND
F8	32	0.25	16	0.5	0.75	IND
F7	16	0.125	16	0.5	0.625	IND
G6	8	0.06	32	1	1.06	IND
G5	4	0.03	32	1	1.03	IND
G4	2	0.015	32	1	1.015	IND
G3	1	0.008	32	1	1.008	IND
G2	0.5	0.004	32	1	1.004	IND
G1	MIC of B	MIC of B	32	MIC of B	MIC of B	MIC of B

APPENDIX 5.12–6

Example of Limited-Series Checkerboard Format for Broth Macrodilution

Concentrations (in micrograms per milliliter) to prepare and test are shaded. The MIC of drug A is 4 µg/ml, and the MIC of drug B is 8 µg/ml. Concentrations are tested for synergism (SYN) and antagonism (ANT).

		Drug A				
Drug B	µg/ml		0.5	1	2	4	8
		0			1/2 MIC	MIC	2× MIC
			A0.5	A1	A2	A4	A8
	1		SYN				ANT
		B1	A0.5+B1				A8+B1
	2			SYN			ANT
		B2		A1+B2			A8+B2
	4	1/2 MIC			SYN		ANT
		B4			A2+B4		A8+B4
	8	MIC					ANT
		B8					A8+B8
	16	2× MIC	ANT	ANT	ANT	ANT	
		B16	A0.5+B16	A1+B16	A2+B16	A4+B16	

APPENDIX 5.12–7

Dilution schematics for two-agent broth macrodilution limited checkerboard

Drug and step no.	Concn (µg/ml)	Source	Vol (ml) of[a]:		Intermediate working concn (µg/ml)[c]
			Source	Diluent[b]	
Drug A					
1	6,400	Stock	1	9	640
2	640	Step 1	0.5	9.5	32
3	32	Step 2	1	1	16
4	32	Step 2	1	3	8
Drug B					
1	1,600	Stock	1	9	160
2	160	Step 1	0.5	9.5	8
3	8	Step 2	1	1	4
4	8	Step 2	1	3	2

[a] These values can be multiplied to obtain sufficient final working volumes.

[b] Diluent is usually CAMHB.

[c] Intermediate working concentration is prepared at four times the concentration stated on schematic.

APPENDIX 5.12–8

Example of broth macrodilution showing synergism (*see* p. 5.12.22)

APPENDIX 5.12–9

Example of broth macrodilution showing antagonism (*see* p. 5.12.23)

APPENDIX 5.12–10

Representing Checkerboards as Isobolograms

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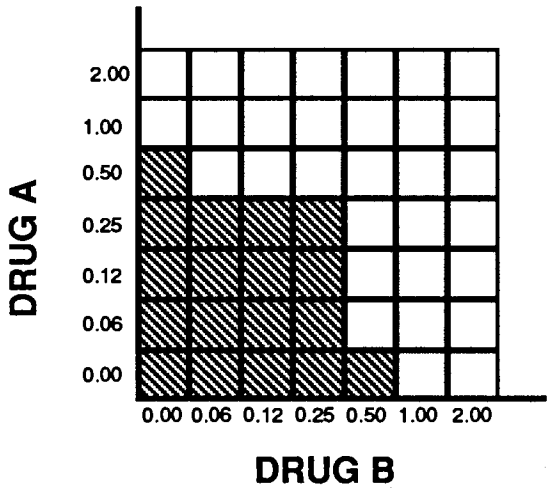

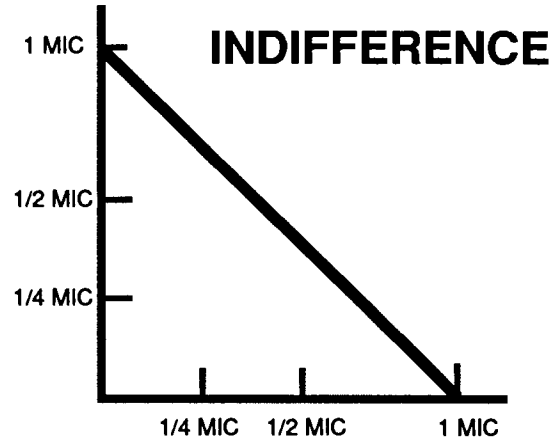

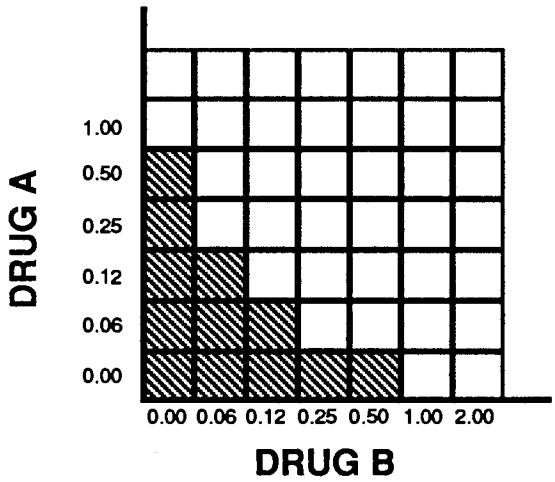

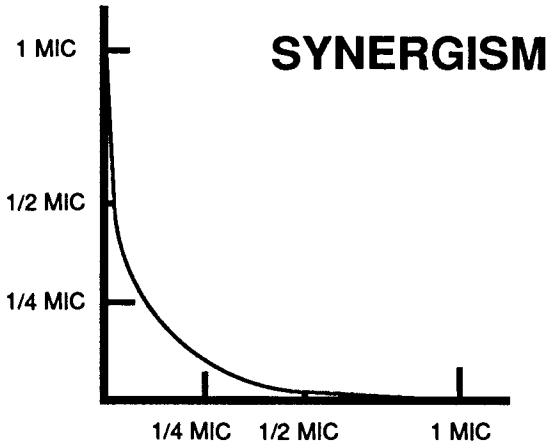

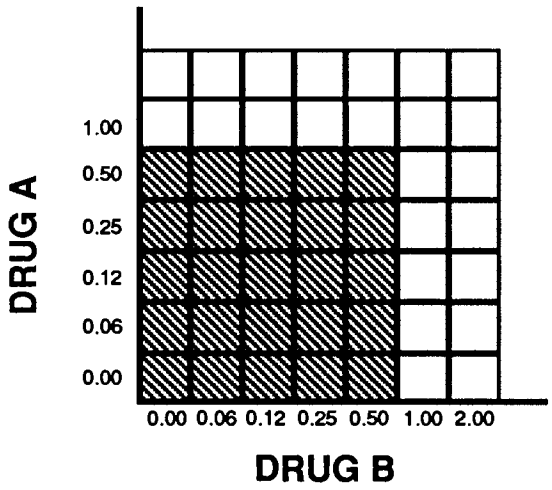

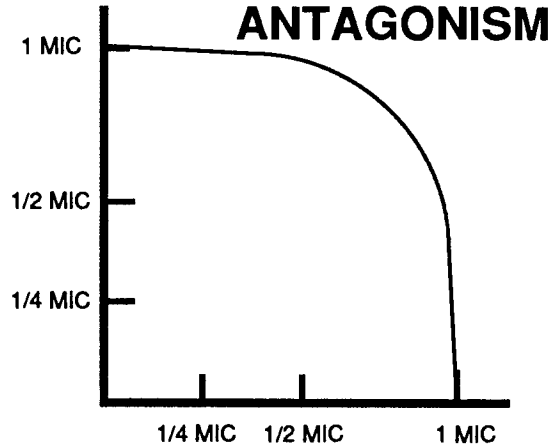

APPENDIX 5.12–10 *(continued)*

Reference

1. **Eliopoulos, G. M., and R. C. Moellering, Jr.** 1991. Antimicrobial combinations, p. 432–492, Fig. 13.2. *In* V. Lorian (ed.), *Antibiotics in Laboratory Medicine,* 3rd ed. The Williams & Wilkins Co., Baltimore, Md.

APPENDIX 5.12–11

Synergy Testing by Disk Agar Diffusion Methods

With disk agar diffusion methods, organisms can be qualitatively screened for synergism, indifference, and antagonism against two antimicrobial agents. Results are best for studying antagonism (1).

Use standard techniques to determine zone diameters for individual antimicrobial agents of interest (*see* procedure 5.1). On another Mueller-Hinton agar plate, place disks separated by a distance equal to the sum of the zone radii for each disk tested separately. After incubation, observe the interface of the zones of inhibition. Theoretically, antagonism shows a truncated zone, indifference shows no change, and synergism shows an enhanced zone. Visualization of results may be difficult, especially if one agent has no zone of inhibition.

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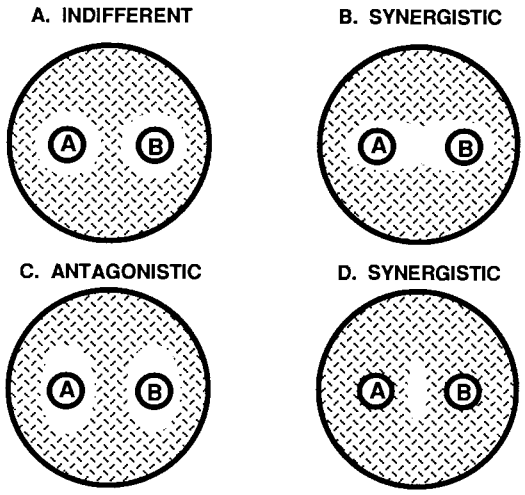


Reference

1. **Eliopoulos, G. M., and R. C. Moellering, Jr.** 1996. Antimicrobial combinations, p. 330–396. *In* V. Lorian (ed.), *Antibiotics in Laboratory Medicine,* 4th ed. The Williams & Wilkins Co., Baltimore, Md.

APPENDIX 5.12–12

Planning Studies

A. Most studies test two antimicrobial agent combinations. Theoretically, more than two agents could be tested, but practicality obviates this choice because of the number of combinations that would be tested and the difficulty of determining the contribution of each agent.

B. Antimicrobial agent concentration ranges to test must be planned on the basis of MICs, percent resistance among organisms tested, achievable concentrations in serum, etc. Numbers of organisms to test and study parameters define the best methods to use.

   1. For example, if the objectives of the study are to study a two-agent combination against 10 genera of gram-negative aerobic bacilli, with each genus composed of 100 species, a 96-well microdilution panel prepared with an automated 96-channel dispenser would be the most efficient. MIC interactions would range over 11 serial twofold concentrations of agent A and 7 serial twofold concentrations of agent B.

APPENDIX 5.12–12 *(continued)*

2. For example, if 25% of the *P. aeruginosa* strains are resistant to piperacillin and 10% are resistant to amikacin according to previously determined MICs, then the last two or three concentrations to be tested in the combination panel should be in the MIC resistance range, i.e., 128, 256, and 512 μg/ml for piperacillin and 32 and 64 μg/ml for amikacin.
3. For example, if study goals are to test for antagonism by using an agent that has been associated with this phenomenon, a limited microdilution or macrodilution checkerboard series can be prepared. Use a fixed concentration at the susceptibility breakpoint (e.g., cefoxitin at 8 or 16 μg/ml) combined with serial twofold dilutions of the second agent (e.g., piperacillin at 1 through 512 μg/ml). From this scheme, isolates for which the FIC ratio of piperacillin MICs in combination with 8 μg of cefoxitin per ml/piperacillin MICs alone was >4 (drug A portion of ΣFIC) would be scored as antagonistic regardless of the drug B portion (cefoxitin) of ΣFIC.
4. Even if the agent MICs are known, test a broad range of concentrations and not just the MIC and one-fourth, one-half, twice, and four times the MIC, because MICs can vary between methods. MICs can also vary within a method, and the magnitude of synergism or antagonism can be determined (e.g., synergism exhibited was ≤ 0.125 for each organism with piperacillin and amikacin in combination).

Example of Broth Macrodilution Showing Synergism

Drug A: PIPERALILLIN
Drug B: AMIKACIN

Organism: *Pseudomonas aeruginosa*
Inoculum colony count = 134 CFU
Final inoculum = 6.7×10^5 CFU/ml

Single agent	Concn (µg/ml)	Turbidity	Outcome
Growth control	0	Yes	Growth
Drug A	2	Yes	1/2 MIC A
Drug A	4	No	MIC A
Drug A	8	No	2× MIC A
Drug B	4	Yes	1/2 MIC B
Drug B	8	No	MIC B
Drug B	16	No	2× MIC B

Drug A (µg/ml)

Drug B (µg/ml)		0.5	1	2	4	8
					MIC A	
1						
2			SYN			
4						
8	MIC B					
16						

Note: Shaded squares indicate growth present in tube.

Combination tubes		Turbidity	FIC A	FIC B	ΣFIC	Outcome
Drug A concn (µg/ml)	Drug B concn (µg/ml)					
0.5	1	Yes	Growth	Growth		
0.5	16	No		>MIC B		
1	2	No	0.25	0.25	0.5	SYN
1	16	No		>MIC B		
2	4	No	>MIC A+B	>MIC A+B		
2	16	No		>MIC B		
4	16	No		>MIC B		
8	1	No	>MIC A			
8	2	No	>MIC A			
8	4	No	>MIC A			
8	8	No	>MIC A			

Example of Broth Macrodilution Showing Antagonism

Drug A: PIPERALILLIN
Drug B: AMIKACIN

Organism: *Pseudomonas aeruginosa*
Inoculum colony count = 134 CFU
Final inoculum = 6.7×10^5 CFU/ml

Single agent	Concn (µg/ml)	Turbidity	Outcome
Growth control	0	Yes	Growth
Drug A	2	Yes	1/2 MIC A
Drug A	4	No	MIC A
Drug A	8	No	2× MIC A
Drug B	4	Yes	1/2 MIC B
Drug B	8	No	MIC B
Drug B	16	No	2× MIC B

Drug A (µg/ml)

Drug B (µg/ml)		0.5	1	2	4	8	>8
	■			■	MIC A		
1		■				■	ANT
2			■				
4	■			IND			
8	MIC B						
16							

Note: Shaded squares indicate growth present in tube.

Combination tubes		Turbidity	FIC A	FIC B	ΣFIC	Outcome
Drug A concn (µg/ml)	Drug B concn (µg/ml)					
0.5	1	Yes	Growth	Growth		
0.5	16	No		>MIC B		
1	2	Yes	Growth	Growth		
1	16	No		>MIC B		
2	4	No	0.5	0.5	1	IND
2	16	No		>MIC B		
4	16	No		>MIC B		
8	1	Yes	Growth	Growth		
8	2	No	>MIC A			
8	4	No	>MIC A			
8	8	No	>MIC A			
16	1	No test	≥4	0.125	≥4.125	ANT

5.13 Quality Assurance Measures for Antimicrobial Susceptibility Testing

I. PRINCIPLE

QC is performed to ensure proper performance of antimicrobial susceptibility tests in order to provide accurate, reproducible, and timely results. The basic QC procedure used in clinical laboratories involves testing reference strains that have defined characteristics of susceptibility to the antimicrobial agent(s) tested. These strains must be properly maintained in order to ensure their reliable performance. Testing the QC strains controls many parameters of the antimicrobial susceptibility test; however, testing the reference strains alone does not always ensure reliable results when testing patients' isolates. Inclusion of supplemental control strains (particularly if recommended in the manufacturer's instructions when using a commercial test system), evaluation of susceptibility profiles on patients' isolates, verifying technologist competency, and review of cumulative susceptibility statistics are some of the additional measures that can be taken to further QC antimicrobial susceptibility tests.

II. REQUIREMENTS OF ACCREDITING AGENCIES FOR QUALITY CONTROL OF ANTIMICROBIAL SUSCEPTIBILITY TESTS

A. CAP ([312] 966-5700; http://www.cap.org/html/ftpdirectory/checklistftp.html)

1. Requirements stated in *CAP Accreditation Checklist* (1)
2. Test organisms in pure cultures only.
3. Check each new lot of disks for activity before use.
4. Establish guidelines for the number and type of antimicrobial agents reported for bacteria isolated from different infection sites.
5. For disk or dilution tests, test control organisms with each new lot or batch of antimicrobial agents or media and each day the test is performed.
6. Control organisms can be tested weekly providing a laboratory can document satisfactory performance with daily control tests (as suggested in NCCLS guidelines).
7. Establish tolerance (out-of-control) limits for antimicrobial potency.
8. For disk diffusion or dilution tests, provide written criteria for interpretation of endpoints or zone sizes.
9. Use a turbidity standard (or equivalent) to standardize the inoculum.
10. Verify unusual or inconsistent results obtained when testing patients' isolates.

B. Centers for Medicare and Medicaid Services, which administers Clinical Laboratory Improvement Amendments of 1988 (CLIA '88) ([202] 512-1530; http://www.cms.gov/clia/)

1. Requirements in CLIA regulations (6)
2. Check each new batch of media and each lot of antimicrobial disks before, or concurrent with, initial use, using approved reference organisms.
3. Zone sizes or MICs for reference organisms must be within established limits before reporting patient results.
4. Each day tests are performed, the laboratory must use the appropriate control organism(s) to check the procedure.

II. REQUIREMENTS OF ACCREDITING AGENCIES FOR QUALITY CONTROL OF ANTIMICROBIAL SUSCEPTIBILITY TESTS *(continued)*

5. Labs that follow NCCLS standards can go to weekly QC. Labs that do not follow NCCLS standards must do daily QC. (*Note:* Methods utilized with Food and Drug Administration [FDA]-cleared commercial systems are comparable to NCCLS standards.)

C. JCAHO ([630] 792-5785; http://www.jcaho.org/accredited + organizations/laboratory + services/index.htm)

1. Requirements stated in standard 8.1 (2).

2. Follows NCCLS guidelines.

D. Some states have specific requirements for QC of antimicrobial susceptibility testing. Obtain information from state laboratory directors.

III. MICROORGANISMS SUGGESTED FOR QUALITY CONTROL OF ANTIMICROBIAL SUSCEPTIBILITY TESTS

A. See Appendix 5.13–1 for a listing of QC strains generally used for antimicrobial susceptibility tests as recommended by the NCCLS (3, 4).

B. Specific notes for the standard aerobic bacterial QC strains

1. *Pseudomonas aeruginosa* ATCC 27853

a. Decreased susceptibility to aminoglycosides as concentrations of Ca^{2+} and Mg^{2+} increase

b. May spontaneously mutate and become increasingly resistant to *Pseudomonas*-active penicillins (e.g., carbenicillin) if repeatedly subcultured.

c. May produce double zone around imipenem disk which is probably due to Mueller-Hinton medium; this is often corrected by using a new lot of medium.

2. *Escherichia coli* ATCC 35218

a. Include when testing beta-lactam–beta-lactamase inhibitor combination agents (amoxicillin-clavulanic acid, ampicillin-sulbactam, ticarcillin-clavulanic acid, piperacillin-tazobactam).

b. This is a beta-lactamase-producing strain (in contrast to *E. coli* ATCC 25922).

3. *Enterococcus faecalis* ATCC 29212

a. Test to make certain medium is sufficiently low in thymidine.

(1) Increased amounts of thymidine interfere with the activity of sulfonamides, trimethoprim, and trimethoprim-sulfamethoxazole, which act by interrupting folate synthesis in bacteria (isolates may appear falsely resistant to these agents).

(2) Enterococci have a unique ability to utilize exogenous thymine (product resulting from enzymatic action of thymidine phosphorylase on thymidine) and can escape the antibacterial action of these drugs, thus appearing resistant if a significant amount of thymidine or thymine is present in medium.

b. Media with blood components (except horse blood, which contains thymidine phosphorylase) contain increased concentrations of thymidine which can result in false resistance for sulfonamides, trimethoprim, and trimethoprim-sulfamethoxazole.

C. When possible, select and test QC organism(s) that most closely resembles the patient isolates to be tested.

D. When performing MIC tests, optimally use QC strains with on-scale endpoints for drugs tested (*see* example in Appendix 5.13–2).

E. When using a commercial antimicrobial susceptibility test system, follow the manufacturer's instructions for QC.

IV. SOURCES OF QUALITY CONTROL STRAINS

A. Obtain directly from the ATCC (http://www.atcc.org/).

B. Obtain from commercial company that sells lyophilized vials (or swabs) containing standard QC strains, such as the following.

1. Becton Dickinson Microbiology Systems (BBL QualiSwab) (http://www.bd.com)

2. MicroBioLogics (LYFO-DISK and KWIK-STIK) (http://www.microbiologics.com/)

3. Remel (Bacti disks) (http://www.remel.com/)

C. Obtain from manufacturer of commercial system used (e.g., Vitek, MicroScan).

V. MAINTENANCE OF QUALITY CONTROL AEROBIC BACTERIAL STRAINS

A. For maintenance of anaerobic bacteria, see section 4.

B. Permanent stock cultures—freezing for long-term storage

1. Materials

a. Media and reagents

(1) Stock bacterial strain obtained from the ATCC or other reliable source. Do not prepare stock cultures from isolates that have been repeatedly subcultured or for which the source is unknown. Store as suggested by the manufacturer.

(2) Brucella broth containing 15% glycerol

(a) Prepare 100 ml of brucella broth according to manufacturer's directions.

(b) Add 15 ml of glycerol to 85 ml of broth and mix well.

(c) Dispense into 250-ml flasks and autoclave according to the manufacturer's directions for brucella broth.

(d) Store at 2 to 8°C.

(3) BAP, CHOC, or other nonselective agar plates
Store at 2 to 8°C.

b. Supplies

(1) Freezer-safe (−60 to −70°C) 1-dram (1 fluidram = 3.697 ml) glass screw-cap vials

(2) Permanent adhesive labels, rectangles (1 by 0.75 in.) and dots (0.5-in. diameter)

(3) Cellophane tape

(4) Sterile cotton-tipped swabs

(5) Sterile polystyrene capped tubes (17 by 100 mm)

c. Equipment

(1) Vortex mixer

(2) −60°C or lower freezer

2. Procedure

a. Subculture stock bacterial strain to a BAP (or other nonselective agar plate), and incubate overnight at 35°C. Subculture for isolation to check purity; subculture a second time.

b. Prepare rectangular label with identification of stock strain and date of preparation of stock culture.

c. Affix rectangular labels to side of vials and cover with cellophane tape.

d. Prepare dot labels with abbreviated stock culture identification.

e. Affix dot labels to lids of vials.

f. Dispense approximately 10 ml of sterile brucella broth with 15% glycerol into 10-ml polystyrene tube.

g. Using a cotton-tipped swab, transfer growth from agar plate to brucella broth containing 15% glycerol, and disperse well, making a heavy suspension.

V. MAINTENANCE OF QUALITY CONTROL AEROBIC BACTERIAL STRAINS *(continued)*

h. Vortex for 15 to 20 s.
i. Dispense approximately 1.0-ml volumes into each of 10 sterile vials.
j. Allow suspension to remain at room temperature for 30 to 60 min.
k. Freeze suspension at −60°C or lower for up to 3 years (storage for 3 years is usually acceptable).
(1) Some organisms may survive and maintain their antimicrobial susceptibility characteristics longer.
(2) Some strains of more fragile organisms such as *Streptococcus pneumoniae* and *Neisseria gonorrhoeae* may not survive 3 years.
(3) Sometimes it is necessary to add antimicrobial agent to the brucella broth containing 15% glycerol to help preserve particular resistance characteristics. For example, it is helpful to add penicillin stock solution to broth (final concentration, 1μg/ml) for storing penicillinase-producing *N. gonorrhoeae.*

C. Working stock cultures
1. Additional materials required
a. Soybean casein digest agar (TSA) slants
b. Other slants such as blood agar or CHOC will be needed for fastidious organisms.
Store at 2 to 8°C.
2. Procedure
a. Remove a small amount of frozen suspension from vial containing permanent stock culture, subculture to a BAP (or other nonselective agar plate), and incubate overnight at 35°C in ambient air. CO_2 may be needed for some fastidious organisms.
(1) One method of obtaining a portion of the frozen suspension is to scrape the top of the suspension with a sterile applicator stick; if the vial is removed from the freezer for less than 1 min and thawing does not occur, the vial can be safely placed back into the freezer.
(2) Alternatively, allow vial to thaw, subculture, and then discard vial.
b. Subculture organisms from three to five isolated colonies to a soybean casein digest agar slant.
c. Incubate overnight at 35°C.
d. Tighten cap, and store slant at 2 to 8°C for up to 1 month.

D. Use of working stock cultures
1. Subculture from slant to a BAP (or other nonselective agar plate), and incubate overnight at 35°C.
2. Test isolated colonies of QC strains according to the standard procedure for testing patient isolates.
3. Frozen permanent stock cultures must be subcultured two times before being used for antimicrobial susceptibility testing.

E. Alternative methods such as freeze drying or lyophilization may be used for long-term storage.

VI. CONTROLLING ANTIMICROBIAL SUSCEPTIBILITY TESTS WITH RECOMMENDED QUALITY CONTROL STRAINS AND LIMITATIONS OF USING THESE STRAINS

A. The test variables that can be controlled to various degrees by testing QC strains are as follows (*see* Appendix 5.13–3 for additional details).
1. Antimicrobial potency
2. Test medium
a. pH
b. Cations (Ca^{2+}, Mg^{2+})
c. Thymidine
d. Medium composition, growth factors
e. Agar depth (disk diffusion)

VI. CONTROLLING ANTIMICROBIAL SUSCEPTIBILITY TESTS WITH RECOMMENDED QUALITY CONTROL STRAINS AND LIMITATIONS OF USING THESE STRAINS *(continued)*

3. Incubation conditions (temperature, atmosphere)
4. Standardization of inoculum and inoculation technique
5. Batch contamination of media or supplies
6. Instrument functioning
7. Measurement of endpoints

B. Some of the test variables not readily controlled are as follows.

1. Individual antimicrobial agent-organism test problem (empty, dry, overfilled, or underfilled disk, well, or tube)
2. Sporadic contamination of broth systems (with MIC tests, purity plate helps avoid problems)
3. Sporadic instrument malfunction
4. NaCl content for oxacillin broth tests with staphylococci
5. Subjective reading of "difficult" endpoints, (e.g., "trailing" or "skipped wells")
6. Interpretation of results, use of appropriate interpretive criteria
7. Transcription errors
8. Individual technical errors
9. Patient isolates that are considerably different from the recommended QC strains whereby testing of these is only modestly controlled by performance testing of the NCCLS-suggested QC reference strains. Some of these include the following.
   a. Organisms with "unusual" growth characteristics (mucoid *P. aeruginosa* or dwarf *E. coli*)
   b. Organisms with unusual resistance characteristics (e.g., organisms with inducible resistance)

C. Because satisfactory performance of the QC strains does not guarantee accurate results for patient isolates, it is important to have additional mechanisms in place to verify individual results obtained from testing bacteria isolated from patient specimens.

VII. VERIFICATION OF SUSCEPTIBILITY RESULTS FOR BACTERIA ISOLATED FROM PATIENT SPECIMENS

A. The antibiogram is the overall antimicrobial susceptibility profile of a bacterial isolate to a battery of antimicrobial agents. Some bacteria have typical profiles, which can be used to do the following.

1. Verify antimicrobial results
2. Verify organism identification

B. In developing an antibiogram check program to aid technologists in consistently identifying atypical (and potentially erroneous) results, provide the following.

1. Descriptions of typical antibiograms for given species (e.g., a common profile for a *Staphylococcus aureus* isolate is resistance to penicillin and susceptibility to clindamycin, erythromycin, oxacillin, and vancomycin). General antibiograms for commonly encountered bacteria are shown in Appendixes 5.13–4a to 5.13–4d.
2. Descriptions of the relatedness of drugs tested (e.g., activity hierarchy). For example, the activity hierarchy of the four generations of cephalosporins against the *Enterobacteriaceae* is fourth > third > second > first.
3. Information on exceptions to "hierarchy" rules such as the following.
   a. Occasional gram-negative bacilli may possess extended-spectrum beta-lactamases (ESBLs) that selectively inactivate cephalosporins.
      (1) **Example:** Occasional *E. coli* organisms may appear susceptible in vitro to cefazolin (first-generation cephalosporin) but resistant to ceftazidime (third-generation cephalosporin).

VII. VERIFICATION OF SUSCEPTIBILITY RESULTS FOR BACTERIA ISOLATED FROM PATIENT SPECIMENS *(continued)*

(2) When this occurs, report all cephalosporin results so clinicians do not extrapolate that the isolate is susceptible to all cephalosporins because the isolate is susceptible to cefazolin. For organisms with ESBLs, follow standard reporting rules and report as resistant to all cephalosporins.

b. Occasional gram-negative bacilli (e.g., *Acinetobacter baumannii*) may possess aminoglycoside-inactivating enzymes that inactivate gentamicin, tobramycin, and amikacin, but not netilmicin. When this occurs, report all aminoglycoside results.

4. Informal updates to advise staff of the prevalence of a particular "atypical" antibiogram at a given time (e.g., increased incidence of nosocomial infections due to gentamicin-resistant *Providencia rettgeri*).

5. Descriptions of the types of antimicrobial resistance (or susceptibility) that when reported (or missed) are likely to impact on patient care. The following are examples.

a. *P. aeruginosa* resistant to amikacin, gentamicin, and tobramycin

b. Oxacillin-resistant *S. aureus*

c. Ceftriaxone-resistant *S. pneumoniae* (particularly from CSF)

C. To verify atypical antibiograms, proceed as follows.

1. Check for transcription errors.

2. Reexamine disk diffusion plate, MIC tray, purity plate, etc., to make certain growth was adequate and appears to represent a pure culture, the test was not misread, and results were correctly interpreted.

a. Subtle problems may not always be detected upon initial examination of the test.

b. Gross examination of test panel (including panels used with automated systems) may reveal blocked or empty well, etc.

c. Too-light growth (e.g., 1 to 2+ in broth system or scanty growth on disk diffusion plate) may result in false-susceptible results.

d. On blood-supplemented Mueller-Hinton agar plates, measure zone of inhibition of growth, *not* zone of inhibition of hemolysis.

e. In broth dilution systems, individually assess each "skipped well or tube" situation and determine whether this can be ignored (versus repeat testing).

3. Check previous reports on the patient: was atypical antibiogram noted and verified previously?

4. Repeat susceptibility tests, identification tests, or both. It may be helpful to use an alternative test method to verify unusual results.

5. Send isolate to a reference lab for confirmation testing using an NCCLS dilution reference method to verify highly atypical results or results that have not been previously reported (e.g., vancomycin-intermediate or -resistant *S. aureus*, cefotaxime-resistant *Haemophilus influenzae*). Consult with the local public health department when highly significant (from a public health perspective) resistance is noted (e.g., vancomycin-intermediate or -resistant *S. aureus*). *Save the isolate.*

D. Examples of results that warrant verification before reporting are listed in Appendix 5.13–5.

VIII. COMPETENCY ASSESSMENT IN ANTIMICROBIAL SUSCEPTIBILITY TESTING

Competency assessment is regulated by CLIA '88 as outlined in the Code of Federal Regulations. Competency assessment of employees should be done within the first 6 months of employment of new personnel and at least annually thereafter.

A. Competency assessment for antimicrobial susceptibility testing should include the following areas.
  1. Testing and reporting of appropriate organisms with appropriate antimicrobial agents
  2. Utilization of correct procedures for testing patient and QC organisms
  3. Verification of antimicrobial susceptibility profiles on patients' organisms
  4. Investigations into abnormal results or profiles
  5. Appropriate documentation of all testing
  6. Appropriate maintenance of instruments utilized for testing

B. Tools that can be utilized to measure and document competency include, but are not limited to, the following.
  1. Direct observation of routine patient testing
  2. Monitoring the recording and reporting of test results
  3. Review of interim results (worksheets, QC, proficiency testing, and preventive maintenance records)
  4. Direct observation of instrument maintenance and function checks
  5. Assessment of problem-solving skills

C. Competency assessment should focus on results that are likely to impact patient care and avoid medical errors.

D. An example of a competency assessment checklist is shown in Appendix 5.13–6.

IX. CUMULATIVE ANTIMICROBIAL SUSCEPTIBILITY TEST DATA REPORTS

Most laboratories analyze results for all routine antimicrobial susceptibility tests and generate a report that presents these cumulative data in a table. The data presented include the percentage of isolates susceptible to specific antimicrobial agents. For example, the report would indicate the percentage of *S. aureus* organisms that are susceptible to oxacillin.

A. The NCCLS has recently published a guideline, *Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data* (NCCLS M39-A), that suggests how to prepare this report (5). The goal of this is to provide antibiogram data to be used by doctors for empiric therapy guidelines.

B. Suggested report format in M39-A
  1. Prepare report annually.
  2. Include species for which there are at least 10 isolates.
  3. For analysis, include only the first isolate/patient/analysis period (irrespective of body site or susceptibility profile) to eliminate duplicate results that may skew the data.
    a. This will not identify a very unusual phenotype if this is not the "first" isolate from the patient; however, such unusual phenotypes should be identified during daily supervisory review activities.
    b. Laboratories can search entire database without excluding results when it is necessary to review isolates rather than patients.
  4. Exclude surveillance isolates such as those obtained during vancomycin-resistant enterococcus (VRE) or methicillin-resistant *S. aureus* (MRSA) surveillance activities.
  5. Include drugs routinely tested.

IX. CUMULATIVE ANTIMICROBIAL SUSCEPTIBILITY TEST DATA REPORTS *(continued)*

6. Do not examine subset of results that are obtained only in certain circumstances (e.g., if imipenem is tested only on cefepime-resistant *Enterobacteriaceae*, do not compare imipenem to drugs routinely tested; imipenem results will be skewed [more resistant]).
7. Calculate and present data for percentage susceptible, except for the following.
   - **a.** *S. pneumoniae:* calculate percent susceptible and percent intermediate for penicillin
   - **b.** *S. pneumoniae:* calculate percent susceptible for meningitis and nonmeningitis for cefotaxime and ceftriaxone
   - **c.** Viridans group streptococci: calculate percent susceptible and percent intermediate for penicillin
   - **d.** *S. aureus:* calculate results from all isolates
   - **e.** *S. aureus:* calculate results from the MRSA subset

REFERENCES

1. **College of American Pathologists.** 2001. *CAP Accreditation Checklist, Microbiology*, College of American Pathologists, Northfield, Ill.
2. **Joint Commission on Accreditation of Healthcare Organizations.** 2002–2003. Section 2: technical functions, quality control, specialty-bacteriology, mycobacteriology, and mycology, p. QC-6. *In Comprehensive Accreditation Manual for Pathology and Clinical Laboratory Services.* Joint Commission on Accreditation of Healthcare Organizations, Chicago, Ill.
3. **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.
4. **NCCLS.** 2003. *Performance Standards for Antimicrobial Disk Susceptibility, 8th ed. Approved* standard M2-A8. NCCLS, Wayne, Pa.
5. **NCCLS.** 2002. *Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data.* Approved guideline M39-A. NCCLS, Wayne, Pa.
6. **U.S. Department of Health and Human Services, Centers for Medicare and Medicaid Services, and Centers for Disease Control and Prevention.** 2003. Medicare, Medicaid, and CLIA programs. *Fed. Regist.* **68:**3708. (42CFR493.1261.)

SUPPLEMENTAL READING

Hindler, J. A. 1990. Nontraditional approaches to quality control of antimicrobial susceptibility tests. *Clin. Microbiol. Newsl.* **12:**65–69.

Livermore, D. M., T. G. Winstanley, and K. P. Shannon. 2001. Interpretative reading: recognizing the unusual and inferring resistance mechanisms from resistance phenotypes. *J. Antimicrob. Chemother.* **48:**87–102.

Von Graevenitz, A. 1991. Use of antimicrobial agents as tools in epidemiology, identification, and selection of microorganisms, p. 723–738. *In* V. Lorian (ed.), *Antibiotics in Laboratory Medicine*, 3rd ed. The Williams & Wilkins Co., Baltimore, Md.

APPENDIX 5.13–1

Suggested QC strains for antimicrobial susceptibility tests[a]

QC strain	Test(s), for which strain is primarily used
Escherichia coli ATCC 25922 (beta-lactamase negative)	Disk diffusion, MIC (Gram − drugs)
Escherichia coli ATCC 35218 (beta-lactamase positive)	Disk diffusion, MIC for beta-lactam–beta-lactamase inhibitor combination drugs
Klebsiella pneumoniae ATCC 700603 (ESBL producer)	ESBL screen and confirmatory tests
Pseudomonas aeruginosa ATCC 27853	Disk diffusion, MIC for aminoglycosides (and other drugs)
Enterococcus faecalis ATCC 29212	MIC (Gram + drugs); vancomycin agar screen; high-level aminoglycoside resistance screen; checking that medium is acceptable for testing sulfonamides, trimethoprim, and trimethoprim-sulfamethoxazole
Enterococcus faecalis ATCC 51299 (resistant to vancomycin and high levels of aminoglycoside)	Vancomycin agar screen; high-level aminoglycoside resistance screen
Staphylococcus aureus ATCC 25923 (beta-lactamase negative)	Disk diffusion (Gram + drugs)
Staphylococcus aureus ATCC 29213 (beta-lactamase positive)	MIC (Gram + drugs)
Staphylococcus aureus ATCC 43300 (oxacillin resistant)	Oxacillin-salt agar screen
Haemophilus influenzae ATCC 49247 (BLNAR)	Disk diffusion, MIC for *Haemophilus* spp.
Haemophilus influenzae ATCC 49766 (beta-lactamase positive).	Disk diffusion, MIC for *Haemophilus* spp. with selected cephalosporins
Haemophilus influenzae ATCC 10211	Checking growth capabilities of medium used for disk diffusion and MIC tests for *Haemophilus* spp.; beta-lactamase
Neisseria gonorrhoeae ATCC 49226 (CMRNG)	Disk diffusion, MIC for *Neisseria gonorrhoeae*
Streptococcus pneumoniae ATCC 49619 (penicillin intermediate)	Disk diffusion, MIC for *Streptococcus pneumoniae* and *Streptococcus* spp.
Bacteroides fragilis ATCC 25285	MIC for anaerobes
Bacteroides thetaiotaomicron ATCC 29741	MIC for anaerobes
Eubacterium lentum ATCC 43055	MIC for anaerobes

[a] BLNAR, beta-lactamase negative, ampicillin resistant; CMRNG, chromosomally mediated (penicillin) resistant *N. gonorrhoeae.*

APPENDIX 5.13–2

Example for Use of QC Strains with "On-Scale" Endpoint

For QC of ciprofloxacin on gram-negative MIC panel

A. Routinely test *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853.
B. Acceptable QC ranges
   1. *E. coli* ATCC 25922: 0.004 to 0.016 μg/ml
   2. *P. aeruginosa* ATCC 27853: 0.25 to 1 μg/ml
C. For a panel that includes test concentrations of 0.12 to 4 μg/ml, *P. aeruginosa* ATCC 27853 has an on-scale endpoint, whereas *E. coli* ATCC 25922 does not.

QC strain	Range of concns tested and acceptable result (μg/ml) for QC strain					
	0.12	0.25	0.5	1.0	2.0	4.0
E. coli ATCC 25922	×[a]					
P. aeruginosa ATCC 27853		×[b]	×[b]	×[b]		

[a] Off-scale endpoint.
[b] On-scale endpoint.

APPENDIX 5.13–3

Primary variables that must be controlled when performing routine disk diffusion and broth microdilution MIC tests[a]

Variable	Standard	Comments
Inoculum	Disk diffusion: 1.5×10^8 CFU/ml Broth microdilution: final concn, 5×10^5 CFU/ml (range, 3×10^5–7×10^5)	Use "adequate" McFarland turbidity standard (0.5 for disk diffusion) or adequate standardizing device; when preparing direct suspensions (without incubation), do not use growth from plates >1 day old.
Media		
Formulation	Mueller-Hinton	Prepare in-house or purchase from reliable source; perform QC to verify acceptability prior to (or concurrent with) testing patient isolates.
Ca^{2+} and Mg^{2+} content	25 mg of Ca^{2+} and 12.5 mg of Mg^{2+}/liter	Increased concentrations result in decreased activity of aminoglycosides against *P. aeruginosa* and decreased activity of tetracyclines against all organisms (decreased concentrations have the opposite effect).
Thymidine content	Minimal or absent	Excessive concentrations can result in false resistance to sulfonamides and trimethoprim.
pH	7.2–7.4	Decreased pH can lead to decreased appearance of potency in aminoglycosides, erythromycin, clindamycin, and quinolones and increased activity of tetracyclines and penicillins (increased pH has the opposite effect).
Agar depth (disk diffusion)	Approximately 4 mm	Possibility for false susceptibility if <3 mm and false resistance if >5 mm
Antimicrobial agents		
Disks	Use disks containing appropriate FDA- or NCCLS-defined concentration of drug	Check NCCLS publication or FDA package insert (accompanying disks) for specifications
	Proper storage	Long-term storage in *non*-frost-free freezer at ≤ −20°C in tightly sealed, desiccated container; short-term storage (≤1 week) at 2–8°C in tightly sealed desiccated container; allow to warm to room temp before opening container
	Proper placement on agar	≤12 disks/150-mm plate; ≤5 disks/100-mm plate (no overlap zones)
Solutions	Prepare from reference standard powders	Pharmacy-grade antimicrobial agents are unacceptable: they may not show antimicrobial activity in vitro.
	Proper storage	Store in *non*-frost-free freezer optimally at ≤ −60°C; never refreeze.

(continued)

APPENDIX 5.13–3 *(continued)*

Primary variables that must be controlled when performing routine disk diffusion and broth microdilution MIC tests[a] *(continued)*

Variable	Standard	Comments
Incubation		
Atmosphere	Humidified ambient air (unless CO_2 specified)	CO_2 incubation decreases pH, which can lead to decreased activity of aminoglycosides, erythromycin, and clindamycin and increased activity of tetracyclines and penicillins.
Temp	35°C	Some MRSA organisms may go undetected if >35°C.
Length	Disk diffusion: 16–18 h. Broth microdilution: 16–20 h (24 h for staphylococci with vancomycin and with oxacillin[b] and for enterococci with vancomycin and high-level gentamicin dilution tests; 48 h for enterococci and high-level streptomycin dilution tests; 20–24 h sometimes needed for fastidious bacteria).	Some MRSA and VISA organisms and VRE and gentamicin HLR enterococci may go undetected if <24 h; some streptomycin HLR enterococci may go undetected if <48 h (dilution tests).
Endpoint measurement		
Disk diffusion	Use reflected light (except for staphylococci and oxacillin[b] and vancomycin and enterococci and vancomycin) and hold plate against or a few inches above a black background; measure zones from back of plate; for opaque plates (e.g., blood-supplemented MHA, remove lid and read from top of plate).	Lawn must be confluent or almost confluent; ignore faint growth of tiny colonies at zone edge. Trimethoprim and sulfonamides: endpoint at ≥80% inhibition; ignore swarm within obvious zone for swarming *Proteus* spp.; retest when colonies seen within zone (except staphylococci and oxacillin[b] and enterococci and vancomycin). For hemolytic organisms on blood-supplemented MHA, measure zone of inhibition of growth and not zone of inhibition of hemolysis.
	Use transmitted light for staphylococci and oxacillin[b] and vancomycin and enterococci and vancomycin	Call resistant if *any* growth within zone (unless possibly artifactual or contaminated). Reproducibility within ±2 mm
Broth microdilution	Use adequate lighting/reading device.	MIC = lowest concentration that inhibits growth (turbidity, haze or pellet); sulfonamides and trimethoprim may trail (ignore trailing <2-mm buttons). Justify "skip wells" or repeat. Staphylococci and penicillin: perform induced beta-lactamase test if MIC is 0.06–0.12 μg/ml. Reproducibility within ±1 two-fold dilution

[a] *See* **J. A. Hindler and L. M. Mann.** 1992. Principles and practices for the laboratory guidance of antimicrobial therapy, p. 548. *In* R. Tilton (ed.), *Clinical Laboratory Medicine.* The C. V. Mosby Co., St. Louis, Mo. Reprinted with permission of Elsevier. Abbreviations: HLR, high-level resistance; VISA, vancomycin-intermediate *S. aureus;* MHA, Mueller-Hinton agar.

[b] Includes all penicillinase-stable penicillins (oxacillin, methicillin, nafcillin).

APPENDIX 5.13–4a

Enterobacteriaceae antibiograms[a]

Organism	AMP	AMP-SULB	CARB GRP	MEZLO GRP	TICAR-CLAV	PIP-TAZO	1ST CEFS	CFMDL GRP	CEFOX	CEFOT	CFTAX GRP	CARBAP GRP	AMINO GRP	CHLOR	TETRA	TMP-SMZ	QUIN GRP	NALID GRP	NITRO-FURAN	POLY B
Aeromonas hydrophila[b]	R*	R	R*	S-R	S-R	S	R	S-R	S-R	S-R	S	S	S	S	S	S	S	S	S	S
Citrobacter diversus	R*	S-R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Citrobacter freundii	R*	S-R	S	S	S	S	R*	S-R	R	S-R	S-R	S	S	S	S	S	S	S	S	S
Enterobacter aerogenes	R*	S-R	S	S	S	S	R*	S-R	R	S-R	S-R	S	S	S	S	S	S	S	S-R	S
Enterobacter cloacae	R*	S-R	S	S	S	S	R*	S-R	R	S-R	S-R	S	S	S	S	S	S	S	S-R	S
Enterobacter agglomerans	S-R	S-R	S	S	S	S	S-R	S-R	S-R	S-R	S	S	S	S	S	S	S	S	S-R	S
Escherichia coli 1	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Escherichia coli 2[c]	R	S-R	S-R	S-R	S-R	S	S-R	S	S	S	S	S	S	S-R	S-R	S	S	S	S	S
Klebsiella spp.[c]	R*	S-R	R*	S-R	S-R	S	S	S	S	S	S	S	S	S	S	S	S	S-R	S-R	S
Morganella morganii	R*	S-R	S	S	S	S	R*	S-R	S-R	S	S	S	S	S	S-R	S	S	S	R*	R*
Proteus mirabilis	S-R	S-R	S	S	S	S	S	S	S	S	S	S	S	S	R*	S	S	S	R*	R*
Proteus vulgaris	R*	S-R	S	S	S	S	R*	R	S-R	S	S	S	S	S	R*	S	S	S	R*	R*
Providencia spp.	R*	S-R	S	S	S	S	R*	S-R	S-R	S	S	S	S	S	R*	S	S	R	R*	R*
Salmonella spp.	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Serratia spp.	R*	R	S	S	S	S	R*	R	S-R	S-R	S	S	S	S	R	S	S	S-R	R*	R*
Shigella spp.	S-R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S-R	S	S	S	S

[a] These antibiograms should serve as guidelines only; exceptions will occur. Although some agents listed here are no longer available in the United States, they may be used in other countries. Abbreviations: AMP, ampicillin; AMP-SULB, ampicillin-sulbactam; CHLOR, chloramphenicol; TETRA, tetracycline; TMP-SMZ, trimethoprim-sulfamethoxazole; NITROFURAN, nitrofurantoin; POLY B, polymyxin B; 1ST CEFS, cefazolin, cephalothin; AMINO GRP, amikacin, gentamicin, netilmicin, tobramycin; CARB GRP, carbenicillin, ticarcillin; CARBAP GRP, imipenem, meropenem; CEFOT, cefotetan; CEFOX, cefoxitin; CFMDL GRP, cefamandole, cefonicid, cefuroxime; CFTAX GRP, aztreonam, cefepime, cefoperazone, cefotaxime, ceftazidime, ceftizoxime, ceftriaxone; MEZLO GRP, mezlocillin, piperacillin; NALID GRP, nalidixic acid, cinoxacin; PIP-TAZO, piperacillin-tazobactam; QUIN GRP, ciprofloxacin, levofloxacin, norfloxacin; TICAR-CA, ticarcillin-clavulanic acid. *, it is very unusual to encounter an isolate susceptible to this drug.

[b] Member of *Vibrionaceae*.

[c] Can produce ESBLs that confer resistance to all penicillins, cephalosporins, and aztreonam.

APPENDIX 5.13–4b

Pseudomonas-Acinetobacter-Stenotrophomonas antibiograms[a]

Organism	AMP	AMP-SULB	CARB GRP	PIP GRP	TICAR-CLAV	PIP-TAZO	1ST 2ND CEFS	CFTAX GRP	CFTAZ GRP	AZTRE	CARBAP GRP	AMINO GRP	CHLOR	TETRA	TMP-SMZ	QUIN GRP	NALID GRP	NRFLX	NITRO-FURAN	POLY B
Acinetobacter baumannii	R	S-R	S	S	S	S	R	S-R	S	R	S	S	R	S-R	S	S	S-R	S-R	R	S
Acinetobacter lwoffii	R	S-R	S	S	S	S	R	S-R	S	R	S	S	S-R	S-R	S	S	S-R	S-R	R	S
Pseudomonas aeruginosa	R*	R*	S-R	S-R	S-R	S-R	R*	R	S-R	S-R	S-R	S-R	R	R	R	S-R	R	S	R	S
Burkholderia cepacia	R*	R	R	S-R	S-R	S-R	R*	S-R	S	S-R	R	R	S	R	S	R	R	R	R	R
Pseudomonas fluor-putida	R*	R*	R	S-R	S-R	S-R	R*	R	S	S-R	S	S	R	S-R	R	S-R	R	S-R	R	S
Stenotrophomonas maltophilia	R*	R*	S-R	S-R	S-R	S-R	R*	R	S-R	R	R*	R	S	R	S	R	R	R	R	S
Pseudomonas stutzeri	S-R	S-R	S-R	S-R	S-R	S-R	R	S-R	S	S-R	S	S	S-R	S	S	S-R	R	S-R	R	S

[a] These antibiograms should serve as guidelines only; exceptions will occur. Although some agents listed here are no longer available in the United States, they may be used in other countries. Abbreviations: 1ST, 2ND CEFS, narrow- and extended-spectrum cephalosporins; AZTRE, aztreonam; CFTAX GRP, cefoperazone, cefotaxime, ceftizoxime, ceftriaxone; CFTAZ GRP, cefepime, ceftazidime; NRFLX, norfloxacin. For other abbreviations, see footnote *a* of Table 5.13–4a. *, it is very unusual to encounter an isolate susceptible to this drug.

APPENDIX 5.13–4c

Gram-positive bacterium antibiograms[a]

Organism	AMP	AMP-SULB	PEN	OXA	1st CEFS	CHLOR	CLIND	ERY	GENT	TETRA	TMP-SMZ	VAN	Q/D	LZD
Staphylococcus aureus 1	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Staphylococcus aureus 2	R	S	R	S	S	S	S	S	S	S-R	S	S	S	S
Staphylococcus aureus 3	R	S	R	S	S	S	R	R	S	S-R	S	S	S	S
Staphylococcus aureus (*mecA*$^+$) 4	R	R	R	R	(R)	S-R	R	R	S-R	S-R	S-R	S	S	S
Staphylococcus aureus (*mecA* negative; acquired OXA-R) 5	R	(S)	R	R	(R)	S	S	S	S	S	S	S	S	S
Coagulase-negative *Staphylococcus*	Variable results											S	S	S
Enterococcus faecalis	S	S	S	R*	R*	S	R	S-R	R*	R	(R)	S	R	S
Enterococcus faecium	R	R	R	R*	R*	S	R	S-R	R*	R	(R)	S-R	S	S
β-Hemolytic *Streptococcus* spp.	S	S	S	S	S	S	S	S	R*	S	S	S	S	S
Viridans group *Streptococcus* spp.	S-R	S-R	S-R	S-R	S-R	S	S	S	R*	S	S	S	S	S
Streptococcus pneumoniae 1	S		S	S	S	S	S	S	R*	S	S	S	S	S
Streptococcus pneumoniae 2	S-R	S-R	S-R	S-R	S-R	S-R	S-R	S-R	R*	S-R	S-R	S	S	S
Corynebacterium spp.[b]	Variable results except for *Corynebacterium* JK and D2[c]											S	S	S
Listeria monocytogenes[b]	S	S	S	S	(R)	S	S	S	S	S	S	S	S	S

[a] These antibiograms should serve as guidelines only; exceptions will occur. Abbreviations: AMP, ampicillin; AMP-SULB, ampicillin-sulbactam; PEN, penicillin; OXA, oxacillin; CEFS, cephems (including cephalosporins); CHLOR, chloramphenicol; CLIND, clindamycin; ERY, erythromycin; GENT, gentamicin; TETRA, tetracycline; TMP-SMZ, trimethoprim-sulfamethoxazole; VAN, vancomycin; Q/D, quinupristin-dalfopristin; LZD, linezolid. Symbol: (R), result often susceptible in vitro, but drug clinically ineffective against noted species. *, it is very unusual to encounter an isolate susceptible to this drug.

[b] Standard NCCLS interpretive criteria not available; data based on various publications.

[c] JK and D2 are usually resistant to these (some organisms ERY-S and/or TETRA-S).

APPENDIX 5.13–4d

Miscellaneous gram-negative bacterium antibiograms[a]

Organism	β-LAC	AMP	AMOX-CLAV	CEFS	ERY	PEN	QUINS	TETRA	TMP-SMZ
Moraxella catarrhalis[b,c]	+	R	S	S	S	R	S	S	S
Moraxella catarrhalis[b,c] (very unusual)	−	S	S	S	S	S	S	S	S

Organism	β-LAC	AMP	AMOX-CLAV	CEFS	CHLOR	QUINS	TMP-SMZ
Haemophilus influenzae 1	+	R	S	S	S	S	S
Haemophilus influenzae 2	−	S	S	S	S	S	S

Organism	AMOX-CLAV AMP-SULB	1ST CEFS	CHLOR	ERY	PEN	TETRA
Pasteurella multocida[b,d]	S	S-R	S	S-R	S	S

Organism	AMOX-CLAV AMP-SULB	AMP	1ST CEFS	CHLOR	CLIND	ERY	PEN	TETRA
Eikenella corrodens[b,e]	S	S	S-R	S	R	S	S	S

[a] These antibiograms should serve as guidelines only; exceptions will occur. Abbreviations: CEFS, narrow-, extended-, and broad-spectrum cephems (including cephalosporins); β-LAC, beta-lactamase; AMOX-CLAV, amoxicillin-clavulanic acid. For other abbreviations, see Table 5.13–4a, footnote *a.* Symbols: +, positive; −, negative.

[b] Standard NCCLS interpretive criteria not available; data based on various publications.

[c] Also resistant to oxacillin and clindamycin and susceptible to aminoglycosides.

[d] Also resistant to clindamycin.

[e] Also resistant to oxacillin and aminoglycosides.

APPENDIX 5.13–5

Suggested Conditions Requiring Verification of Identification and/or Antimicrobial Susceptibility Results

A. Verify by repeat testing—unless patient had some isolate from another recent culture (1).
   1. *S. aureus*
      a. Linezolid nonsusceptible (NS)
      b. Oxacillin resistant (R)
      c. Quinupristin-dalfopristin intermediate (I) or R
      d. Vancomycin I or R
   2. Coagulase-negative staphylococci
      a. Linezolid NS
      b. Vancomycin I or R
   3. Any *Enterococcus* spp.: linezolid R
   4. *E. faecalis*
      a. Ampicillin or penicillin R
      b. Quinupristin-dalfopristin susceptible (S)
   5. *Enterococcus faecium:* quinupristin-dalfopristin R
   6. *S. pneumoniae*
      a. Fluoroquinolone I or R
      b. Linezolid NS
      c. Vancomycin NS
   7. Viridans group *Streptococcus* spp.
      a. Linezolid NS
      b. Vancomycin NS

APPENDIX 5.13–5 *(continued)*

8. *Streptococcus*, beta-hemolytic
   a. Ampicillin or penicillin NS
   b. Third-generation cephalosporin NS
   c. Linezolid NS
   d. Vancomycin NS
9. Other gram-positive organisms: vancomycin I or R (except *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., or *Erysipelothrix rhusiopathiae*)
10. Any *Enterobacteriaceae:* carbapenem I or R (except *Proteus* spp.)
11. Other *Enterobacteriaceae*
    a. ESBL-producing *Klebsiella* spp. or *E. coli*
    b. Ampicillin-, cefazolin-, or cephalothin-S *Enterobacter* spp., *Citrobacter freundii*, or *Serratia marcescens*
    c. Ampicillin-S *Klebsiella* spp., *Proteus vulgaris*, or *Providencia* spp.
    d. Third-generation cephalosporin-I or -R *Salmonella* spp.
    e. Fluoroquinolone-I or -R *Salmonella* spp.
12. *Stenotrophomonas maltophilia*
    a. Trimethoprim-sulfamethoxazole R
    b. Carbapenem S
13. *H. influenzae*
    a. Third-generation cephalosporin NS
    b. Ampicillin I or R (beta-lactamase negative) or amoxicillin-clavulanic acid I or R
    c. Aztreonam NS
    d. Carbapenem NS
    e. Fluoroquinolone NS
14. *N. gonorrhoeae*
    a. Third-generation cephalosporin R
    b. Fluoroquinolone I or R
15. Other
    a. Isolate resistant to all drugs routinely reported.
    b. Isolate for which results for related drugs do not correlate (e.g., resistance to third-generation cephalosporin and susceptible to first-generation cephalosporin in *Enterobacteriaceae*)

B. Verify by reexamination of test and, if necessary, repeat testing—unless patient had same isolate from another recent culture (1).
1. *Enterococcus* spp
   a. Gentamicin high-level aminoglycoside R; sterile body site isolates
   b. Vancomycin R
2. *S. pneumoniae:* penicillin I or R
3. Viridans group *Streptococcus* spp.: penicillin I or R
4. *Enterobacteriaceae*
   a. Amikacin R
   b. Fluoroquinolone R
5. Non-*Enterobacteriaceae:* gentamicin, tobramycin, and amikacin R (except *Burkholderia cepacia*, *S. maltophilia*)

Reference

1. **NCCLS.** 2002. *Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data.* Approved guideline M39-A. NCCLS, Wayne, Pa.

APPENDIX 5.13–6

Competency Assessment Checklist—Antimicrobial Susceptibility Testing

Competency Period: Initial (6 mos) _____ Annual _____ (please check one)

EMPLOYEE: _____ DEPT #: _____

TITLE: Medical Technologist

VALIDATOR: _____ DEPT #: _____

TITLE: Supervisor

NOTE: This is a sampling of the competencies necessary for safe, effective performance.

KEYS

HOW COMPETENCY MEASURED

A. Direct observation of routine patient testing
B. Monitoring the recording & reporting of test results
C. Review of interim results (worksheets, QC, PT & PM records)
D. Direct observation of instrument maintenance & function checks
E. Assessment of problem solving skills

_____

Employee Signature/Date

POSSESSES AND DEMONSTRATES THE ABILITIES NECESSARY FOR THE QUALITY PERFORMANCE OF THIS JOB

LEVEL OF COMPETENCE

1. Little or no experience
2. Some experience (may require practice and/or assistance)
3. Competent and can perform independently
4. Competent and can perform independently, and is able to assess the competency of others.

DATE	HOW MEASURED	VALIDATOR INITIALS/COMMENTS	COMPETENCIES	LEVEL OF COMPETENCE
	A and/or B		Appropriately determines which organisms require testing	
	A		Utilizes appropriate inoculum preparation method for organism and AST procedure	
	A		Prepares inoculum correctly	
	A and/or B		Tests antimicrobial agents or panel appropriate for the organism	
	A		Inoculates test correctly	
	A		Incubates tests correctly	
	A		Confirms purity of test prior to reading and recording results	
	A and B		Reads, records, and interprets results correctly	
	B		Verifies antimicrobial susceptibility profile and organism identification prior to reporting	
	A and/or B		Takes appropriate action to confirm any unusual or atypical results	
	B		Reports appropriate antimicrobial agent(s) and comment(s) for the organism and specimen	
	A and B		Utilizes computer appropriately for reporting process	

A and/or B	Notifies supervisor, infection control, physician, etc., as appropriate for confirmed atypical and/or noteworthy results
A and/or B	Reviews AST reports from previous day's work
A and/or B	Consults with physicians on special AST requests
A and/or B	Handles STAT AST requests and testing
A and/or E	Handles AST problems with microbiology staff members
A	Trains new microbiology staff, medical staff and students in AST
A	Maintains and subcultures QC strains appropriately
A and/or C	Runs appropriate daily or weekly QC with AST
B and/or C	Reads and documents QC results appropriately
E	Identifies and troubleshoots out-of-control results
C and E	Takes and documents appropriate corrective action for out-of-control results
E	Performs problem solving in AST
C	Maintains appropriate inventory of supplies needed for AST
A and/or B	Handles all testing materials appropriately
D	Performs/documents AST instrument maintenance
A and/or B	Locates the current NCCLS standards

NA = not applicable

5.14.1 McFarland Standards

I. PRINCIPLE

McFarland turbidity standards are used to standardize the approximate number of bacteria in a liquid suspension by visually comparing the turbidity of a test suspension with the turbidity of a McFarland standard. McFarland standards are prepared by adding barium chloride to sulfuric acid to obtain a barium sulfate precipitate. By adjusting the volumes of these two reagents, standards of varying degrees of turbidity can be prepared to represent several different concentrations of bacteria. The standard most commonly used in the clinical microbiology laboratory for routine antimicrobial susceptibility tests (1, 2) is 0.5, which represents 1.5×10^8 (generally, range is 1.0×10^8 to 2.0×10^8) bacteria/ml. McFarland standards are commercially available from several sources. As an alternative to the traditional barium sulfate standards, McFarland standards prepared from latex particles have recently become commercially available.

II. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

1. Sulfuric acid, 1% (vol/vol) (H_2SO_4)
   - **a.** Add approximately 90 ml of deionized water to a 100-ml volumetric flask.
   - **b.** Using a 1.0-ml volumetric pipette, add 1.0 ml of concentrated H_2SO_4 to the flask.
   - **c.** Bring to 100 ml with deionized water, and mix.
   - **d.** Store in a screw-cap glass bottle for up to 1 year at 25°C.
2. Barium chloride, 1.175% (wt/vol) ($BaCl_2 \cdot 2H_2O$)
   - **a.** Weigh out 1.175 g of $BaCl_2 \cdot 2H_2O$ and place in a 100-ml volumetric flask.
   - **b.** Add approximately 50 ml of deionized water, and mix well to dissolve.
   - **c.** Bring to 100 ml with deionized water, and mix.
   - **d.** Store in a screw-cap glass bottle for up to 1 year at 25°C.
3. Sterile deionized reagent-grade water
   Store in a screw-cap glass bottle for up to 1 year at 25°C.

B. Supplies

1. Sterile 1.0-liter and 10.0-ml serological pipettes and pipette bulb
2. Acid-washed glass screw-cap tubes of a diameter comparable to that used for inoculum preparation (e.g., 13 by 100 mm)
3. Parafilm or paraffin

C. Equipment

1. 100-ml volumetric flasks
2. 0.5- and 1.0-ml volumetric pipettes for 0.5 standards
   See Appendix 5.14.1–1 for sizes of volumetric pipettes needed for preparation of other standards. $BaCl_2 \cdot 2H_2O$ should be dispensed with a volumetric pipette.
3. Magnetic stirrer and stirring rod
4. Vortex
5. Spectrophotometer

III. QUALITY CONTROL

A. At time of preparation of standards

Check optical density of McFarland standard at wavelength of 625 nm and record results. The acceptable range for a McFarland 0.5 standard is 0.08 to 0.10. For standards other than 0.5, establish acceptable ranges in-house.

III. QUALITY CONTROL *(continued)*

B. Additional checks

1. Check in-use standards with each use for visible evidence of clumping, and periodically compare in-use standard with a standard that has not been in use. If clumping is apparent, discard the standard.

2. Check standards for any evidence of evaporation. This can be accomplished by drawing a line on the tube at the meniscus when the tubes are filled. A drop in liquid level below this line indicates that evaporation has occurred. If evaporation is evident, discard the standard.

3. Check the optical density of a representative standard (that has not been in use) at 3 months to make certain that the optical density of the lot is still within control limits. If in control, the shelf life of the lot can be extended for another month.

4. Repeat optical density check monthly. If in control, shelf life can be extended for another month for up to 12 months.

IV. PROCEDURE

A. Refer to Appendix 5.14.1–1 for volumes of 1% H_2SO_4 and 1.175% $BaCl_2 \cdot 2H_2O$ needed to prepare different grades of McFarland standards (representing various concentrations of bacteria), and substitute these volumes into the procedure below when preparing standards other than 0.5.

B. To prepare McFarland 0.5 standards

1. Add approximately 85 ml of 1% H_2SO_4 to a 100-ml volumetric flask.

2. Using a 0.5-ml volumetric pipette, add 0.5 ml of 1.175% $BaCl_2 \cdot 2H_2O$ dropwise to the H_2SO_4 while constantly swirling the flask.

3. Bring to 100 ml with 1% H_2SO_4.

4. Place magnetic stirring bar in flask and place on magnetic stirrer for approximately 3 to 5 min.

5. Examine solution visually to make certain it appears homogeneous and free of visible clumps; check optical density and record on QC sheet.

6. Providing QC is acceptable, dispense 2- to 7-ml volume (depending on volume routinely used in inoculum suspension preparation) into each tube.

7. Cap tubes tightly.

8. Seal with Parafilm or paraffin.

9. Store in the dark at room temperature for 3 months or longer (*see* item III above).

V. USE OF McFARLAND STANDARDS

A. Mix McFarland standard and test suspension well on a vortex mixer prior to examination.

B. In the presence of good lighting, visually compare turbidity of test suspension to the McFarland standard.

1. Hold both tubes in front of a Wickerham card (Appendix 5.14.1–2).

2. If it is more difficult to see lines on Wickerham card through test suspension than through the McFarland standard, the suspension is too dense and must be diluted.

3. If dilution is necessary, use a sterile pipette to add additional sterile broth or saline to the tube to obtain turbidity comparable to that of the McFarland standard.

4. Before subsequent examinations, vortex test suspension and McFarland standard well.

5. If test suspension is too light, inoculate with additional organisms or reincubate suspension (depending on inoculum preparation protocol) until turbidity reaches that of the standard.

VI. PROCEDURE NOTES

A. Test suspension and McFarland standard should be contained in tubes of the same diameter.

B. McFarland standards are more likely to clump and evaporate if exposed to air. Thus, it is imperative that caps be tightly sealed with Parafilm or paraffin.

C. Standards in use clump and evaporate more rapidly than those in storage, and in-use standards must be examined regularly. Discard standards with evidence of clumping or evaporation regardless of stated shelf life.

D. When glass tubes are used, acid washing helps minimize clumping.

E. A practical storage container for the McFarland standards is a test tube mailing container. Alternatively, wrap tube in aluminum foil.

F. An alternative to using a spectrophotometer to QC McFarland standards is performing colony counts on bacterial suspensions (e.g., *Escherichia coli* ATCC 25922) that have been standardized against a particular lot of standards.

1. Prepare six successive 10-fold dilutions of test suspension in 0.85% NaCl (the first dilution tube is labeled 10^{-2}; plating 0.1 ml from the suspension standardized to the McFarland 0.5 would yield the 10^{-1} dilution). (TNTC, too numerous to count.)

Dilution	Expected no. of colonies (with 0.1-ml aliquots)
10^{-1}	TNTC
10^{-2}	TNTC
10^{-3}	TNTC
10^{-4}	TNTC
10^{-5}	TNTC
10^{-6}	150
10^{-7}	15

2. Plate 0.1-ml aliquots of dilutions expected to yield approximately 30 to 300 colonies (in this example, plate 10^{-6} and 10^{-7}) to BAP (in duplicate) and spread evenly to distribute using a loop or sterile "hockey stick."

3. Colony counts should fall within 10% of expected numbers.

G. Actual numbers of viable bacteria present in McFarland standardized-suspensions may vary depending on size, viability, and clumping of the particular bacteria tested.

H. When using commercially available McFarland standards prepared from latex particles, follow manufacturer's directions for use and storage.

I. Other devices are satisfactory for standardizing bacterial suspensions. These include the following:

1. Wand-type inoculum devices that pick up a standardized volume of cell paste (from colonies grown overnight on an agar plate) that is subsequently suspended in a standardized volume of liquid (BBL Prompt inoculation system, [Becton Dickinson Microbiology Systems, Sparks, Md., or Dade MicroScan, West Sacramento, Calif.).

2. Photometers or colorimeters such as the following

a. Vitek colorimeter (bioMérieux Vitek, Inc., Durham, N.C.)

b. MicroScan turbidity meter (Dade MicroScan)

VII. LIMITATIONS

Suspensions standardized to McFarland standards will contain the estimated number of bacteria only if high-quality McFarland standards are used. Standards that have clumped or evaporated will yield unsatisfactory results.

REFERENCES

1. **NCCLS.** 2003. *Performance Standards for Antimicrobial Disk Susceptibility Tests,* 8th ed. Approved standard M2-A8. NCCLS, Wayne, Pa.
2. **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.

SUPPLEMENTAL READING

Barry, A. L. 1976. *The Antimicrobic Susceptibility Test: Principles and Practices*, p. 72–74. Lea and Febiger, Philadelphia, Pa.

McFarland, J. 1907. Nephelometer: an instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. *JAMA* **14:**1176–1178.

Washington, J. A., E. Warren, and A. G. Karlson. 1972. Stability of barium sulfate turbidity standards. *Appl. Microbiol.* **24:**1013.

APPENDIX 5.14.1–1

Preparation of McFarland Standards

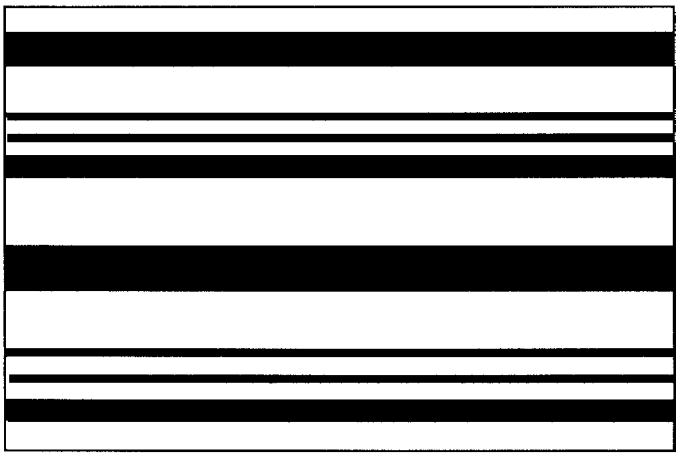
Adapted from **K. C. Chapin and T. Lauderdale.** 2003. Reagents, stains, and media: bacteriology, p. 358. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.

Standard no.	Vol (ml)		No. of bacteria/ml (10^8) represented
	$BaCl_2 \cdot 2H_2O$ (1.175%)	H_2SO_4 (1%)	
0.5	0.5	99.5	1.5
1	1.0	99.0	3
2	2.0	98.0	6
3	3.0	97.0	9
4	4.0	96.0	12
5	5.0	95.0	15
6	6.0	94.0	18
7	7.0	93.0	21
8	8.0	92.0	24
9	9.0	91.0	27
10	10.0	90.0	30

APPENDIX 5.14.1–2

Wickerham Card

L. J. Wickerham. 1951. Taxonomy of yeasts, p. 11. *In* Technical bulletin no. 1029. U.S. Department of Agriculture, Washington, D.C.



5.14.2 Antimicrobial Stock Solutions

I. PRINCIPLE

Aliquots of antimicrobial stock solutions are incorporated into broth or agar medium for antimicrobial susceptibility tests. Stock solutions are prepared from laboratory reference standard-grade antimicrobial powders. These are assayed for the amount of active agent present, and this is expressed as activity or potency. Potency or activity is usually defined in terms of micrograms of active drug per milligram (e.g., 912 μg/mg) or percentage of active drug (e.g., 91.2%). The activities of some drugs are expressed as international units per milligram of powder. *Pharmacy-grade preparations should not be used, as these often contain stabilizers or blocking agents which render them inactive until metabolized in vivo.* An analytical balance is used to weigh out an appropriate amount of antimicrobial powder, which is dissolved in a solvent specific for that powder and subsequently diluted with water or buffer. Antimicrobial stock solutions are optimally stored at −70°C or lower.

II. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

1. Antimicrobial reference standard powders
   - **a.** Obtain from the U.S. Pharmacopeia, chemical supplier, or pharmaceutical company that manufactures that drug.
   - **b.** Store according to manufacturer's specifications. Opened vials or any nonvacuum vials must be stored in a desiccator (preferably in a vacuum).
   - **c.** Potency (activity), lot number, and expiration date must be specified. If they are not, contact the supplier.
2. Solvents and diluents (*see* Appendix 5.14.2–1)
   - **a.** Sterile deionized water
   - **b.** Saturated sodium bicarbonate powder
   - **c.** Sodium carbonate powder
   - **d.** 95% Ethanol
   - **e.** 100% Methanol
   - **f.** Sodium hydroxide (various normalities)
   - **g.** Hydrochloric acid (various normalities)
   - **h.** Phosphate buffers
   - **i.** Dimethyl sulfoxide

B. Supplies

1. Sterile polypropylene, polystyrene, or polyethylene capped tubes (12 by 75 mm and 16 by 100 mm) appropriate for freezing
2. Sterile 1-, 2-, 5-, and 10.0-ml serological pipettes and pipette bulb
3. Weigh boats or weighing papers
4. Spatulas
5. Sterile volumetric flasks (50, 100, 250 ml)
6. Sterile Erlenmeyer flasks (50, 100, 250 ml)
7. Adhesive labels, 1 by 0.75 in. (that withstand −70°C storage)

◩ **NOTE:** *All* glassware should be acid washed.

C. Equipment

1. Calibrated analytical balance sensitive to 0.0001 g
2. Refrigerator (2 to 8°C) (if part of refrigerator-freezer combination, must not be self-defrosting)
3. Forceps
4. −20°C non-frost-free freezer
5. −70°C (or lower) freezer
6. Vortex
7. Desiccator and desiccant

III. QUALITY CONTROL

A. Record stock solution preparation information in QC log. Include preparation date, technologist's initials, concentration of solution, and expiration date of solution. For the powder, record manufacturer, lot number, potency, and expiration date. See Appendix 5.14.2–2.

B. Perform QC of stock solutions by testing the particular stock solution in the test system with reference bacterial strains with known susceptibility profiles.

C. Other methods for QC of stock solution, such as antimicrobial assay by high-pressure liquid chromatography, may be indicated in some situations (e.g., when stock solution is used in preparing controls for assay of antimicrobial agent in patient serum).

IV. PROCEDURE

A. Remove desiccator from refrigerator or freezer, and allow it to warm to room temperature before opening to avoid condensation of water.

B. Plan stock solution preparation (*see* Appendix 5.14.2–3).

1. Determine concentration (in micrograms per milliliter) of stock solution needed.

2. Determine volume of stock solution needed.

3. Calculate amount of powder and diluent (including solvent) needed. Prepare stock solution at 1,000 μg/ml or more.

4. Obtain information from the manufacturer, and check NCCLS tables (1) for appropriate solvent and diluent.

C. Weighing the powder

1. Using a forceps, place a piece of weigh paper or a weigh boat on analytical balance pan and tare.

2. Using a spatula for transferring powder, weigh out powder.

3. Transfer powder to flask, and rinse weigh paper or weigh boat with a small amount of solvent.

a. If a set amount of powder is weighed and the final volume of solution is predetermined, use a volumetric flask, if one is available.

b. If a variable amount of powder is weighed and the amount of solvent and diluent will be adjusted to that weight, use an Erlenmeyer flask.

D. Dissolve and dilute the powder.

1. Add just enough solvent to dissolve powder (will generally require a few drops to a few milliliters).

2. Use solvent to remove every speck of powder from weigh paper or weigh boat.

3. Add diluent to obtain desired final volume.

4. Mix well.

E. Dispense and store stock solutions.

1. Prepare label to specify antimicrobial agent's name, concentration, preparation date, expiration date, and technologist's initials. Affix label to tube.

2. Dispense stock solution in tubes at volumes appropriate for subsequent use.

3. Cap tubes tightly, and store at −70°C or lower.

4. Most antimicrobial stock solutions are stable for 6 months at −70°C or lower. *Exceptions include imipenem, clavulanic acid, cefaclor, and rifampin, which are very labile and should not be stored longer than 1 month (at −70°C or lower).* Contact the manufacturer of the antimicrobial agent for additional preparation and stability information.

F. Use stock solutions.

1. Allow stock solution to thaw at room temperature. Thawing can be expedited by placing tube of frozen stock solution in a beaker of tap water (not hot). Do not allow water to reach the cap of the tube.

2. Never refreeze a stock solution after it has been thawed.

V. PROCEDURE NOTES

A. Antimicrobial powders (1, 2)

1. The activity or potency of an antimicrobial powder may vary from lot to lot.
2. Some powders may have >100% potency. For example, vancomycin powder is now produced in a purer form than the reference standard powder initially characterized many years ago. Consequently, on a weight basis, current preparations are often more active (>100% potency) than the original reference standard powder.
3. Some powders will have a retest date in lieu of an expiration date. The retest date indicates when the manufacturer will reassay the powder to see if it has maintained potency. If the powder has maintained potency, the manufacturer will extend the expiration date (sometimes a manufacturer gives a powder a second retest date).
4. Moisture considerations
   - **a.** Moisture becomes a greater problem and poses a greater risk of water condensation on the powders at lower storage temperatures. Do not store powders at temperatures lower than that specified by the manufacturer.
   - **b.** It is not necessary to dry standard reference-grade powders unless specifically recommended by the manufacturer.
   - **c.** If possible, remove moist air within desiccators that have been in a refrigerator or freezer by evacuation.
   - **d.** Aminoglycoside powders are very stable at room temperature but are very hygroscopic. Consequently, some recommend that they be stored at room temperature in a desiccator.

B. Procedure

1. Whenever possible, weigh >100 mg of powder.
2. It is best to weigh out slightly more powder than is required. Then calculate the volume of diluent needed. This avoids multiple adjustments of the powder and minimizes exposure to moisture.
3. It is best to weigh out a precalculated amount of powder if an organic solvent is used.
4. Rifampin may deteriorate following exposure to sunlight and should be stored in the dark.
5. It is best to prepare stock solutions that are 10 times or more the highest concentration tested and at concentrations of 1,000 μg/ml or greater.
6. Make volume measurements point to point on a pipette. When appropriate, use a volumetric flask.
7. To avoid stock solution storage tubes from cracking at −70°C, place filled tubes at −20°C for 4 to 5 h before transferring them to −70°C. Polystyrene tubes are more likely to crack than polypropylene tubes.
8. It is usually unnecessary to filter stock solutions prepared at high concentrations, since the antimicrobial should sterilize the solution. As a precaution, however, use sterile materials (e.g., glassware, solvents and diluents, etc.) in preparing stock solutions, whenever possible. *If filter sterilization is necessary, use membrane filters only. Other types of filters (e.g., asbestos, glass, paper) may absorb antimicrobial agent and must not be used.* Assay all filtered solutions prior to use.

C. Antimicrobial stock solutions

1. Imipenem stock solutions are very unstable and must be stored at −70°C or lower. Other more labile antimicrobial solutions include clavulanic acid, cefaclor, and rifampin. The beta-lactam agents in general tend to be more temperature labile.
2. Some stock solutions such as ampicillin, trimethoprim, and others prepared in phosphate buffers may precipitate during storage. To get drug back in solution, vortex well.

VI. LIMITATIONS

A. Stock solutions at high concentrations (e.g., 12,800 μg/ml) can be satisfactorily prepared for most antimicrobial agents, although there are some drugs (e.g., chloramphenicol) that will not go into solution at high concentrations.

B. Stock solutions will maintain the specified potency when prepared and stored according to the manufacturer's directions.

REFERENCES

1. **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically,* 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.
2. **Shungu, D. L.** 1996. Chemical and physical properties of antibiotics: preparation and control of antibiotic susceptibility disks and other devices containing antibiotics, p. 766–792. *In* V. Lorian (ed)., *Antibiotics in Laboratory Medicine,* 4th ed. The Williams and Wilkins Co., Baltimore, Md.

APPENDIX 5.14.2–1

Preparation of Solvents and Diluents

A. Obtain dimethyl sulfoxide, anhydrous sodium carbonate, and methanol from chemical suppliers.

B. 1 N HCl
   1. Add 8.33 ml of concentrated HCl to a 100-ml volumetric flask containing approximately 90 ml of deionized water and bring to 100 ml with deionized water.
   2. Mix well.
   3. Dispense into a screw-cap bottle.
   4. Store at 25°C for up to 1 year.
   5. 0.04 N HCl
      a. Add 4.0 ml of 1 N HCl to a 100-ml volumetric flask containing approximately 90 ml of deionized water and bring to 100 ml with deionized water.
      b. Mix well.
      c. Dispense into a screw-cap bottle.
      d. Store at 25°C for up to 1 year.
   6. 0.05 N HCl
      a. Add 5.0 ml of 1 N HCl to a 100-ml volumetric flask containing approximately 90 ml of deionized water and bring to 100 ml with deionized water.
      b. Mix well.
      c. Dispense into a screw-cap bottle.
      d. Store at 25°C for up to 1 year.

C. 1 N NaOH
   1. Add 4 g of NaOH pellets to a 100-ml volumetric flask containing approximately 50 ml of deionized water, mix to dissolve, and bring to 100 ml with deionized water.
   2. Mix well.
   3. Dispense into a screw-cap bottle.
   4. Store at 25°C for up to 1 year.

D. 0.1 N NaOH
   1. Add 10.0 ml of 1 N NaOH to a 100-ml volumetric flask containing approximately 90 ml of deionized water and bring to 100 ml with deionized water.
   2. Mix well.
   3. Dispense into a screw-cap bottle.
   4. Store at 25°C for up to 1 year.

E. 2.5 N NaOH
   1. Add 10 g of NaOH pellets to a 100-ml volumetric flask containing approximately 50 ml of deionized water, mix to dissolve, and bring to 100 ml with deionized water.
   2. Mix well.
   3. Dispense into a screw-cap bottle.
   4. Store at 25°C for up to 1 year.

F. 95% Ethanol
   1. Add 95 ml of absolute ethanol to a 100-ml volumetric flask and bring to 100 ml with deionized water.
   2. Mix well.
   3. Dispense into a screw-cap bottle.
   4. Store at 25°C for up to 1 year.

APPENDIX 5.14.2–1 *(continued)*

G. Saturated sodium bicarbonate (Na_2HCO_3)
1. Add 100 ml of water to a small beaker, place magnetic stirring bar in beaker, and place beaker on stirring unit.
2. Gradually add sodium bicarbonate powder to water while stirring constantly.
3. Continue adding powder until it no longer goes into solution.
4. Dispense into a screw-cap bottle.
5. Autoclave for 15 min at 121°C (alternatively, filter through a 0.22-μm-pore-size membrane filter).
6. Store at 25°C for up to 1 year.

H. 0.1 M sodium bicarbonate (Na_2HCO_3)
1. Add 2.1 g of Na_2HCO_3 to a beaker.
2. Add 250 ml of water, place magnetic stirring bar in beaker, and place beaker on stirring unit.
3. Mix until dissolved.
4. Dispense into a screw-cap bottle.
5. Autoclave for 15 min at 121°C (alternatively, filter through a 0.22-μm-pore-size membrane filter).
6. Store at 25°C for up to 1 year.

I. Phosphate buffers
1. Prepare stock buffers (1).
   a. Stock buffer A: KH_2PO_4 (0.2 M)
      (1) Add 27.22 g of KH_2PO_4 (0.2 mol) (monobasic potassium phosphate, anhydrous) to a 1,000-ml volumetric flask.
      (2) Add approximately 200 ml of deionized water.
      (3) Shake to dissolve.
      (4) Bring to 1,000 ml with deionized water.
      (5) Dispense into 500-ml screw-cap bottles.
      (6) Autoclave for 15 min at 121°C (alternatively, filter through a 0.22-μm-pore-size membrane filter).
   b. Stock buffer B: K_2HPO_4 (0.2 M)
      (1) Add 45.64 g of $K_2HPO_4 \cdot 3H_2O$ (0.2 mol) (dibasic potassium phosphate) to a 1,000-ml volumetric flask.
      (2) Add approximately 200 ml of deionized water.
      (3) Shake to dissolve.
      (4) Bring to 1,000 ml with deionized water.
      (5) Dispense into 500-ml screw-cap bottles.
      (6) Autoclave for 15 min at 121°C (alternatively, filter through a 0.22-μm-pore-size membrane filter).
   c. Store sterile stock buffers at 2 to 8°C for up to 1 year.
2. Prepare 0.1 M phosphate working buffers by adding the following volumes of stock buffers A and B to 100-ml volumetric flasks to prepare working buffer at respective pHs.

pH	Stock buffer A (ml)	Stock buffer B (ml)
6.0	43.85	6.15
7.0	19.5	30.5
8.0	2.65	47.35

3. Bring to 100 ml with deionized water.
4. Check pH with a pH meter. It should be close to desired value. If necessary, adjust with 1 N NaOH or 0.1 M phosphoric acid (if more than a few drops of base or acid are necessary to adjust pH, remake buffer).
5. To prepare 0.01 M phosphate buffer (pH 7.0), make a 1:10 dilution of the 0.1 M buffer.
6. Dispense approximately 150 ml into screw-cap bottles.
7. Autoclave for 15 min at 121°C (alternatively, filter through a 0.22-μm-pore-size membrane filter).
8. Store sterile working buffers at 25°C for up to 1 year.

Reference

1. **Costilow, R. N.** 1981. Biophysical factors in growth, p. 67–69. *In* P. Gerhardt (ed.), *Manual of Methods for General Bacteriology.* American Society for Microbiology, Washington, D.C.

APPENDIX 5.14.2–2

Worksheet for antimicrobial stock solution QC (*see* p. 5.14.2.7)

APPENDIX 5.14.2–3

Formulas for Preparation of Antimicrobial Stock Solutions

A.

$$\text{weight (mg)} = \frac{\text{volume (ml)} \times \text{concentration (µg/ml)}}{\text{assay potency (µg/mg)}}$$

B.

$$\text{volume (ml)} = \frac{\text{weight (mg)} \times \text{assay potency (µg/mg)}}{\text{concentration (µg/ml)}}$$

C. Definitions

1. Assay potency (micrograms per milligram) is the activity or potency specified by manufacturer of reference standard powder (usually appears on the label).
2. Concentration (micrograms per milliliter) is the desired concentration of stock solution.
3. Volume (milliliters) is the desired volume of stock solution.
4. Weight (milligrams) is the weight of powder needed to prepare the desired volume of stock solution at the desired concentration.

D. Example using formula A

1. Apply when weighing an exact amount of powder.

Antimicrobial:	cephalothin
Desired concentration:	1,280 µg/ml
Desired volume:	100 ml
Assay potency:	931 µg/mg

$$x \text{ mg} = \frac{100 \text{ ml} \times 1{,}280 \text{ µg/ml}}{931 \text{ µg/mg}} = 137.5 \text{ mg}$$

2. Weigh exactly 137.5 mg (0.1375 g) and place it in a 100-ml volumetric flask.
3. Dissolve in approximately 2 ml of phosphate buffer (0.1 M pH 6.0), and bring to 100 ml with sterile deionized water.

E. Example using formula B

1. Use when weighing powder to a weight slightly in excess of that needed. Then calculate the exact volume of diluent needed to obtain the concentration desired.

Antimicrobial:	cephalothin
Desired concentration:	1,280 µg/ml
Desired volume:	approximately 50 ml
Assay potency:	931 µg/mg
Approximate amount to weigh:	>140 mg (approximately 141 to 160 mg)

$$x \text{ ml} = \frac{142.4 \text{ mg} \times 931 \text{ µg/mg}}{1{,}280 \text{ µg/ml}} = 103.57 \text{ ml}$$

2. Weigh approximately 141 to 160 mg (0.141 to 0.160 g). For example purposes, this weight is 142.4 mg (0.1424 g). Place in a 125-ml Erlenmeyer flask.
3. Dissolve in exactly 2 ml of phosphate buffer (0.1 M, pH 6.0) and add exactly 101.57 ml of sterile deionized water.

Antimicrobial Stock Solution QC

Antimicrobial Solution (µg/ml)	Tech	Date Made (Lot #)	Exp. Date	Powder Information			
				Activity (µg/mg)	Exp. Date	Date Opened	Manufacturer and Lot #

5.14.3

Preparation of Agar and Broth Media Used in Routine Antimicrobial Susceptibility Tests

I. PRINCIPLE

Specific agar and broth media (generally Mueller-Hinton with or without supplements) used for routine disk diffusion (1), agar dilution, and broth dilution (2) susceptibility tests are prepared from commercially available dehydrated components. Although these media have shown fairly good reproducibility through many years of testing, they are undefined media, and there may be subtle performance differences among batches and lots.

II. MATERIALS

Include QC information on reagent container and in QC records.

A. Media and reagents

1. Distilled (or deionized) water
Store at 25°C.

2. Dehydrated Mueller-Hinton agar (sometimes referred to as Mueller-Hinton medium)
Store at 25°C.

3. Dehydrated Mueller-Hinton broth
Store at 25°C.

4. Dehydrated GC agar base
Store at 25°C.

5. Additives or supplements (*see* specific media to determine which of these are needed, and *see* Appendix 5.14.3–1 for preparation)

a. Defibrinated sheep blood
Store at 2 to 8°C.

b. Defibrinated horse blood
Store at 2 to 8°C.

c. NaCl
Store at 25°C.

d. Thymidine phosphorylase (Sigma-Aldrich, St. Louis, Mo).
Store at 2 to 8°C.

e. Bovine hematin, *not* hemin (Sigma Chemical Co., St. Louis, Mo.)
Store at 2 to 8°C.

f. Yeast extract powder
Store at 25°C.

g. β-NAD solution powder
Store at 25°C.

h. GC agar supplement
Store at 2 to 8°C.

i. Calcium chloride ($CaCl_2 \cdot 2H_2O$)
Store at 2 to 8°C.

j. Magnesium chloride ($MgCl_2 \cdot 6H_2O$)
Store at 2 to 8°C.

k. 1 N HCl
Store at 25°C.

l. 1 N NaOH
Store at 25°C.

B. Supplies

1. Weigh paper or weigh boats

2. Spatulas or tongue depressors

3. Stoppers for flasks

4. Petri dishes (100 and 150 mm)

5. Tubes, flasks, or bottles of various sizes to contain prepared media

6. 1-, 5-, and 10-ml sterile serological pipettes and pipette bulb

7. Sterile tubes that withstand high centrifugal force, 12,000 × *g* (for preparation of lysed horse blood)

8. Membrane filters (0.22- and 0.45-μm pore size)

C. Equipment

1. Graduated cylinders

2. Erlenmeyer flasks

3. Volumetric flasks

4. Calibrated probe marked at 3, 4, and 5 mm (for checking agar depth)

5. Autoclave

6. 48 ± 2°C water bath

7. Magnetic stirrer and stirring bars

II. MATERIALS *(continued)*

8. Hot plate or other flat burner
9. Bunsen burner
10. Refrigerator (2 to 8 °C); *non*-self-defrosting if using refrigerator-freezer combination
11. Automated dispensing apparatus (optional)
12. 35 ± 2°C ambient-air incubator
13. Analytical balance
14. pH meter
15. Large, flat, level surface for pouring plates
16. High-speed (12,000 × *g*) centrifuge (for preparation of lysed horse blood)

III. QUALITY CONTROL

Perform the following checks when preparing media.

A. Sterility checks

1. Test medium for sterility by incubating a 5 to 10% sample from each newly prepared batch of media in ambient air at 35°C for 48 h.
2. Disregard sporadic contamination, e.g., one piece of medium.
3. Discard entire batch if significant contamination (≥10% of the medium incubated) is noted.
4. Discard all media used for sterility checks.

B. pH

1. Check the pH of each new lot of prepared media after it has cooled to room temperature.
2. Check the pH of agar by one of the following methods.
   a. Use a surface electrode.
   b. Macerate the medium in neutral distilled water, and use an immersion electrode.
   c. Allow the agar to solidify around the electrodes of a pH meter.
3. For broth, place an immersion electrode into the liquid.
4. Acceptable range is 7.2 to 7.4 for Mueller-Hinton-based media and GC agar base.

C. Agar depth (for media used for the disk diffusion test)

1. Check the depths of two to five plates from each batch of plates.
2. Push a thin, sterile calibrated probe marked at 3, 4, and 5 mm into the agar to measure depth.
3. Acceptable range is 3 to 5 mm for disk diffusion testing.

D. Cation (Ca^{2+} and Mg^{2+}) content

1. For broth, measure the concentration of each of these divalent cations following completion of broth preparation (send an aliquot to the chemistry laboratory for analysis of calcium and magnesium content)
2. For agar and broth, do a performance check with *Pseudomonas aeruginosa* ATCC 27853 and aminoglycosides (*see* disk diffusion and broth microdilution MIC test procedures [procedures 5.1 and 5.2])
3. Acceptable ranges (atomic absorption method or equivalent) are as follows.
   a. Ca^{2+}, 20 to 25 mg/liter (equivalent to 2.0 to 2.5 mg/dl)
   b. Mg^{2+}, 10 to 12.5 mg/liter (equivalent to 0.83 to 1.04 meq/liter)

E. Thymidine content

See procedures 5.1 and 5.2 for descriptions of performance testing of *Enterococcus faecalis* ATCC 29212. Satisfactory performance of this isolate with trimethoprim-sulfamethoxazole indicates acceptable thymidine content (zone of ≥20 mm or MIC of ≤0.5/9.5 μg/ml).

F. NaCl content (cation-adjusted Mueller-Hinton broth [CAMHB] plus 2% NaCl)

1. Measure concentration of NaCl following completion of broth preparation (send an aliquot to the chemistry laboratory for analysis of sodium and/or chloride).

III. QUALITY CONTROL
(continued)

2. Acceptable range (ion selective electrode method) is 450 to 480 mmol of Na^+ and/or Cl^-. Sources of ions are from both the Mueller-Hinton broth (naturally occurring) and addition of NaCl (20 g/liter) to broth medium.

G. Growth-supporting capabilities and performance checks

Determine growth-supporting capabilities, antimicrobial compatibility, and performance by testing QC reference strains (*see* procedures 5.1 and 5.2 and Appendix 5.14.3–2)

H. Appearance (*see* Appendix 5.14.3–2)

Prior to each use, examine media for evidence of discoloration, drying or cracking (agar), insufficient volume, or other signs of deterioration. Do not use media that do not appear acceptable.

IV.A. PROCEDURE: AGAR

A. Mueller-Hinton agar

The following procedure describes preparation of 1 liter of Mueller-Hinton agar from commercially available dehydrated Mueller-Hinton agar (medium) powder; for other volumes, modify accordingly. Additionally, this procedure assumes that dehydrated powder contains acceptable concentrations of Ca^{2+} and Mg^{2+}. In establishing shelf life, see procedure 14.2.

1. Weigh appropriate amount of dehydrated Mueller-Hinton agar powder (follow manufacturer's instructions on bottle) and place in a 2-liter flask.
2. Add 1 liter of distilled water and swirl to disperse powder.
3. Place over a hot plate with magnetic stirrer (or other heating device) and heat until powder is dissolved (bring to a gentle boil). *Do not boil vigorously, and do not apply direct heat without stirring, as medium will burn.*
4. Carefully remove agar from heat and dispense in desired aliquots into containers of choice (e.g., dispense 250-ml volumes into 500-ml Erlenmeyer flasks).
5. Loosely cover containers (e.g., insert stopper into mouth of flask).
6. Autoclave at 121°C for 15 min.
7. Allow to cool in a 48°C water bath.
8. Arrange petri plates on a level surface.
9. For disk diffusion tests, pour accurately measured volumes of molten agar into plates.
   a. 60 to 70 ml/150-mm plate
   b. 25 to 30 ml/100-mm plate
10. Eliminate bubbles on the molten agar surface by quickly (and carefully) passing the flame from a Bunsen burner over the agar.
11. Allow plates to solidify at room temperature with plate lids slightly ajar.
12. Store prepared plates at 2 to 8°C in tightly sealed packages.

B. Sheep blood (5%)-supplemented Mueller-Hinton agar

1. Prepare 1 liter Mueller-Hinton agar as described above.
2. Add 50 ml of sterile defibrinated sheep blood to molten and cooled (48°C) agar.
3. Gently swirl to mix and pour plates as described above.
4. Store prepared plates at 2 to 8°C in tightly sealed packages.

C. *Haemophilus* test medium (agar)

1. Weigh appropriate amount of dehydrated Mueller-Hinton agar powder (follow manufacturer's instructions on bottle) and place in a 2-liter flask.
2. Add 5 g of yeast extract.
3. Add 967 ml of distilled water, and swirl to disperse powder.
4. Place over a hot plate with magnetic stirrer (or other heating device) and heat until powder is dissolved (bring to a gentle boil). *Do not boil vigorously, and do not apply direct heat without stirring because medium will burn.*

IV.A. PROCEDURE: AGAR *(continued)*

5. Add 30 ml of freshly prepared hematin stock solution and mix well.
6. Dispense and autoclave at 121°C for 15 min.
7. Allow to cool in a 48°C water bath.
8. Aseptically add 3 ml of sterile β-NAD stock solution and mix well.
9. Pour plates as described above.
10. Store prepared plates at 2 to 8°C in tightly sealed packages.

D. GC agar base with 1% defined supplement

1. Weigh out appropriate amount of dehydrated GC agar base powder (follow manufacturer's instructions on bottle) and place in a 2-liter flask.
2. Add 999 ml of distilled water and swirl to disperse powder.
3. Place over a hot plate with magnetic stirrer (or other heating device) and heat until powder is dissolved (bring to a gentle boil). *Do not boil vigorously, and do not apply direct heat without stirring, as medium will burn.*
4. Autoclave at 121°C for 15 min.
5. Allow to cool in a 48°C water bath.
6. Aseptically add 10 ml of sterile defined supplement solution, and mix well.
7. Pour plates as described above.
8. Store prepared plates at 2 to 8°C in tightly sealed packages.

IV.B. PROCEDURE: BROTH

A. CAMHB

The following procedure describes preparation of 1 liter of CAMHB from commercially available dehydrated Mueller-Hinton broth powder; for other volumes, modify accordingly. In establishing shelf life, see procedure 14.2.

1. Weigh appropriate amount of dehydrated Mueller-Hinton broth powder (follow manufacturer's instructions on bottle) and place in a 2-liter flask.
2. Add 1 liter of distilled water and swirl to disperse powder.
3. Place over a hot plate with magnetic stirrer (or other heating device) and heat until powder is dissolved (bring to a gentle boil). *Do not boil vigorously, and do not apply direct heat without stirring because medium will burn.*
4. Carefully remove broth from heat and dispense in desired aliquots into containers of choice (e.g., dispense accurately measured 250-ml volumes into 500-ml Erlenmeyer flasks).
5. Loosely cover containers (e.g., insert stopper into mouth of flask).
6. Autoclave at 121°C for 15 min.
7. Allow to cool, and then chill at 2 to 8°C for several hours.
8. Check the cation content for the specific lot of medium that is printed on the label.
   - **a.** Calculate the amount of calcium stock solution needed.
   - **b.** The following is an example.
     (1) Specified amount of calcium already present in medium = 10.0 mg/liter
     (2) Desired amount per liter of broth = 22.5 mg (20 to 25.0 mg/liter)
     (3) Add 0.1 ml of calcium stock solution per liter for each increment of 1 mg/liter needed to attain desired concentration.

$$\begin{array}{l} 22.5 \text{ mg/liter} \\ -\,10.0 \text{ mg/liter (already present in broth)} \\ \hline 12.5 \text{ mg/liter needed to reach desired concentration} \end{array}$$

$$12.5 \times 0.1 \text{ ml/liter} = 1.25 \text{ ml}$$

Add 1.25 ml of calcium stock solution to each liter of broth.

   - **c.** Calculate the amount of magnesium stock solution needed.

IV.B. PROCEDURE: BROTH
(continued)

d. The following is an example.

(1) Specified amount of magnesium already present in medium = 7.0 mg/liter

(2) Desired amount per liter of broth = 11.25 mg (10 to 12.5 mg/liter)

(3) Add 0.1 ml of magnesium stock solution per liter for each increment of 1 mg/liter needed to attain desired concentration.

11.25 mg/liter
−7.0 mg/liter (already present in broth)
4.25 mg/liter needed to reach desired concentration
4.25 × 0.1 ml/liter = 0.425 ml

Add 0.425 ml of magnesium stock solution to each liter of broth.

9. Remove flasks from the refrigerator, aseptically add the appropriate volume of each *sterile* cation stock solution to the chilled broth, and mix well (medium must be cold to prevent precipitation of the cations).

10. If testing sulfonamides or trimethoprim-sulfamethoxazole, add thymidine phosphorylase to obtain a final concentration of 0.2 IU/ml.

11. Store prepared CAMHB at 2 to 8°C in sealed containers.

B. CAMHB supplemented with 2% NaCl

To the flask containing the dehydrated powder (step IV.B.A.1 above), add 20 g of NaCl per liter. Follow remaining steps as given above.

C. CAMHB supplemented with 2 to 5% lysed horse blood

1. Prepare CAMHB as described above.

2. Aseptically add the appropriate volume of 50% lysed horse blood to CAMHB to obtain a final concentration of 2 to 5% lysed horse blood.

a. Add 4 to 10 ml of 50% lysed horse blood to obtain a final volume of 100 ml of CAMHB.

b. When supplementing broth microdilution panels, add 5 µl (0.005 ml) of 50% lysed horse blood to each well containing 100 µl (0.1 ml) of antimicrobial agent and broth.

D. *Haemophilus* test medium (broth)

1. Weigh appropriate amount of dehydrated Mueller-Hinton broth powder to prepare 1 liter of medium (follow manufacturer's instructions on bottle) and place in a 2-liter flask.

2. Add 5 g of yeast extract.

3. Add 967 ml of distilled water, and swirl to disperse powder.

4. Place on a hot plate with magnetic stirrer (or other heating device) and heat until powder is dissolved (bring to a gentle boil). *Do not boil vigorously, and do not apply direct heat without stirring because medium will burn.*

5. Add 30 ml of freshly prepared hematin stock solution, and mix well.

6. Dispense, and autoclave at 121°C for 15 min.

7. Allow to cool, and chill at 2 to 8°C for several hours or overnight.

8. Aseptically add the appropriate volume of each sterile cation solution to the chilled broth, and mix well (*see* item IV.B.A above).

9. Aseptically add 3 ml of sterile β-NAD stock solution (to 1 liter of broth), and mix well.

10. If testing sulfonamides or trimethoprim-sulfamethoxazole, add thymidine phosphorylase to obtain a final concentration of 0.2 IU/ml.

11. Store prepared broth at 2 to 8°C in sealed containers.

V. PROCEDURE NOTES

A. General

1. Dehydrated media

a. Store bottles of dehydrated media tightly capped and in a cool, dry place to prevent the powder from hardening.

b. Read directions carefully, especially if a new lot of medium is received.

c. Add powder to flask, and then add water.

2. Water

Use only distilled or deionized water. Impurities in tap water may affect medium performance.

3. Glassware

a. All glassware should be clean and free of soap residue.

b. Rinse all glassware with distilled or deionized water after washing and prior to use.

4. Heating and autoclaving

a. Heat medium to a gentle boil to achieve an even solution.

b. When the medium begins to clear and runs in a sheet down the side of the flask when the flask is swirled, it is ready to be autoclaved.

c. Do not autoclave medium longer or at higher temperatures than specified by the manufacturer.

5. When using prepared media, it is expected that the manufacturer has performed extensive QC to include but not be limited to the following.

a. Sterility checks

b. Measurement of pH

c. Measurement of some specific components (e.g., Ca^{2+}, Mg^{2+})

d. Measurements of fill (volume, depth, etc.)

e. Performance checks

f. Shelf life studies

B. Agar

1. Mueller-Hinton agar

a. The NCCLS has developed a protocol to assist manufacturers of commercially prepared Mueller-Hinton agar in producing a product that performs reliably (3).

b. The correct depth of plated medium for disk diffusion testing is critical. Failure to dispense accurate volumes may result in agar that is too thin (often yielding false-susceptible results) or too thick (often yielding false-resistant results).

2. In the preparation of GC agar with 1% supplement, do not use supplements containing cysteine because cysteine may inactivate newer beta-lactam antimicrobial agents. A chemically defined "XV-like" supplement without cysteine is recommended. This supplement is most like BBL IsoVitaleX but has no cysteine and is composed of (per liter of medium) 1.1 g of L-cystine, 0.03 g of guanine HCl, 3 mg of thiamine HCl, 13 mg of *p*-aminobenzoic acid, 0.01 g of vitamin B_{12}, 0.1 g of cocarboxylase, 0.25 g of NAD, 1.0 g of adenine, 10 g of L-glutamine, 100 g of glucose, and 0.02 g of ferric nitrate.

C. Mueller-Hinton broth

1. The NCCLS has developed a protocol to assist manufacturers of commercially prepared Mueller-Hinton agar in producing a product that performs reliably (4).

2. Cations (Ca^{2+} and Mg^{2+})

a. The following alternative procedure is satisfactory for the addition of cations (adding cations prior to autoclaving).

(1) Dissolve Mueller-Hinton broth powder as described above.

(2) As soon as broth has cooled slightly (approximately 15 min), chill in a refrigerator at 2 to 8°C (or in an ice bath). Small volumes (500 ml)

V. PROCEDURE NOTES
(continued)

will be sufficiently cool after 4 to 6 h; larger volumes may require overnight chilling.
(3) Add appropriate volumes of cation solutions as described above.
(4) Dispense into desired containers, and autoclave as described above.
(5) If water is sufficiently pure, precipitation does not occur. Although some do not feel that it is acceptable to hold medium for more than a few hours prior to autoclaving (even if refrigerated), one of us has been holding medium overnight at 2 to 8°C (for preparation of CAMHB), without problems, during 20 years of testing.

b. The addition of cations to Mueller-Hinton broth primarily affects activities of aminoglycosides and *P. aeruginosa* (isolates appear more resistant to aminoglycosides as cation content increases). Similarly, the activity of tetracycline against all isolates decreases as cation content increases.

3. CAMHB supplemented with 2% NaCl is limited to testing staphylococci with the penicillinase-stable penicillins (oxacillin, methicillin, and nafcillin).

4. Thymidine content

a. Most currently manufactured dehydrated Mueller-Hinton broth and agar powders are relatively free of thymidine.

1. Excessive concentrations of thymidine will interfere with testing of sulfonamides, trimethoprim, and trimethoprim-sulfamethoxazole (isolates will appear more resistant).

2. If the MIC of trimethoprim-sulfamethoxazole for *E. faecalis* ATCC 29212 is ≤0.5/9.5 μg/ml and/or the inhibition zone is ≥20 mm and free of fine colonies, the medium is sufficiently free of thymidine and acceptable for use (1, 2).

b. Thymidine phorphorylase enzyme can be added to CAMHB to eliminate thymidine. Add sufficient volumes of sterile solution to autoclaved broth to obtain a final concentration of 0.2 U/ml.

5. The pH of Mueller-Hinton broth increases slightly (approximately 0.1 to 0.2 pH units) following autoclaving. Adjusting the preautoclave pH to approximately 7.1 results in a final pH within the acceptable range of 7.2 to 7.4. Adjust pH with 1 N HCl or 1 N NaOH. It generally takes approximately 0.5 ml of 1 N HCl to decrease the pH of 1 liter of broth by 0.1 pH unit. However, this will vary with different lots of medium and water.

6. Lysed horse blood

a. Successful use of lysed horse blood relates to clarity of the lysed horse blood preparation. Only a high-speed centrifuge adequately precipitates cell debris. Repeated centrifugation or passage of supernatant through a filter may be necessary to obtain a clear solution.

b. When performing a broth dilution test using CAMHB with lysed horse blood, always include an uninoculated negative control tube (or well) of CAMHB with lysed horse blood to use for comparison with inoculated solutions.

c. *Always* check durability of tubes by centrifugation of a sample tube containing water prior to centrifugation of blood.

VI. LIMITATIONS

A. Not all clinically significant aerobic isolates that require antimicrobial susceptibility testing grow satisfactorily in the Mueller-Hinton agars or broths described here.

VI. LIMITATIONS *(continued)*

B. Sometimes supplements may have an inactivating effect on the antimicrobial agents tested. Supplements that have not been extensively studied should be used with caution. Always test QC reference strains in the medium used for testing patient isolates. Results must fall into specified ranges for the test to be acceptable.

C. There may be variations in performance of media obtained from different manufacturers or different lots from the same manufacturer.

REFERENCES

1. **NCCLS.** 2003. *Performance Standards for Antimicrobial Disk Susceptibility Tests,* 8th ed. Approved standard M2-A8. NCCLS, Wayne, Pa.
2. **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically,* 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.
3. **NCCLS.** 1996. *Protocols for Evaluating Dehydrated Mueller-Hinton Agar.* Approved standard M6-A. NCCLS, Wayne, Pa.
4. **NCCLS.** 2001. *Evaluation of Lots of Mueller-Hinton Broth for Antimicrobial Susceptibility Testing.* Proposed guideline M32-P. NCCLS, Wayne, Pa.

APPENDIX 5.14.3–1

Preparation of Additives and Supplements

A. Calcium stock solution (10 mg of Ca^{2+} per ml)
   1. Add 3.68 g of $CaCl_2 \cdot 2H_2O$ to a 100-ml volumetric flask and bring to 100 ml with deionized water.
   2. Mix well to dissolve.
   3. Dispense into 100-ml screw-cap bottles (approximately 50 ml per bottle) and autoclave for 15 min at 121°C (or filter sterilize through a 0.22-μm-pore-size filter).
   4. Store at 2 to 8°C for up to 1 year.

B. Magnesium stock solution (10 mg of Mg^{2+} per ml)
   1. Add 8.36 g of $MgCl_2 \cdot 6H_2O$ to a 100-ml volumetric flask and bring to 100 ml with deionized water.
   2. Mix well to dissolve.
   3. Dispense into 100-ml screw-cap bottles (approximately 50 ml per bottle) and autoclave for 15 min at 121°C (or filter sterilize through a 0.22-μm-pore-size filter).
   4. Store at 2 to 8°C for up to 1 year.

C. 1 N HCl
   1. Add 8.33 ml of concentrated HCl to a 100-ml volumetric flask containing approximately 90 ml of deionized water and bring to 100 ml with deionized water.
   2. Mix well.
   3. Dispense into a screw-cap bottle.
   4. Store at 25°C for up to 1 year.

D. 1 N NaOH (and 0.01 N NaOH)
   1. Add 4 g of NaOH pellets to a 100-ml volumetric flask containing approximately 50 ml of deionized water, mix to dissolve, and bring to 100 ml with deionized water.
   2. Mix well.
   3. Dispense into a screw-cap bottle.
   4. Store at 25°C for up to 1 year.
   5. For preparation of 0.01 N NaOH, add 1.0 ml of 1 N NaOH to a volumetric flask and bring to 100 ml with deionized water.

E. Hematin stock solution
   Do not use hemin.
   1. Add 50 mg of bovine hematin powder and 100 ml of 0.01 N NaOH to a 250-ml Erlenmeyer flask.
   2. Add magnetic stirring bar, and place over low heat on a hot plate until dissolved (takes about 15 min).
   3. Prepare fresh each time of use, and discard any unused reagent.

F. β-NAD stock solution
   1. Add 50 mg of β-NAD powder and 10 ml of deionized water to a 25-ml Erlenmeyer flask.
   2. Mix well to dissolve.
   3. Filter sterilize by passing solution through a 0.22-μm-pore-size membrane filter.

APPENDIX 5.14.3–1 *(continued)*

4. Dispense into a sterile screw-cap tube.
5. Store at 2 to 8°C for up to 1 month.

G. Lysed horse blood

1. Dispense defibrinated horse blood into freezer-safe tubes, and freeze-thaw a minimum of three times until the RBCs are completely lysed. Freezing at −20°C is preferred because this temperature disrupts RBCs more efficiently than −70°C. Thaw at room temperature.
2. Add equal volumes of lysed blood and sterile deionized water (e.g., 5 ml of blood and 5 ml of water; solution is now 50% lysed horse blood).
3. Dispense into tubes that will safely withstand the centrifugal force and centrifuge at 12,000 × *g* for 20 min.
4. Decant supernatant into another sterile tube, and discard the sediment.
5. Centrifuge supernatant to further remove cell debris, if necessary.
6. Decant supernatant into another sterile tube, and discard the sediment.
7. Sometimes it may be helpful to pass solution through a 0.22- or 0.45-μm-pore-size membrane filter to clarify it further. However, this is usually unnecessary if centrifugation is satisfactory.
8. Store at −20°C for up to 6 months.

Supplemental Reading

NCCLS. 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically,* 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.

APPENDIX 5.14.3–2

Notes and QC parameters for media[a]

Medium or reagent	Appearance	QC strain(s) for performance test	Notes
Mueller-Hinton agar (unsupplemented)[b]	Straw colored, translucent	*Escherichia coli* ATCC 25922 *Staphylococcus aureus* ATCC 25923 *Staphylococcus aureus* ATCC 29213 (agar dilution) *Pseudomonas aeruginosa* ATCC 27853 *Enterococcus faecalis* ATCC 29212 *Escherichia coli* ATCC 35218	Susceptibility results must fall within NCCLS-defined ranges (1, 2).
Mueller-Hinton agar with 5% sheep blood[b]	Bright red, opaque (no hemolysis apparent)	*Streptococcus pneumoniae* ATCC 49619	Susceptibility results must fall within NCCLS-defined ranges (1).
Haemophilus test medium agar[b]	Pale yellow or tan, translucent	*Haemophilus influenzae* ATCC 49247 *Haemophilus influenzae* ATCC 49766 *Haemophilus influenzae* ATCC 10211	Susceptibility results must fall within NCCLS-defined ranges (1). ATCC 10211 is tested to assess growth-supporting capabilities.
GC agar with defined supplement[b]	Straw, translucent	*N. gonorrhoeae* ATCC 49226	Susceptibility results must fall within NCCLS-defined ranges (1, 2).
CAMHB (Mueller-Hinton broth supplemented with 20–25 mg of Ca^{2+} and 10–12.5 mg of Mg^{2+} per liter)	Sparkling clear, crisp gold	*Escherichia coli* ATCC 25922 *Staphylococcus aureus* ATCC 29213 (broth dilution) *Pseudomonas aeruginosa* ATCC 27853 *Enterococcus faecalis* ATCC 29212 *Escherichia coli* ATCC 35218	Susceptibility results must fall within NCCLS-defined ranges (2).
CAMHB with 2% NaCl	Sparkling clear, crisp gold	*Staphylococcus aureus* ATCC 43300 *Staphylococcus aureus* ATCC 25923	Currently, NCCLS recommends use of ATCC 43300, which is an MRSA,[c] for oxacillin-salt agar screen plates only. There are no MIC QC ranges for this strain (2).
CAMHB with 2–5% lysed horse blood	Sparkling clear, crisp burgundy	*Streptococcus pneumoniae* ATCC 49619	Susceptibility results must fall within NCCLS-defined ranges (2).
Haemophilus test medium broth	Pale yellow or tan, translucent	*Haemophilus influenzae* ATCC 49247 *Haemophilus influenzae* ATCC 49766 *Haemophilus influenzae* ATCC 10211	Susceptibility results must fall within NCCLS-defined ranges (2). ATCC 10211 is tested to assess growth-supporting capabilities.

[a] For additional information, see QC information in procedures 5.1 and 5.2, and also see procedure 5.13.

[b] Agar depth is critical for disk testing; must be 3 to 5 mm.

[c] MRSA, methicillin-resistant *S. aureus.*

References

1. **NCCLS.** 2003. *Performance Standards for Antimicrobial Disk Susceptibility Tests,* 8th ed. Approved standard M2-A8. NCCLS, Wayne, Pa.
2. **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically,* 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.

5.15 Preparation of Broth Microdilution MIC Trays

I. PRINCIPLE

Broth microdilution MIC trays may be prepared in-house for performance of quantitative antimicrobial susceptibility studies. If large numbers of trays are required, dilutions of the antimicrobial agents are made in large volumes and generally dispensed in 0.1-ml amounts into microdilution trays by using a commercial dispensing apparatus. For individual test trays, dilutions of the required antimicrobial agents may be accomplished with an eight-channel micropipette. The procedure described here is based on 96-well trays containing 0.1 ml per well. Not every aspect of broth microdilution tray preparation has been standardized, and for some steps, there are multiple ways of accomplishing identical results. The method presented here conforms closely to that described by the NCCLS (2) and is the procedure that has been successfully used with a Quick Spense (Sandy Springs Instrument Co.) dispenser at the clinical microbiology laboratories of the University of California—Los Angeles for nearly two decades. It assumes that the layout of the tray in terms of positioning respective concentrations of each antimicrobial agent has been previously determined. The method is detailed for preparation of approximately 1,900 trays, but recommendations for preparing fewer trays are included.

II. MATERIALS

Include QC information on reagent container and in QC records.

See procedure 5.2 for materials required for QC.

A. Media and reagents

1. Cation-adjusted Mueller-Hinton broth (CAMHB) and CAMHB supplemented with 2% NaCl for penicillinase-stable penicillins (e.g., oxacillin, nafcillin) (*see* procedure 5.14 and Appendix 5.15–1)
Store at 2 to 8°C.

2. Antimicrobial stock solutions (*see* procedure 5.14)
Store at −70°C.

3. Thymidine phosphorylase, if trimethoprim-sulfamethoxazole, trimethoprim, or a sulfonamide is included
Store at 2 to 8°C.

4. Sterile deionized water
Store at 25°C.

5. Dye solution

a. 1% phenol red for gram-negative bacteria trays

(1) Dissolve the following.

phenol red powder ……0.5 g
0.1 N NaOH …… 14 ml

(2) Add 36 ml of deionized water.

(3) Filter sterilize through a 0.45-µm-pore-size filter, and store in a glass bottle.

(4) For use as a marker in the MIC trays, add 1.0 ml of this solution per 200 ml of CAMHB.

Store at 25°C.

b. 1.6% Bromcresol purple for gram-positive bacteria trays

(1) Dissolve the following.

bromcresol purple ……0.8 g
0.1 N NaOH ……. 14.8 ml

(2) Add 35.2 ml of 95% ethanol.

(3) Filter sterilize through a 0.45-µm-pore-size filter, and store in a glass bottle.

(4) For use as a marker in the MIC trays, add 0.2 ml of this solution per 200 ml of CAMHB.

Store at 25°C.

B. Supplies

1. Large containers (e.g., 12-liter Florence flasks or stainless steel pots to hold 12 liters of broth)

II. MATERIALS *(continued)*

2. 250-ml Erlenmeyer flasks (approximately 20)
3. Foam plugs to fit 250-ml Erlenmeyer flasks
4. Sterile serological pipettes (1.0, 2.0, 5.0, 10, and 25 [or 50] ml) and pipette bulb
5. Sterile plastic capped tubes (16 by 100 mm)
6. Round labels, 0.5-in. diameter
7. Two or three large sterile towels or drapes (approximately 24 by 48 in.)
8. Sterile 96-well polystyrene U-bottom broth microdilution trays
9. Microdilution tray plate sealers
10. Plastic storage bags with seals, 10 by 13 in.
11. 100-mm petri plates or other sterile reservoir (for preparation of individual trays)
12. 0.45-μm-pore-size filter

C. Equipment

1. Commercial microdilution tray dispensing apparatus (Appendix 5.15–2)
   a. Large reservoir tubes with volumetric gradations (to fit dispensing apparatus) that have been acid washed
   b. Two large racks that each hold 96 large reservoir tubes in a format identical to that of the microdilution tray (12 columns by 8 rows)
2. pH meter
3. Vortex mixer
4. Analytical balance (sensitive to 0.1 g)
5. −70°C freezer
6. Multichannel micropipette and sterile tips (for preparation of individual trays)

III. QUALITY CONTROL

Document all QC results (*see* example forms in Appendixes 5.15–3 and 5.15–4). See procedure 5.14 also.

A. Check sterility and pH of CAMHB. Also check Ca^{2+} and Mg^{2+} concentrations by sending representative samples of broth to the chemistry laboratory for analysis.

1. Sterility
   Incubate one flask and one tube of CAMHB at 35°C for 48 h (preferably prior to performing the run).
2. pH: acceptable range, 7.2 to 7.4 at room temperature
3. Ca^{2+} (atomic absorption method or equivalent): acceptable range is 20 to 25 mg/liter (equivalent to 2.0 to 2.5 mg/dl)
4. Mg^{2+} (atomic absorption method or equivalent): acceptable range is 10 to 12.5 mg/liter (equivalent to 0.83 to 1.04 meq/liter)

B. Check potency of all antimicrobial stock solutions prior to their use in the preparation of MIC trays by performing MIC tests with appropriate NCCLS-recommended ATCC reference strains.

1. Prepare dilutions of stock antimicrobial agent encompassing acceptable MIC ranges as illustrated in Appendix 5.15–4.
2. Perform a MIC test for each drug with a QC organism that has an on-scale MIC endpoint (*see* procedure 5.2).

C. Confirm the sterility of the dispensing apparatus by using an approved autoclave indicator.

D. Check the accuracy of the dispensed volume (must be within 10% of ideal volume or weight) as follows.

1. Volume check by volume
   Perform at beginning, middle, and end of run.
   a. Dispense three “shots” of solution into a single tray. (Each shot should contain approximately 0.1 ml.)
   b. Visually inspect menisci, checking for an even fill.
   c. Using a 1.0-ml serological pipette, aspirate the entire contents of the well showing the lowest fill, and note volume.

III. QUALITY CONTROL *(continued)*

d. Repeat step III.D.1.c for well showing the highest fill.
e. Acceptable volume reading = 0.3 ± 0.03 ml

2. Volume check by weight
Perform at beginning of run and after every 100 trays dispensed.
a. Using the analytical balance, tare in the weight of an empty tray.
b. Fill the tray with one shot of solution, and weigh.
c. Acceptable weight = 9.6 ± 0.5 g

E. Check sterility of prepared trays by incubating five trays each from the beginning and end of the run at 35°C for 48 h.

F. Performance testing using QC strains

1. Test each new lot of prepared trays in duplicate and in parallel with an old lot of trays by using the recommended ATCC reference strains. It is desirable to use strains with on-scale endpoints. See sample listing of drugs in Appendix 5.15–5 and the recommended ATCC reference strains that have on-scale endpoints for the drug concentrations included (Appendix 5.15–6).

2. Test additional organisms (usually in-house-selected strains) that would complement the ATCC reference strains in controlling preparation of the trays. The number of additional control organisms included depends on the number and variety of antimicrobial agents included in the tray.

3. In selecting supplemental QC strains
a. Include strains to control antimicrobial agents for which none of the ATCC strains have on-scale endpoints (e.g., in example in Appendix 5.15–6, trimethoprim-sulfamethoxazole).
b. Include strains that control significant antimicrobial concentrations that may not be optimally controlled by the ATCC strains (e.g., include an isolate for which the gentamicin MIC is near the breakpoint of susceptibility [MIC, 4 to 8 μg/ml]).
c. Include strains that detect types of resistance that may not be detected with the ATCC reference strains, such as the following.
(1) Oxacillin-resistant *Staphylococcus aureus*
(2) Erythromycin-resistant *S. aureus*
(3) Aminoglycoside-resistant *Pseudomonas aeruginosa*
(4) Cefotaxime-resistant *Enterobacter* spp.

4. For handling and testing of QC organisms and additional QC information, see procedure 5.13.

IV. PROCEDURE

This procedure is for preparation of 500 to 2,000 trays using a 200-ml capacity dispenser. For preparation of trays using a smaller automated dispensing apparatus (e.g., one that is designed for preparation of up to 200 trays), see Appendix 5.15–7. For preparation of a small number of individual trays, see Appendix 5.15–8. See procedure 5.14 for alternative methods for preparation of CAMHB.

A. Fill reservoir tubes with CAMHB (96 of the tubes should be positioned in a 96-tube rack).

1. Dispense an amount of prepared (but not autoclaved) CAMHB just short of the required volume into calibrated screw-cap reagent tubes supplied with the dispensing apparatus. Cap tubes loosely, and place in 96-tube rack supplied for use with the dispensing apparatus.

2. Dispense remaining broth (in approximately 150-ml amounts) into 250-ml Erlenmeyer flasks. Plug flasks.

3. Autoclave filled tubes and flasks for 15 min at 121°C.

4. Allow broth to cool to room temperature. Tighten caps.

IV. PROCEDURE *(continued)*

B. Label trays, storage bags, and CAMHB reservoir tubes.

1. Label the required numbers of empty broth microdilution trays and plastic storage bags with lot number and preparation date. A quick and efficient way to do so is to arrange the trays in stacks of 30 and run a permanent-ink marking pen down one side. This colored stripe(s) ("code") is referenced on the plastic storage bag and on the QC sheets with the preparation date. The preparation date serves as the lot number.

2. For each CAMHB reservoir tube, prepare a label that indicates the antimicrobial agent, concentration of antimicrobial solution required, and volume of antimicrobial solution to add (*see* Appendix 5.15–7).

3. Place labels on caps of the appropriate tubes containing CAMHB, and make certain that all tubes are positioned in the rack in the exact order required for the antimicrobial tray layout.

C. Add antimicrobial agents. (All measurements made must be point to point when using serological pipettes.)

1. Prepare intermediate dilutions of antimicrobial stock solutions (*see* Appendix 5.15–7). If using scheme in Table 5.15–A1, use sterile deionized water as the diluent. If using scheme in Table 5.15–A2, use CAMHB as the diluent.

2. If thymidine phosphorylase is required (trimethoprim, sulfonamides, or trimethoprim-sulfamethoxazole), add the required amount to the appropriate tube(s) to achieve a final concentration of 0.2 IU/ml.

3. Dispense the exact volume of the indicated concentration of antimicrobial solution into the appropriate tube of CAMHB.

4. Mix by inverting the tube several times.

5. Following the addition of antimicrobial solution, transfer each tube to a separate rack, making certain that the tube maintains the same position it held in the first rack.

6. Continue with one tube at a time until all antimicrobial solutions have been added. Note that negative and positive control tubes will not contain antimicrobial agent.

7. If used, add indicator dye to dye marker tube (volume may vary depending on the dye).

8. Fill all tubes to final volumes by using the CAMHB in Erlenmeyer flasks.

9. Invert each tube several times to mix.

10. To ensure that the contents of each tube have been thoroughly mixed, transfer filled tubes back to the original rack following mixing, and make certain that they are replaced in their appropriate positions.

D. Fill trays.

1. Place rack of filled tubes into the dispensing apparatus, and assemble and prime according to manufacturer's instructions.

2. Insert first empty tray into dispensing apparatus, and fill with one shot of solution. Continue filling additional trays.

3. Set aside the first five and last five trays filled to serve as sterility checks. Seal the top tray of each stack of five trays with a plate sealer (either sealing tape or empty tray), and incubate at 35°C for 72 h. Examine daily.

4. Stack filled trays five high, and place in plastic bags (four stacks of five per bag). (The numbers of trays stacked and placed in a bag will depend on the bag dimensions.)

5. Freeze at −70°C as rapidly as possible. *Note:* Storage of trays at −20°C may be acceptable for some antimicrobial agents. *Imipenem and combinations containing clavulanic acid must be stored at −70°C or lower. It is preferable to store all beta-lactams at −70°C or lower.*

IV. PROCEDURE *(continued)*

6. Perform weight checks approximately every 100 trays. Perform volume checks at beginning, middle, and end of run.
7. At completion of run, flush and clean dispensing apparatus according to manufacturer's instructions.
   - **a.** Flush a minimum of 40 liters of hot water (≈95°C) through the tubing. *(Flushing with hot water is essential to prevent carryover of agents such as quinolones.)*
   - **b.** Rinse all tubes and caps thoroughly (five or six times) with distilled water. *Do not use soap. Acid wash tubes or use equivalent cleaning procedure, and rinse them with distilled water.*

V. VALIDATION OF PRODUCT

A. The finished product is valid for use for routine testing providing that all QC parameters have been met.

B. Broth microdilution MIC trays containing antimicrobial agents currently in use in routine clinical laboratories have a minimum shelf life of 1 year when stored at −70°C (1, 3). (For additional information, obtain drug stability data from the manufacturer of the drug.) *Never refreeze trays following thawing.*

VI. PROCEDURE NOTES

A. Materials

1. CAMHB is recommended for susceptibility testing of commonly isolated rapidly growing nonfastidious bacteria. It has demonstrated fairly good batch-to-batch reproducibility for susceptibility testing; is low in sulfonamide, trimethoprim, and tetracycline inhibitors; and yields satisfactory growth of most pathogens (2). CAMHB may be supplemented to support the growth of fastidious organisms (*see* procedure 5.2).
2. Test penicillinase-stable penicillins (e.g., oxacillin, methicillin, nafcillin, and dicloxacillin) in CAMHB supplemented with 2% NaCl.
3. Antimicrobial stock solutions may be prepared in advance, frozen at −70°C, and used within 1 week of preparation. They should be quality controlled prior to use. Fresh solutions can be used, but the potency cannot be validated prior to use in tray preparation.
4. The pH of CAMHB tends to increase after autoclaving. A preautoclave pH of 7.1 to 7.15 usually results in a pH of 7.2 to 7.4 after autoclaving.
5. Dispense several extra tubes of broth so that in the event of an error (e.g., incorrect amount or wrong drug added, broken tube), materials to easily rectify the situation are on hand.
6. If high concentrations of aminoglycosides (e.g., gentamicin and streptomycin at 500 or 1,000 μg/ml) are included, add weighed antimicrobial powder directly to the broth tubes. It is important to maintain dedicated tubes for these high concentrations of antimicrobial agents to avoid any possibility of carryover of the antimicrobial agents from one run to the next.

B. Labeling

1. The use of a dye marker in one well (without antimicrobial agent) is helpful in distinguishing one type of MIC tray from another if several types are prepared in the laboratory. This also helps orient positioning of antimicrobial dilutions in trays.
2. Food colors can be used as dye markers.
3. Small round stickers containing information on antimicrobial agent, concentration, and volume of antimicrobial solution to add are convenient for labeling the caps of large 200-ml tubes. In addition, these labels may be saved on a plastic sheet protector for future use.

VI. PROCEDURE NOTES *(continued)*

C. QC

1. The maximum acceptable weight allowance of liquid in the 96-well MIC tray (containing 0.1 ml per well) is generally 9.6 ± 0.5 g if the 10% error rule is used (2), and this can be easily accomplished.

2. A second person to act as a spotter, who observes the critical steps of the procedure (e.g., all measuring steps), may be helpful in ensuring that the MIC trays have been prepared properly.

3. A practical supplemental method for QC of high concentrations of aminoglycosides (i.e., gentamicin or streptomycin at 500 or 1,000 μg/ml) is assay by a chemical or immunoassay method (performed in many clinical chemistry laboratories). Dilute the sample so that the expected concentration is within the limits of detection.

4. An on-scale endpoint will have a defined MIC within the range of concentrations tested (*not* less than or equal to the lowest concentration and not more than the highest concentration). For example, if 0.5, 1.0, 2.0, 4.0, 8.0, 16, and 32 μg/ml are tested, on-scale endpoints would be 1.0, 2.0, 4.0, 8.0, 16, and 32.

5. CAMHB containing 2% NaCl will yield 450 to 480 mmol of Na^+ and/or Cl^- when assayed by the ion-selective electrode method. The sources of the ions are both the Mueller-Hinton broth (naturally occurring) and the NaCl (20 g/liter) added to the broth medium.

D. Storage

1. Fill standard −70°C freezers no more than 25% full (or less, depending on the particular freezer) with bagged MIC trays. It may be less stressful on the freezer if panels are cooled in a refrigerator or −20°C freezer for a short time before they are transferred to the −70°C freezer. Specifically designed freezers (those containing a double cooling system) that can accommodate the thermal load are optimal.

2. Storage of trays at −20°C may be acceptable for some antimicrobial agents. Imipenem is unstable at −20°C. Imipenem and combinations containing clavulanic acid must be stored at −70°C or lower. It is preferable to store all beta-lactams at −70°C or lower (1, 3).

3. It is important to use non-frost-free freezers for storage of antimicrobial solutions and prepared trays. Dehydration of liquid caused by frost-free freezers adversely affects antimicrobial solution volumes and concentrations.

E. Refer to microdilution tray layout blank form and examples in Appendix 5.15–9 when designing tray layout.

F. Refer to Appendix 5.15–10 for a quick checklist of required tasks for preparation of broth microdilution MIC trays and the appropriate times to perform them.

VII. LIMITATIONS

The procedure described has been shown to work successfully with the Quick Spense but may require some modification if other dispensing apparatus is used.

REFERENCES

1. **Hwang, J. M. D., T. E. Piccinini, C. J. Lammel, W. K. Hadley, and G. F. Brooks.** 1986. Effect of storage temperature on the stability of antimicrobial agents in MIC trays. *J. Clin. Microbiol.* **23:**959–961.

2. **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.

3. **Nickolai, D. J., C. J. Lammel, B. A. Byford, J. H. Morris, E. B. Kaplan, W. K. Hadley, and G. F. Brooks.** 1985. Effects of storage temperature and pH on stability of 11 beta-lactam antibiotics in MIC trays. *J. Clin. Microbiol.* **21:**366–370.

APPENDIX 5.15–1

Ordering media for broth microdilution MIC tray preparation (*see* p. 5.15.12) (instructions for medium preparation)

APPENDIX 5.15–2

Commercial Source for Broth Microdilution MIC Tray Dispensing Apparatus

Quick Spense
Sandy Springs Technologies
21131 Georgia Ave.
Brookeville, MD 20833
(301) 260-1220

APPENDIX 5.15–3

QC log for broth microdilution MIC tray preparation (*see* p. 5.15.13 and 5.15.14)

APPENDIX 5.15–4

QC of stock solution: form and example (*see* p. 5.15.15 and 5.15.16)

APPENDIX 5.15–5

Sample listing of antimicrobial agents, abbreviations, and concentrations that might be included in microdilution MIC trays

Antimicrobial agent	Abbreviation	Concn range (μg/ml)[a]
Amikacin	AK	0.5–32
Ampicillin	AMP	0.5–32
Ampicillin-sulbactam	AM-SUL	1/0.5–32/16
Aztreonam	AZTR	0.5–32
Cefazolin	CFAZ	0.5–32
Cefepime	CPM	0.5–32
Cefotaxime	CTAX	0.5–32
Cefoxitin	CFOX	0.5–32
Cefpodoxime	CFPD	1–4[b]
Ceftazidime	CTAZ	0.5–32
Ceftriaxone	CTRX	0.5–32
Cefuroxime	CFUR	0.5–32
Chloramphenicol	CHLR	0.5–32
Ciprofloxacin	CIPR	0.12–8
Clindamycin	CLIN	0.25–8
Erythromycin	ERY	0.25–16
Gentamicin	GM	0.5–10[c]
Imipenem	IPM	0.25–8
Oxacillin	OX	0.25–16
Penicillin	PEN	0.03–4
Piperacillin	PP	8–512
Rifampin	RIF	0.06–4
Ticarcillin	TIC	8–512
Tobramycin	TB	0.5–10[c]
Trimethoprim-sulfamethoxazole	TMP-SMX	0.5/9.5–4/76
Vancomycin	VANC	0.5–32

[a] Generally, serial twofold dilutions are tested.

[b] For extended-spectrum beta-lactamase screening test.

[c] Additional dilutions may be tested to define endpoint more exactly.

APPENDIX 5.15–6

ATCC QC organisms that have on-scale endpoints for the agents and concentrations depicted in example in Appendix 5.15–5

Drug[a]	Endpoint on scale				
	Staphylococcus aureus ATCC 29213	*Enterococcus faecalis* ATCC 29212	*Escherichia coli* ATCC 25922	*Pseudomonas aeruginosa* ATCC 27853	*Escherichia coli* ATCC 35128
AK	×		×	×	
AMP	×	×	×		
AM-SUL			×		×
AZTR				×	
CFAZ	×		×		
CFOX	×		×		
CFPD					
CFUR	×		×		
CHLR	×	×	×		
CIPR	×	×		×	
CLIN		×			
CPM	×		×	×	
CTAX	×			×	
CTAZ	×			×	
CTRX	×			×	
ERY	×	×			
GM	×	×	×	×	
IPM		×		×	
OX	×	×			
PEN	×	×			
PP	×	×	×	×	
RIF		×			
TB	×	×	×	×	
TIC	×	×	×	×	
TMP-SMX					
VANC	×	×			

[a] See Appendix 5.15–5 for definitions of abbreviations. For QC values, see **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*, 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.

APPENDIX 5.15–7

Preparation of Antimicrobial Dilutions for Broth Microdilution MIC Trays

Table 5.15–A1 Preparation of antimicrobial dilutions: primary scheme

Final concn (μg/ml) in reservoir tube and well	Dilution (μg/ml) used	Vol (ml) of antimicrobial dilution added to reservoir tube to prepare[a]:				
		20 ml	50 ml	100 ml	150 ml	200 ml
0.03	10	0.06	0.15	0.3	0.45	0.6
0.06	10	0.12	0.3	0.6	0.9	1.2
0.12	10	0.24	0.6	1.2	1.8	2.4
0.25	100	0.05	0.12	0.25	0.38	0.5
0.5	100	0.1	0.25	0.5	0.75	1.0
1.0	100	0.2	0.5	1.0	1.5	2.0
2.0	100	0.4	1.0	2.0	3.0	4.0
4.0	1,000	0.08	0.2	0.4	0.6	0.8
6.0	1,000	0.12	0.3	0.6	0.9	1.2
8.0	1,000	0.16	0.4	0.8	1.2	1.6
10.0	1,000	0.2	0.5	1.0	1.5	2.0
16.0	1,000	0.32	0.8	1.6	2.4	3.2
32.0	1,000	0.64	1.6	3.2	4.8	6.4
64.0	10,000	0.13	0.32	0.64	0.96	1.28
128.0	10,000	0.26	0.64	1.28	1.92	2.56
256.0	10,000	0.51	1.28	2.56	3.84	5.12
512.0	10,000	1.02	2.56	5.12	7.68	10.24

[a] Sufficient volumes for preparation of trays: 20 ml for approximately 150 trays (0.1 ml per well), 50 ml for 400 trays, 100 ml for 900 trays, 150 ml for 1,400 trays, and 200 ml for 1,900 trays.

Table 5.15–A2 Preparation of antimicrobial dilutions: alternate scheme[a]

Concn (μg/ml) of antimicrobial solution[b]	Vol of[c]:		Concn (μg/ml) of intermediate antimicrobial solution	Final concn (μg/ml)[e]
	Antimicrobial solution	CAMHB[d]		
5,120	1	1	2,560	256
5,120	1	3	1,280	128
5,120	1	7	640	64
640	1	1	320	32
640	1	3	160	16
640	1	7	80	8
80	1	1	40	4
80	1	3	20	2
80	1	7	10	1
10	1	1	5	0.5
10	1	3	2.5	0.25
10	1	7	1.25	0.125

[a] Data are based on information in **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.

[b] 5.12 ml of 10,000-μg/ml antimicrobial solution + 4.88 ml of CAMHB = 5,120 μg/ml.

[c] Use multiples of these figures to obtain desired volumes of intermediate antimicrobial solutions.

[d] Use CAMHB rather than deionized water (as in Table 5.15–A1) as diluent to avoid the diluting effect of large volumes of water in CAMHB.

[e] Add intermediate solution and CAMHB to achieve final concentrations in reservoir tube as follows.

Vol (ml) of:		
Final antimicrobial solution	Intermediate antimicrobial solution	CAMHB
20	2	18
50	5	45
100	10	90
150	15	135
200	20	180

APPENDIX 5.15–8

Preparation of Individual Broth Microdilution MIC Trays

A. Prepare 1.0-ml amounts of antimicrobial agent solutions at the highest concentrations to be tested.
B. Using a repeating or multichannel pipette, dispense 0.1 ml of CAMHB or other appropriate broth medium into each well of a U-bottom microdilution tray except for wells in the first column.
C. Test concentrations from 128 to 0.25 µg/ml (or other concentrations as needed). Include two wells without antimicrobial agent to serve as positive and negative controls.
D. Add 0.1 ml of each antimicrobial agent at 128 µg/ml to the first two wells (labeled 128 and 64 µg/ml) of each row.
E. Using a multichannel pipette, aspirate the contents of the second well in each row up and down several times to mix well.
F. Transfer 0.1 ml from the second well in each row to the third well in each row.
G. Continue mixing and serially transferring the antimicrobial solutions until the last desired concentrations are achieved.
H. Discard 0.1 ml from the last well in each row.
I. Prepare additional trays as needed for QC.
J. Inoculate trays as outlined in procedure 5.2.
K. *Note:* For this particular procedure, it is not necessary to change tips between dilutions.

APPENDIX 5.15–9

Worksheet for microdilution MIC trays and sample sheet (*see* p. 5.15.17 and 5.15.18)

APPENDIX 5.15–10

Broth Microdilution MIC Tray Preparation Checklist

A. 3 weeks before run
   Order empty microdilution trays from vendor.
B. 2 weeks before run
   1. Prepare and QC antimicrobial stock solutions.
   2. Inform medium preparation area of scheduled tray preparation, and order media.
C. 1 week before run
   1. Check with medium preparation area to verify schedule.
   2. All stock solutions should be quality controlled and ready for use.
   3. Acid wash large reservoir tubes.
   4. Label plastic bags for packaging of prepared trays. Include preparation date and color code.
D. 3 days before run
   1. Get supplies in order.
   2. Prepare large flasks of media and refrigerate.
   3. Add Ca^{2+} and Mg^{2+} to cold Mueller-Hinton broth, and adjust pH.
   4. Dispense broth into tubes and flasks.
   5. Autoclave broth.
   6. Check final pH.
   7. Incubate one tube and one flask of broth for sterility check.
E. 2 days before run
   1. Organize freezers for storage of prepared trays.
   2. Subculture QC organisms from frozen stocks.
   3. Send samples of broth to chemistry laboratory for Ca^{2+}, Mg^{2+}, and NaCl analysis.
F. 1 day before run
   1. Label tubes, and dispense sterile water for intermediate antimicrobial dilutions.
   2. Subculture QC organisms a second time.
   3. Assemble and label materials for QC of new trays.
   4. Place dots (labels) on large reservoir tubes, and recheck tray layout.
   5. Weigh out gentamicin and streptomycin (for runs of gram-positive trays).
   6. Begin preparation of dispensing apparatus.

APPENDIX 5.15–10 *(continued)*

7. Assemble supplies for next morning (including sterile towels, which may require autoclaving).
8. Unwrap, stack, and mark empty trays (open wells down). Cover with sterile towel to prevent contamination.

G. Day of run
1. Disinfect all work areas.
2. Remove stock solutions from freezer.
3. Add thymidine phosphorylase to appropriate reservoir tubes.
4. Prepare intermediate antimicrobial solution dilutions (in presence of spotter).
5. Check positioning of tubes, and add antimicrobial solutions (in presence of spotter).
6. Fill all tubes to final volume with CAMHB.

H. Immediately before dispensing
1. Recheck positions of tubes.
2. Prepare dispensing apparatus.
3. Remove labels from caps.
4. Remove caps from tubes.
5. Place rack of tubes in reservoir, and finish assembling the dispensing apparatus.
6. Proceed with volume and weight QC checks prior to filling trays.
7. Place filled trays in freezers.

I. After run
1. Clean dispensing apparatus according to manufacturer's instructions.
2. Rinse large tubes and caps six to eight times with deionized (or distilled) water. Do not use soap of any kind on tubes or caps.
3. Wash reservoir tubes with acid or use another equivalent cleaning procedure.

Broth Microdilution MIC Tray Preparation Medium Ordering

For 1,900-tray run:

Please prepare for _____ (date). Color code _____

24.0 liters of CAMHB

1. Prepare MHB according to manufacturer's directions.

2. Dispense into two 12.0-liter volumes and chill to 2–8°C.

3. Add Ca^{2+} and Mg^{2+}, adjust pH, and dispense broth as follows.
   a. If CAMHB–2% NaCl not required:

      105 tubes to contain 195 ml
      15 flasks to contain 150 ml

   b. If CAMHB–2% NaCl required (gram-positive run):

      88 tubes to contain 195 ml of CAMHB
      10 flasks to contain 150 ml of CAMHB

      Add 60 g of NaCl to 3.0 liters of MHB during medium preparation step, add Ca^{2+} and Mg^{2+}, adjust pH, and dispense broth as follows:

      11 tubes to contain 195 ml of CAMHB–2% NaCl
      2 flasks to contain 150 ml of CAMHB–2% NaCl

Broth Microdilution MIC Tray Preparation QC Log

Date of Run (Lot #) _____ No. of Trays Made _____ Tech _____ (Primary)

Expiration Date _____ Lot # of Plastic Trays _____ Tech _____ (Spotter)

DYE MARKER:

Lot _____ Expiration Date _____

CAMHB:

Lot _____ Expiration Date _____ Manufacturer _____

Sterility (Incubate for 48 h)

pH Pre Autoclave (Pre Adjust) _____ Flask _____

(Post Adjust) _____ Tube _____

Post Autoclave _____ Trays Begin _____

(range: 7.2–7.4) Trays End _____

Adjustments: 1 N HCl:

Lot# _____ Amount Added _____

1 N NaOH:

Lot# _____ Amount Added _____

CALCIUM:

Stock Solution Lot # _____

Expiration Date _____

Final Assayed Conc. _____ mg/liter

(range: 20–25)

MAGNESIUM:

Stock Solution Lot # _____

Expiration Date _____

Final Assayed Conc. _____ mg/liter

(range: 10–12.5)

THYMIDINE PHOSPHORYLASE:

Lot # _____ Expiration Date _____

(continued)

DISPENSER:

A. By Volume (3 Shots) (range: 0.27–0.31 ml)

Beginning of Run	Low	_____
	High	_____
Mid-Run	Low	_____
	High	_____
End of Run	Low	_____
	High	_____

B. By Weight (range: 9.1–10.1 g)

1) _____	6) _____	11) _____	16) _____
2) _____	7) _____	12) _____	17) _____
3) _____	8) _____	13) _____	18) _____
4) _____	9) _____	14) _____	19) _____
5) _____	10) _____	15) _____	20) _____

CAMHB–2% NaCl:

Lot # MHB _____ Expiration Date _____ Manufacturer _____

Lot # NaCl _____ Expiration Date _____ Manufacturer _____

Sterility (Incubate for 48 h)

pH Pre Autoclave (Pre Adjust) _____ Flask _____

(Post Adjust) _____ Tube _____

Post Autoclave _____ Trays Begin _____

(range: 7.2–7.4) Trays End _____

Adjustments: 1 N HCl:

Lot # _____ Amount Added _____

1 N NaOH:

Lot # _____ Amount Added _____

NaCl Concentration = _____ mmol/liter

(range: 450–480)

CALCIUM:

Stock Solution Lot # _____

Expiration Date _____

Final Assayed Conc. _____ mg/liter

(range: 20–25)

MAGNESIUM:

Stock Solution Lot # _____

Expiration Date _____

Final Assayed Conc. _____ mg/liter

(range: 10–12.5)

Stock Solution QC

TRAY TYPE _____

LOT # (DATE) _____

COLOR CODE _____

	AMT NEEDED (ml)	ANTIMICROBIAL AGENT	CONC (µg/ml)	STOCK LOT #	STOCK EXP	DATE QC	TECH	QC ORGANISM (ATCC #)	ACCEPTABLE MIC RANGE (µg/ml)	MIC (µg/ml)
1										
2										
3										
4										
5										
6										
7										
8										
9										
10										
11										
12										
13										
14										
15										

Example
Stock Solution QC

TRAY TYPE Gram-negative

LOT # (DATE) _____

COLOR CODE _____

	AMT NEEDED (ml)	ANTIMICROBIAL AGENT	CONC (μg/ml)	STOCK LOT #	STOCK EXP	DATE QC	TECH	QC ORGANISM (ATCC #)	ACCEPTABLE MIC RANGE (μg/ml)[a]	MIC (μg/ml)
1		Ampicillin	10,000					*E. coli* (25922)	2–8	
2		Amikacin	10,000					*P. aeruginosa* (27853)	0.5–4	
3		Cefazolin	10,000					*E. coli* (25922)	1–4	
4		Cefepime	10,000					*P. aeruginosa* (27853)	1–8	
5		Ceftazidime	10,000					*S. aureus* (29213)	4–16	
6		Cefotaxime	10,000					*S. aureus* (29213)	1–4	
7		Cefpodoxime	1,000					*S. aureus* (29213)	1–8	
8		Ceftriaxone	10,000					*S. aureus* (29213)	1–8	
9		Ciprofloxacin	1,000					*P. aeruginosa* (27853)	0.25–1	
10		Gentamicin	1,000					*P. aeruginosa* (27853)	0.5–2	
11		Imipenem	1,000					*P. aeruginosa* (27853)	1–4	
12		Piperacillin	10,000					*E. coli* (25922)	1–4	
13		Tobramycin	1,000					*P. aeruginosa* (27853)	0.25–1	
14		Trimethoprim-sulfamethoxazole	1,000 10,000					*E. cloacae* (UCLA U)	2/38–4/76	
15										

[a] Except for data on *E. cloacae* (UCLA U), values are from **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically,* 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.

Worksheet for Microdilution MIC Trays

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Example of microdilution MIC tray layout for gram-negative organisms[a,b,c]

	1	2	3	4	5	6	7	8	9	10	11	12
A	NEG CONT	POS CONT	TMP-SMX 0.25/4.75	TMP-SMX 0.5/9.5	TMP-SMX 1.0/19	TMP-SMX 2/38	TMP-SMX 4/76	TMP-SMX 8/152	CFPD 1.0	CFPD 2.0	CFPD 4.0	Dye marker
B	AMP 0.5	PP 8.0	GM 0.5	TB 0.5	AK 0.5	CFAZ 0.5	CPM 0.5	CTAX 0.5	CTAZ 0.5	CIPR 0.12	IPM 0.25	CTRX 0.5
C	AMP 1.0	PP 16.0	GM 1.0	TB 1.0	AK 1.0	CFAZ 1.0	CPM 1.0	CTAX 1.0	CTAZ 1.0	CIPR 0.25	IPM 0.5	CTRX 1.0
D	AMP 2.0	PP 32.0	GM 2.0	TB 2.0	AK 2.0	CFAZ 2.0	CPM 2.0	CTAX 2.0	CTAZ 2.0	CIPR 0.5	IPM 1.0	CTRX 2.0
E	AMP 4.0	PP 64.0	GM 4.0	TB 4.0	AK 4.0	CFAZ 4.0	CPM 4.0	CTAX 4.0	CTAZ 4.0	CIPR 1.0	IPM 2.0	CTRX 4.0
F	AMP 8.0	PP 128.0	GM 6.0	TB 6.0	AK 8.0	CFAZ 8.0	CPM 8.0	CTAX 8.0	CTAZ 8.0	CIPR 2.0	IPM 4.0	CTRX 8.0
G	AMP 16.0	PP 256.0	GM 8.0	TB 8.0	AK 16.0	CFAZ 16.0	CPM 16.0	CTAX 16.0	CTAZ 16.0	CIPR 4.0	IPM 8.0	CTRX 16.0
H	AMP 32.0	PP 512.0	GM 10.0	TB 10.0	AK 32.0	CFAZ 32.0	CPM 32.0	CTAX 32.0	CTAZ 32.0	CIPR 8.0	IPM 16.0	CTRX 32.0

[a] This represents a sample layout only and is not a recommendation from the author.

[b] See Appendix 5.15–5 for antimicrobial agents represented by abbreviations.

[c] Numbers are final concentrations (micrograms per milliliter).

5.16 Selecting Antimicrobial Agents for Testing and Reporting

I. INTRODUCTION

Developing protocols for antimicrobial susceptibility testing and reporting is best done with input from the infectious disease service, infection control, and the pharmacy and therapeutics committee. The goals are to provide clinically relevant information that will support cost-effective utilization of antimicrobial agents and to avoid reporting results that may adversely affect patient care. Because of differences in hospital formularies and laboratory functions, it is impossible to make specific recommendations and the suggestions described herein can only serve as a guide to the decision making processes. A list of commonly used antibacterial agents is shown in Appendix 5.16–1.

II. BASIC STEPS IN DEVELOPING A TESTING AND REPORTING PROTOCOL

A. Assess resources available (test system[s], numbers of drugs practically tested in respective system, laboratory reporting capabilities [e.g., computer reporting], manpower)

B. Determine needs of the infectious disease service, pharmacy and therapeutics committee, infection control, and others.

C. Determine which drugs are on the formulary.

D. Determine which drugs will be included in routine testing "batteries." Define, for example, batteries for the following.

1. Gram-negative bacilli (e.g., *Enterobacteriaceae*)

2. Gram-positive cocci (e.g., *Staphylococcus* spp., *Enterococcus* spp.)

3. *Pseudomonas* spp.

4. *Haemophilus* spp.

5. *Streptococcus pneumoniae* and *Streptococcus* species

6. Urine isolates

7. Very resistant isolates (include "broad spectrum" battery and/or strategy for testing agents not routinely tested; this may involve use of a reference laboratory)

E. Determine which drugs will be routinely reported for specific organisms and organism groups.

F. Determine if selective reporting will be implemented.

G. Develop a protocol for determining how to report test results obtained following special requests by physicians. For example, if a physician requests a drug be reported that is not reported routinely, qualify with a comment such as, "Drug X reported upon special request by Dr. Jones."

III. PRIMARY RESOURCES

A. Tables 1 and 1A in NCCLS documents M7-A6 and M2-A8 (3, 4), "Suggested Groupings of U.S. FDA-Approved Antimicrobial Agents That Should be Considered for Routine Testing and Reporting by Clinical Microbiology Laboratories"

B. *The Medical Letter* (1) and *The Sanford Guide to Antimicrobial Chemotherapy* (2) provide recommendations for the drugs of first choice and alternative agents for treating infections caused by specific bacteria.

C. Other medical publications that address antimicrobial prescribing choices

IV. SELECTIVE DRUG REPORTING (CASCADE REPORTING) OPTION

A. Test first- and second-line drugs routinely.

B. Base reporting on organism identification, overall antimicrobial susceptibility profile of the isolate, and site of infection.

C. Report second-line agents only if first-line agents appear inappropriate (inactive, inappropriate for specific infection or infecting species, etc.)

D. Make all results available for unusual clinical situations.

E. Examples of selective reporting based on organism identification and overall antibiogram

1. *Enterobacteriaceae*

a. *If* isolate is resistant to first-generation cephalosporins, *Then* report second-generation cephalosporins.

b. *If* isolate is resistant to gentamicin, *Then* report tobramycin or amikacin or both.

2. *Pseudomonas aeruginosa*

If isolate is resistant to ceftazidime, piperacillin, and aminoglycosides, *Then* report imipenem.

F. Examples of selective reporting according to body site

1. Report results for drugs (e.g., cefotaxime, ceftriaxone) that cross the blood-brain barrier on CSF isolates, if appropriate for the organism group.

Do not report agents administered only by the oral route or aminoglycosides, first- and second-generation cephalosporins, clindamycin, fluoroquinolones, macrolides, or tetracyclines, as these are ineffective in treating meningitis.

2. Report results for agents (e.g., nitrofurantoin, norfloxacin) that are used only for treating urinary tract infections on urine isolates only.

3. Some antimicrobial agents are inappropriate for treatment of infections caused by certain bacteria, even if the in vitro results indicate that the isolate is susceptible. These include the following.

a. Beta-lactams (penicillins, cephalosporins, beta-lactam–beta-lactamase inhibitor combinations, imipenem) for methicillin-resistant staphylococci

b. Cephalosporins, aminoglycosides (except high-level screens), clindamycin, and trimethoprim-sulfamethoxazole for enterococci

c. Cephalosporins for *Listeria* spp.

d. First- or second-generation cephalosporins and aminoglycosides for *Salmonella* spp. and *Shigella* spp.

e. Penicillins, cephalosporins, and aztreonam on extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella* spp.

G. Selective reporting may not be practiced by institutions where there are other mechanisms in place to prevent physicians from prescribing antimicrobial agents inappropriately.

REFERENCES

1. **Abramowicz, M. (ed).** 2001. The choice of antibacterial drugs. *Med. Lett.* **43:**69–78.
2. **Gilbert, D. N., R. C. Moellering, Jr., and M. A. Sande.** 2003. *The Sanford Guide to Antimicrobial Chemotherapy,* 33rd ed. Antimicrobial Therapy, Inc., Hyde Park, Vt.
3. **NCCLS.** 2003. *Performance Standards for Antimicrobial Disk Susceptibility Tests,* 8th ed. Approved standard M2-A8. NCCLS, Wayne, Pa.
4. **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically,* 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.

APPENDIX 5.16–1

Reference chart for commonly used antimicrobial agents[a]

Drug family and generic name (route[s] of administration)	Product name(s)[b]	Primary spectrum of activity		Mode of action	Clinical use; comments
		Gram positive	Gram negative		
Penicillins					
Natural penicillins					
Penicillin G (i.m., i.v.)	Various product names	×		Inhibition of cell wall synthesis	Community-acquired meningitis in normal hosts due to pneumococci, meningococci, or *Listeria* spp.; serious streptococcal infection (e.g., endocarditis, bacteremia, and osteomyelitis), serious enterococcal infections (given in combination with gentamicin); anaerobic infections other than those caused by *Bacteroides fragilis; Pasteurella* spp.
Penicillin V (p.o.)	Various product names	×		Inhibition of cell wall synthesis	Streptococcal pharyngitis/otitis, skin and soft tissue infections
Extended-spectrum penicillins					
Aminopenicillins		×	×	Inhibition of cell wall synthesis	Same as penicillin G plus infections caused by *E. coli, Salmonella* spp., *Haemophilus influenzae* (beta-lactamase negative), and *Proteus mirabilis;* amoxicillin is used for less serious infections.
Ampicillin (p.o., i.m., i.v.)	Various product names				
Amoxicillin (p.o.)	Various product names				
Carboxypenicillins			×	Inhibition of cell wall synthesis	Used in combination with an aminoglycoside for serious infections due to susceptible *Enterobacteriaceae* (*Klebsiella* spp. are resistant) and *P. aeruginosa;* oral carbenicillin is used exclusively for urinary tract infections.
Carbenicillin (p.o., i.m., i.v.)	Geopen, Geocillin, Pyopen				
Ticarcillin (i.m., i.v.)	Ticar				
Ureidopenicillins			×	Inhibition of cell wall synthesis	Same as carboxypenicillins, with enhanced activity against *Enterobacteriaceae* (including *Klebsiella* spp.), *P. aeruginosa*, and *Bacteroides* spp.
Azlocillin (i.v.)	Azlin				
Mezlocillin (i.m., i.v.)	Mezlin				
Piperacillin (i.m., i.v.)	Pipracil				
Penicillinase-stable penicillins		×		Inhibition of cell wall synthesis	Staphylococcal infections due to strains susceptible to penicillinase-stable penicillins; will cover most streptococci (but not enterococci); oral agents (cloxacillin, dicloxacillin) used for less serious infections.
Methicillin (i.m., i.v.)	Staphcillin				
Nafcillin (p.o., i.m., i.v.)	Nafcil, Nalipen, Unipen				
Isoxazolyl penicillins					
Oxacillin (p.o., i.m., i.v.)	Prostaphlin				
Cloxacillin (p.o.)	Tegopen				
Dicloxacillin (p.o.)	Dynapen, Pathocil				
Amidinopenicillin			×	Inhibition of cell wall synthesis	Acute uncomplicated urinary tract infections
Mecillinam (p.o.)	CoActin				

(continued)

APPENDIX 5.16–1 *(continued)*

Reference chart for commonly used antimicrobial agents[a] *(continued)*

Drug family and generic name (route[s] of administration)	Product name(s)[b]	Primary spectrum of activity		Mode of action	Clinical use; comments
		Gram positive	Gram negative		
Beta-lactam–beta-lactamase inhibitor combinations		×	×	Inhibition of cell wall synthesis	Moderate to severe mixed infections due to aerobic and anaerobic bacteria (e.g., intra-abdominal and pelvic infections, aspiration pneumonia, etc.); amoxicillin-clavulanic acid is used for upper and lower respiratory tract infections due to *H. influenzae, Moraxella catarrhalis, Staphylococcus aureus,* and anaerobes; the beta-lactamase-inhibiting component facilitates activity against beta-lactamase-producing *Staphylococcus* spp. (methicillin susceptible), *H. influenzae,* and *Bacteroides* spp.
Amoxicillin-clavulanic acid (p.o.)	Augmentin				
Ticarcillin-clavulanic acid (i.v.)	Timentin				
Ampicillin-sulbactam (i.v.)	Unasyn				
Piperacillin-tazobactam (i.v.)	Zosyn				
Cephems,[d] parenteral					
Cephalosporins					
Narrow spectrum (first generation)		×	×	Inhibition of cell wall synthesis	Surgical prophylaxis; community-acquired pulmonary, skin and soft tissue, and urinary tract infections; staphylococcal osteomyelitis and soft tissue infection; active against many gram-positive cocci other than methicillin-resistant staphylococci and enterococci; active against some *Enterobacteriaceae,* including many strains of *E. coli, P. mirabilis, Klebsiella* spp., and most anaerobes (other than *B. fragilis*).
Cefazolin (i.m., i.v.)					
Cephalothin (i.m., i.v.)					
Cephapirin (i.m., i.v.)	Kefzol, Zolicef				
Cephradine (p.o., i.m., i.v.)	Keflin Cefadyl Anspor, Velosef				
Extended spectrum (second generation)		×	×	Inhibition of cell wall synthesis	Community-acquired pulmonary, skin, and soft tissue infections, and exacerbations of chronic obstructive pulmonary disease; slightly more active than first-generation agents against *Enterobacteriaceae* (increased beta-lactamase stability) and less active against staphylococci
Cefamandole (i.m., i.v.)	Mandol				
Cefuroxime-sodium (i.m., i.v.)	Zinacef, Kefurox				
Cefonicid (i.m., i.v.)	Monocid				
Cephamycins[f]					The cephamycins are used for mild to moderate mixed infections (e.g., intra-abdominal and pelvic infections, aspiration pneumonia, etc.) and surgical prophylaxis in gastrointestinal, obstetric, and gynecological procedures. The cephamycins have good activity against *B. fragilis.*
Cefotetan (i.v.)	Cefotan				
Cefoxitin (i.m., i.v.)	Mefoxin				
Cefmetazole (i.v.)	Zefazone				
Extended spectrum (third generation)		×	×	Inhibition of cell wall synthesis	Used in combination with aminoglycoside for serious infections caused by susceptible *Enterobacteriaceae* and *P. aeruginosa*; used alone for childhood meningitis due to *H. influenzae* and gram-negative bacillary meningitis. Ceftriaxone is used for gonorrhea and Lyme disease. Have greatly increased activity against gram-negative bacilli because of increased beta-lactamase stability. Compared to other cephems, most have activity against *P. aeruginosa* (particularly ceftazidime) and CSF penetration; however, they are not as active against staphylococci.
Cefoperazone (i.m., i.v.)	Cefobid				
Cefotaxime (i.m., i.v.)	Claforan				
Ceftizoxime (i.m., i.v.)	Cefizox				
Ceftriaxone (i.m., i.v.)	Rocephin				
Ceftazidime (i.m., i.v.)	Fortaz, Tazicef, Tazidime				

APPENDIX 5.16–1 *(continued)*

Drug family and generic name (route[s] of administration)	Product name(s)[b]	Primary spectrum of activity		Mode of action	Clinical use; comments
		Gram positive	Gram negative		
Extended spectrum (fourth generation) Cefepime (i.v.)	Maxipime	×	×	Inhibition of cell wall synthesis	Similar to third-generation cephalosporins, with enhanced activity against organisms that produce inducible beta-lactamases (e.g., *Enterobacter cloacae*); activity against gram-positive bacteria is similar to that of cefotaxime and activity against gram-negative bacteria is similar to that of ceftazidime, to include good antipseudomonal activity.
Cephems, p.o.					
Cephalosporins		×	×	Inhibition of cell wall synthesis	Community-acquired upper respiratory tract, ear, urinary tract, skin, and soft tissue infections; cefaclor, loracarbef, cefprozil, and cefuroxime are more active than the others against *Haemophilus* spp. and *M. catarrhalis.*
Cefaclor	Ceclor				
Cefadroxil	Duricef, Ultracef				
Cefdinir	Omnicef				
Cefditoren	Spectracef				
Cefixime	Suprax				
Cefpodoxime	Vantin				
Cefprozil	Cefzil				
Cefuroxime-axetil	Ceftin				
Cephalexin	Keflex, Biocef				
Cephradine	Anspor, Velosef				
Ceftibuten	Cedax				
Carbacephem					
Loracarbef	Lorabid				
Monobactam					
Aztreonam (i.v.)	Azactam		×	Inhibition of cell wall synthesis	Infections caused by aerobic gram-negative bacilli in patients allergic to other beta-lactams; activity is comparable to that of third-generation cephalosporins against *P. aeruginosa* and *Enterobacteriaceae,* inactive against gram-positive organisms and anaerobes.
Carbapenems					
Ertapenem (i.m., i.v.)	Invanz	×	×	Inhibition of cell wall synthesis	Imipenem and meropenem are used for severe mixed infections (e.g., intra-abdominal and pelvic infections and aspiration pneumonia) and infections caused by multiply resistant gram-negative bacilli, including *P. aeruginosa;* highly active against most gram-positive organisms (but not against methicillin-resistant staphylococci, *Enterococcus faecium,* or diphtheroids); and the most active beta-lactams against *Enterobacteriaceae* and *P. aeruginosa.* Like the cephalosporins, carbapenems have poor activity against *Stenotrophomonas maltophilia* and *Burkholderia cepacia;* active against *B. fragilis.* Ertapenem has no activity against *P. aeruginosa* and slightly less activity against the other organisms mentioned above; is primarily for intra-abdominal and pelvic infections.
Imipenem (i.v.)	Primaxin				
Meropenem (i.v.)	Merrem				

(continued)

APPENDIX 5.16–1 *(continued)*

Reference chart for commonly used antimicrobial agents[a] *(continued)*

Drug family and generic name (route[s] of administration)	Product name(s)[b]	Primary spectrum of activity		Mode of action	Clinical use; comments
		Gram positive	Gram negative		
Aminocyclitols[e]				Interference with protein synthesis at the 30S ribosomal subunit	Gonorrhea only; however, largely replaced by ceftriaxone or a quinolone
Spectinomycin (i.m.)	Trobicin				
Trospectinomycin (i.m., i.v.)					
Aminoglycosides		×	×	Interference with protein synthesis at the 30S ribosomal subunit	
Gentamicin (i.m., i.v.)	Garamycin, Gentocin				Moderate to severe infections caused by aerobic gram-negative bacilli; often used in combination with a beta-lactam agent, e.g., in combination with ampicillin or penicillin (or sometimes vancomycin) for synergistic killing of enterococci, streptococci, and staphylococci causing endocarditis.
Tobramycin (i.m., i.v.)	Nebcin, Tobrex				Gram-negative infections, indications similar to those for gentamicin; compared with gentamicin, has slightly increased activity against *P. aeruginosa* and sometimes decreased activity against *Serratia marcescens.*
Amikacin (i.m., i.v.)	Amikin				Gram-negative infections, indications similar to those for gentamicin; has increased activity against gentamicin- and tobramycin-resistant isolates.
Netilmicin (i.m., i.v.)	Netromycin				Gram-negative infections, indications similar to those for gentamicin; slightly less active than other aminoglycosides against *P. aeruginosa* and *S. marcescens.*
Neomycin (p.o., topical)	Mycifradin, Neobiotic				Oral and topical use only for gastrointestinal tract decontamination and superficial infections, respectively
Kanamycin (p.o.)	Kantrex, Klebcil				Oral and topical use, similar to neomycin; parenteral forms infrequently used
Streptomycin (i.m., i.v.)	Streptomycin				Used in combination with ampicillin or penicillin (or sometimes vancomycin) for synergistic killing of enterococci or streptococci causing endocarditis.
Ansamycin		×		Inhibition of DNA transcription	Used in combination with antistaphylococcal agents to enhance antibacterial activity, especially in osteomyelitis and endocarditis; first-line agent for tuberculosis in combination with isoniazid; prophylaxis for meningococcal disease; rifabutin for mycobacterial infections only
Rifampin (p.o., i.v.)	Rifadin, Rimactane				
Quinolones (narrow spectrum)		×	×	Inhibition of DNA gyrase activity	Cinoxacin and nalidixic acid, lower urinary tract infection only; are inactive against *P. aeruginosa* and have limited activity against gram-positive bacteria. Garenoxacin is like fluoroquinolones in its spectrum against respiratory pathogens; is active against *Enterobacteriaceae* and has some activity against *P. aeruginosa.*
Cinoxacin (p.o.)	Cinobac				
Garenoxacin (p.o., i.v.)[c]					
Nalidixic acid (p.o.)	NegGram				

APPENDIX 5.16–1 *(continued)*

Drug family and generic name (route[s] of administration)	Product name(s)[b]	Primary spectrum of activity		Mode of action	Clinical use; comments
		Gram positive	Gram negative		
Fluoroquinolones (broader spectrum)		×	×	Inhibition of DNA gyrase activity	Norfloxacin and ofloxacin, uncomplicated and complicated urinary tract infections, gonorrhea, and prostatitis. Ciprofloxacin, moderate to severe infections caused by gram-negative bacilli (especially pulmonary, bone, and joint infections); has limited activity against gram-positive bacteria; is active against *P. aeruginosa.* The other drugs have enhanced activity against gram-positive bacteria; used for respiratory tract infections, including acute exacerbations of chronic bronchitis caused by common respiratory pathogens (*H. influenzae, M. catarrhalis,* and *S. pneumoniae*) and also atypical organisms (*Legionella* spp., *Mycoplasma pneumoniae, Chlamydia pneumoniae*); have little activity against *P. aeruginosa* (except levofloxacin, which is also active against gram-negative bacteria)
Norfloxacin (p.o.)	Noroxin				
Ofloxacin (p.o., i.v.)	Floxin				
Ciprofloxacin (p.o., i.v.)	Cipro				
Gatifloxacin (p.o., i.v.)	Tequin				
Levofloxacin (p.o., i.v.)	Levaquin				
Lomefloxacin (p.o.)	Maxaquin				
Moxifloxacin (p.o., i.v.)	Avelox				
Folate pathway inhibitors					
Sulfonamides (p.o., i.v.)	Various product names	×	×	Competitive inhibition of folic acid synthesis	Uncomplicated lower urinary tract infections and nocardiosis
Trimethoprim-sulfamethoxazole (p.o., i.v.)	Bactrim, Septra		×	Competitive inhibition of folic acid synthesis	Acute and chronic urinary tract infections; bacterial diarrhea; infections caused by *Enterobacteriaceae;* acute and chronic community-acquired upper respiratory tract infections; also used to treat *Pneumocystis carinii* infections.
Trimethoprim (p.o.)	Proloprim, Trimpex		×	Inhibition of folic acid synthesis	Uncomplicated lower urinary tract infections and prophylaxis for recurrent urinary tract infections
Fosfomycin					
Fosfomycin (p.o.)	Monurol	×	×	Inhibition of pyruvyl transferase enzyme	Acute uncomplicated urinary tract infections due to *E. coli* and *Enterococcus faecalis*
Ketolide					
Telithromycin[c] (p.o.)	Ketek	×	×	Binds to 50S ribosomal subunit, blocking the initiation of peptide chains	Respiratory infections caused by common and atypical and intracellular or cell-associated respiratory pathogens, including those that are resistant to beta-lactam and/or macrolide-lincosamide-streptogramin B antimicrobials
Lincosamide					
Clindamycin (p.o., i.m., i.v.)	Cleocin	×		Binds to 50S ribosomal subunit, blocking the initiation of peptide chains	Anaerobic infections (including those caused by *B. fragilis*); gram-positive bone and joint infections in children

(continued)

APPENDIX 5.16–1 *(continued)*

Reference chart for commonly used antimicrobial agents[a] *(continued)*

Drug family and generic name (route[s] of administration)	Product name(s)[b]	Primary spectrum of activity		Mode of action	Clinical use; comments
		Gram positive	Gram negative		
Lipopeptide					
Daptomycin[c] (i.v.)	Cidecin	×		Disruption of multiple aspects of bacterial plasma membrane function	Bacteremia, endocarditis, and skin and soft tissue infections caused by gram-positive bacteria; active against VRE and MRSA.
Macrolides		×		Binds to 50S ribosomal subunit, blocking the initiation of peptide chains	Upper and lower respiratory tract infections caused by gram-positive organisms (pneumococci or group A streptococci) in penicillin-allergic patients; pertussis, *Campylobacter, M. pneumoniae, Chlamydia trachomatis, Legionella* spp., and *H. influenzae* (azithromycin and clarithromycin); less serious staphylococcal infections; clarithromycin is used in combination with other agents for some mycobacterial infections.
Azithromycin (p.o., i.v.)	Zithromax				
Clarithromycin (p.o.)	Biaxin				
Erythromycin (p.o., i.v.)	Various product names				
Dirithromycin[c] (p.o.)	Dynabac				
Nitrofuran					
Nitrofurantoin (p.o.)	Furadantin, Macrodantin	×	×	Inhibition of a variety of bacterial enzyme systems	Acute and chronic lower urinary tract infections; inactive against *S. marcescens, Proteus* spp., and *P. aeruginosa*
Nitroimidazole					
Metronidazole (p.o., i.v.)	Flagyl			Inhibition of DNA replication	Anaerobic infection; vaginitis caused by *Gardnerella vaginalis* and *Trichomonas vaginalis; Clostridium difficile* toxin-associated diarrhea and colitis
Oxazolidinone					
Linezolid (p.o., i.v.)	Zyvox	×		Inhibition of initiation of bacterial protein synthesis	Complicated and uncomplicated skin and soft tissue infections; community- and hospital-acquired pneumonia; drug-resistant gram-positive infections (including MRSA and VRE)
Glycopeptides		×		Inhibition of cell wall synthesis	Methicillin-resistant staphylococcal infection and gram-positive infection in penicillin-allergic patients; oral form for *C. difficile* toxin-associated diarrhea/colitis; is inactive against *Lactobacillus* spp., *Pediococcus* spp., *Leuconostoc* spp., and *Erysipelothrix* spp.
Vancomycin (p.o., i.v.)	Vancocin				
Teicoplanin[c] (i.m., i.v.) (lipoglycopeptide)	Targocid				
Phenicol					
Chloramphenicol (p.o., i.v.)	Chloromycetin	×	×	Prevents mRNA from attaching to ribosomes	Childhood meningitis (e.g., that caused by *S. pneumoniae, H. influenzae,* and *Neisseria meningitidis*); typhoid fever; anaerobic infections (especially brain abscesses); salmonellosis

APPENDIX 5.16–1 *(continued)*

Drug family and generic name (route[s] of administration)	Product name(s)[b]	Primary spectrum of activity		Mode of action	Clinical use; comments
		Gram positive	Gram negative		
Streptogramin Quinupristin-dalfopristin	Synercid	×		Inhibition of substrate attachment and inhibition of peptide chain elongation (50S ribosome)	Complicated skin and skin structure infections caused by *S. aureus* and group A streptococci; *E. faecium* (inactive against *E. faecalis*)
Tetracyclines Short-acting tetracycline Tetracycline (p.o., i.m., i.v.) Long-acting tetracyclines Doxycycline (p.o., i.v.) Minocycline (p.o.)	 Various product names Vibramycin Minocin, Vectrin	×	×	Interference with protein synthesis at the 30S ribosomal subunit	Not often used as directed therapy for common bacterial infections; however, used for brucellosis and chlamydial, mycoplasmal, and rickettsial infections.

[a] i.m., intramuscularly; p.o., orally; i.v., intravenously; VRE, vancomycin-resistant enterococci; MRSA, methicillin-resistant *S. aureus.*

[b] Denotes the product name(s) according to *Facts and Comparisons* or according to the manufacturer.

[c] Investigational agent; not Food and Drug Administration approved as of January 2003.

[d] Cephems have no activity against methicillin-resistant staphylococci, enterococci, or *Listeria* spp.

[e] Active against *Neisseria gonorrhoeae.*

[f] These agents are cephems but *not* cephalosporins.

5.17 Evaluating Antimicrobial Susceptibility Test Systems

I. PRINCIPLE

A wide variety of antimicrobial susceptibility test systems are available to microbiologists today. This procedure provides guidance on evaluating and choosing a system or method appropriate for your laboratory. The primary focus should be on determination of the systems' capabilities, assessment of costs and work flow, and examination of performance data.

Obviously, not every laboratory has the resources for performing the detailed evaluation described here. When smaller laboratories are faced with decision making regarding a new antimicrobial test system, they must rely primarily on published data, information supplied by the manufacturer, and information supplied by other users of the system. However, every laboratory should undertake a limited study (at least 50 isolates of various species, various antimicrobial susceptibility profiles, and NCCLS QC strains should be tested) so that performance can be assessed on a small scale and, more important, so that technologists and supervisory personnel can get a feel for how the system works and how it would fit into the laboratory's work flow.

II. MATERIALS

A. Marketing literature from manufacturers

B. Package inserts from systems being evaluated

C. Pertinent scientific journal articles and abstracts

D. QC organisms

E. Clinical isolates

F. Challenge isolates

G. Media for storage of QC and test isolates (2) (*see* procedure 5.13)

H. Appropriate agar media
Store at 2 to 8°C.

I. McFarland standards (store at room temperature in the dark) or turbidity standard method recommended by the manufacturer (e.g., nephelometer or colorimeter)

J. Materials necessary to set up reference system (usually agar dilution [2], broth microdilution [2], or disk diffusion)

K. Materials necessary to set up each test system according to instructions in the package insert (e.g., cytochrome oxidase, sterile saline, disposable test tubes, coagulase test method)

L. Required storage equipment for materials and disposables (e.g., −70°C freezer, −20°C non-frost-free freezer, 2 to 8°C refrigerator)

III. PROCEDURE

A. Consider the following when choosing an antimicrobial susceptibility test system to evaluate.

1. Performance

a. Examine independent evaluations in peer-reviewed journals. Note the following to determine validity of study.

(1) Number of isolates tested
(2) Specific organism-antimicrobial agent combinations tested
(3) Number of clinically significant resistant isolates included
(4) Number of institutions included in study
(5) Reference method used for comparison
(6) Method used for data correlation
(7) Date of study (Changes may have been made since study was performed.)

b. Examine recent data provided by manufacturer.

c. Abstracts may provide additional information on product performance, but these are not subjected to peer review, and methods are often sketchy.

2. Cost

a. Capital equipment
b. Per test
c. Maintenance
d. Technical assistance (e.g., training)
e. Software updates

3. Practical considerations

a. Space requirements
b. Electrical requirements
c. Instrument noise
d. Instrument capacity
e. Current customer satisfaction
f. Ease of use, personnel requirements
g. Biohazardous waste generated
h. Shelf lives of disposable materials
i. Storage requirements of disposable materials
j. Environmental requirements

(1) Temperature
(2) Humidity

k. Organism identification capabilities of system
l. Instrument downtime

4. Software

a. Computer interface capabilities
b. Data management capabilities
c. Frequency of updates
d. Ease of updates
e. Availability of selective reporting

5. Manufacturer's support

a. Service response time
b. Availability of technical assistance
c. Order response time

6. Technical considerations

a. Range of dilutions reported per antimicrobial agent
b. Flexibility of antimicrobial agent selection
c. QC required
d. Response time for adding new antimicrobial agents

III. PROCEDURE
(continued)

e. Range of organisms tested by system (*Haemophilus* spp., other fastidious organisms, anaerobes, etc.)
f. Number and type of disclaimed antimicrobial agent-organism combinations
g. Compliance with NCCLS recommendations
h. Result reported
(1) Quantitative (MIC)
(2) Qualitative (breakpoint)
i. Food and Drug Administration cleared
j. Backup method requirement
k. Length of time before results can be reported
l. Compatibility with identification system
m. Number of antimicrobial agents available

7. Work flow considerations
a. Inoculum
(1) Source
(a) Age of culture
(b) Medium
(2) Inoculation fluid
(3) Preincubation
(4) Standardization
(a) Visual
(b) Instrument assisted
(5) Inoculation process
(a) Manual
(b) Automated
(6) Same suspension for identification and susceptibility?
b. Labeling
(1) Manual
(2) Bar codes
(3) Other (e.g., computer keyboard entry)
c. Data-test entry
d. Time before reports become available

B. Devise a strategy for evaluating antimicrobial susceptibility test systems.
1. Determine systems to be compared.
2. Determine reference method.
a. NCCLS-approved standard method (2) (recommended)
b. Laboratory's current method or other test system with NCCLS-approved standard method to resolve discrepancies
3. Review methods to be tested.
4. Determine antimicrobial agents to be tested.
5. Determine strains to be tested.
a. Test a minimum of 50 fresh and recent clinical isolates (1 isolate of a given species per patient) representing a wide variety of species. Fresh isolates are those which have never been frozen and have been on agar for less than 7 days.
b. Test a minimum of 50 challenge strains (organisms with varied resistance patterns for each antimicrobial agent to be evaluated).
(1) Beta-lactam antimicrobial agents
(a) Organisms resistant to extended-spectrum cephalosporins
i. *Enterobacter cloacae*
ii. *Enterobacter aerogenes*
iii. *Citrobacter freundii*
iv. *Serratia marcescens*

III. PROCEDURE
(continued)

v. *Pseudomonas aeruginosa*
vi. Indole-positive *Proteeae* (e.g., *Morganella morganii* and *Providencia* spp.)
vii. Extended-spectrum beta-lactamase-producing *Klebsiella* spp. and *Escherichia coli*

(b) Imipenem-resistant *P. aeruginosa*
(c) Ampicillin-resistant *E. coli*
(d) Piperacillin-resistant *Klebsiella* spp.
(e) Methicillin-resistant staphylococci
i. *Staphylococcus aureus*
ii. Coagulase-negative staphylococci
(f) Ampicillin-resistant *Enterococcus faecium*

(2) Aminoglycosides
(a) Gentamicin-resistant organisms
i. Members of the family *Enterobacteriaceae*
ii. Staphylococci
iii. *P. aeruginosa*
(b) Amikacin-resistant organisms
i. *Enterobacteriaceae*
ii. *P. aeruginosa*
(c) Tobramycin-resistant *P. aeruginosa*
(d) High-level aminoglycoside-resistant enterococci

(3) Fluoroquinolone-resistant organisms
(a) *Enterobacteriaceae*
(b) Staphylococci
(c) *P. aeruginosa*

(4) Vancomycin-resistant enterococci
(5) Clindamycin-resistant staphylococci
(6) Erythromycin-resistant staphylococci
(7) For fastidious organism panels
(a) *Streptococcus pneumoniae*
i. Penicillin resistant and intermediate
ii. Extended-spectrum cephalosporin resistant and intermediate
iii. Fluoroquinolone resistant
(b) *Streptococcus* species
i. Penicillin-resistant and -intermediate viridans group streptococci
ii. Macrolide-resistant *Streptococcus* species
iii. Clindamycin-resistant *Streptococcus* species

c. The evaluation process may be improved if more than 50 to 100 isolates are tested. It is not unreasonable for a larger laboratory to test 100 to 300 isolates.

d. Test a minimum of five strains in triplicate for 3 days to determine reproducibility of system.
(1) Choose a variety of species.
(2) Choose strains with a history of reproducible results by another method.
(3) Choose resistant isolates and isolates for which the MICs are on-scale.

6. Define procedural parameters.

a. General techniques
(1) Subculture stock isolates to nonselective media twice before testing.
(2) Pick one colony from the first subculture to make the second subculture.

III. PROCEDURE
(continued)

(3) Use fresh isolates (less than 24 h old).
(4) Run test system and reference method in parallel on the same day from the same inoculum if possible.
(5) Run recommended NCCLS QC strains (2) along with manufacturer's recommended QC strains daily.
(6) Plate inoculum to check purity.
(7) Save all isolates tested.
(a) For short-term storage (<2 weeks), use soybean casein digest agar slants at 2 to 8°C (2).
(b) For long-term storage, use brucella broth with 15% glycerol and freeze at −20°C or lower (preferably −70°C).
(8) Retest isolates with insufficient growth. Confirmed growth failures of >10% for any genus or species tested indicate that the system might be unreliable for that specific group.

b. Procedural techniques defined by each system
(1) Inoculum
(2) Storage of test kits
(3) Required reagents and media
(4) Acceptable organisms to test in specific system
(5) Proper incubation time and temperature

C. Compare test result with reference result (providing QC is acceptable).

1. MIC (quantitative) comparison
A valid quantitative comparison can be made only when more than three consecutive twofold dilutions are tested (1)

a. Essential agreement
Test result is ±1 doubling dilution from reference result. For example, if the reference result is 2 μg/ml, the test result could be 1, 2, or 4 μg/ml and be in essential agreement.

b. Exact agreement
Test result and reference result have the same MIC.

2. Category (qualitative) comparison (*see* Appendix 5.17–1)

a. Agreement
Category results are identical.

b. Very major error
Test result shows susceptibility, and reference result shows resistance.

c. Major error
Test result shows resistance, and reference result shows susceptibility.

d. Minor error
Test result shows resistance or susceptibility and reference result shows intermediate, or test result shows intermediate and reference result shows resistance or susceptibility.

3. Essential versus category agreement (example)

a. Breakpoints for tobramycin
(1) Susceptible = ≤4 μg/ml
(2) Intermediate = 8 μg/ml
(3) Resistant = ≥16 μg/ml

b. Dilutions tested: 0.5, 1, 2, 4, 8, and 16 μg/ml

c. Essential but not category agreement
(1) Reference = 8 μg/ml (intermediate)
(2) Test = 4 μg/ml (susceptible)

d. Category but not essential agreement
(1) Reference = 4 μg/ml (susceptible)
(2) Test = 0.5 μg/ml (susceptible)

III. PROCEDURE
(continued)

4. Resolution of discrepancies
- **a.** Repeat all tests with major and very major errors in triplicate for both systems (1)
- **b.** Repeat tests with significant minor errors.
  - (1) Antimicrobial agents with >10% minor errors
  - (2) Minor errors that may influence antimicrobial therapy
- **c.** Repeat all tests with MIC errors (i.e., that do not have essential agreement or that are $> \pm 1$ doubling dilution from reference).
- **d.** Use NCCLS standard method (2) to resolve discrepancies that exist after both methods have been repeated.
- **e.** Use results obtained from retests to correlate data.

D. Correlate data.

1. Percent essential agreement =

$$\frac{\text{number of tests within } \pm 1 \text{ doubling dilution from reference}}{\text{number of organisms tested}} \times 100$$

2. Calculate essential agreement for the following.
- **a.** Overall (all organisms and all antimicrobial agents)
- **b.** Each antimicrobial agent with all organisms
- **c.** Each organism with all antimicrobial agents
- **d.** Each antimicrobial agent for each group of organisms

3. Percent category agreement =

$$\frac{\text{number of tests with no category errors}}{\text{number of organisms tested}} \times 100$$

4. Calculate category agreement for the following.
- **a.** Overall (all organisms and all antimicrobial agents)
- **b.** Each antimicrobial agent with all organisms
- **c.** Each organism with all antimicrobial agents
- **d.** Each antimicrobial agent for each group of organisms

5. Percent minor (or very major or major) errors =

$$\frac{\text{number of tests with minor (or very major or major) errors}}{\text{number of organisms tested}} \times 100$$

6. Calculate minor (or very major or major) errors for the following.
- **a.** Overall (all organisms and all antimicrobial agents)
- **b.** Each antimicrobial agent with all organisms
- **c.** Each organism with all antimicrobial agents
- **d.** Each antimicrobial agent for each group of organisms

E. Record results on display sheet that summarizes total performance of a single antimicrobial agent for the various organism groups, and include overall performance of the drug (Appendix 5.17–2).

F. Record results on display sheet that summarizes essential and categoric agreement for each drug and each organism group, and include overall performance (Appendix 5.17–3).

IV. INTERPRETATION OF CORRELATIONS

A. Essential agreement should be >90% for each organism group-antimicrobial agent combination (1).

B. For reproducibility testing, results for each antimicrobial agent should be within ±1 doubling dilution 95% of the time (1).

C. Recommended acceptable category guidelines are as follows.

1. Food and Drug Administration guidelines for manufacturers (1)

a. Very major errors

(1) Very major errors must be ≤1.5%.

(2) Calculate very major errors from the number of resistant isolates.

$$\%\ \text{very major errors} = \frac{\text{number of very major errors}}{\text{total number of resistant isolates}} \times 100$$

(3) Test at least 20 resistant strains representing each known mechanism of resistance for each antimicrobial agent. For example, for methicillin-resistant staphylococci, test organisms with altered penicillin-binding proteins and test hyper-beta-lactamase producers.

b. Major errors

(1) Major errors must be ≤3.0%.

(2) Calculate major errors from the number of susceptible isolates.

$$\%\ \text{major errors} = \frac{\text{number of major errors}}{\text{total number of susceptible isolates}} \times 100$$

D. Significance of errors

1. Even though tolerance levels have been established for errors, examine all errors closely to identify any trends that may have a significant clinical impact. Some results may be more significant than others, depending on the particular medical community's needs.

2. Minor errors, examples

a. Ceftriaxone versus *Enterobacter* species

(1) Errors

(a) Very major, 0.2%

(b) Major, 0.1%

(c) Minor, 4%

(2) Example of minor error: reference = >32 μg/ml (resistant) and test = 16 μg/ml (intermediate)

(3) Concern

Laboratories servicing physicians involved in at-home intravenous antimicrobial therapy programs require reliable results with this agent. Therefore, if most of the errors are similar to the example, a 4% minor error rate might be unacceptable.

b. Tobramycin versus *P. aeruginosa*

(1) Errors

(a) Very major, 0.3%

(b) Major, 0.2%

(c) Minor, 3%

(2) Example of minor error: reference = 8 μg/ml (intermediate) and test = 2 μg/ml (susceptible)

(3) Other aminoglycoside results

(a) Amikacin = 8 μg/ml (susceptible)

(b) Gentamicin = >16 μg/ml (resistant)

IV. INTERPRETATION OF CORRELATIONS *(continued)*

(4) Concern

A physician prescribing an aminoglycoside would select amikacin on the basis of reference results but tobramycin or amikacin on the basis of test results. If most minor errors are similar to the example, a 3% minor error rate might be unacceptable.

3. Major errors, examples

a. Oxacillin versus *S. aureus*

(1) Errors

(a) Very major, 0.2%

(b) Major, 3%

(2) Example of major error: reference = 2 μg/ml (susceptible) and test = 8 μg/ml (resistant)

(3) Concern

A physician would select oxacillin on the basis of the reference result but probably vancomycin on the basis of the test result. This would result in increased patient care costs due to patient isolation and the expense of the antimicrobial agent. In this case, a 3% major error rate might be unacceptable.

b. Cefazolin versus *Klebsiella pneumoniae*

(1) Errors

(a) Very major, 0.5%

(b) Major, 2%

(2) Example of major error: reference = 4 μg/ml (susceptible) and test = 32 μg/ml (resistant)

(3) Concern

A physician may choose cefazolin on the basis of the reference result but an aminoglycoside or a broad-spectrum cephalosporin on the basis of the test result. This increases patient risk because of the toxic effects of aminoglycosides and also increases the cost of therapy.

4. Very major errors, examples

Very major errors are the most significant type of error. With certain organism-antimicrobial agent combinations, some of these errors have a great impact on patient therapy and prognosis. This is especially true when the antimicrobial agent is the drug of choice and/or the infection is serious (e.g., endocarditis, osteomyelitis, septicemia). Examples of very major errors that have a high likelihood of resulting in clinical failure are as follows.

a. Oxacillin versus *S. aureus*

b. Tobramycin versus *P. aeruginosa*

c. Cefotaxime versus *Enterobacter* spp.

E. Putting performance data in perspective

1. Some antimicrobial agents are not recommended for treatment of infections caused by certain organisms regardless of in vitro test results. Major and very major errors with these combinations may therefore not be as significant.

a. Trimethoprim-sulfamethoxazole versus enterococci

b. Ampicillin versus *P. aeruginosa*

c. Cephalothin versus methicillin-resistant *S. aureus*

2. If most errors involve isolates that are not commonly encountered in your laboratory setting

a. Weigh the inconvenience and cost of using another method for those isolates against the overall advantages of the test system.

b. Utilize selective reporting of antimicrobial agent-organism combinations that have low correlations.

IV. INTERPRETATION OF CORRELATIONS *(continued)*

3. If small numbers of resistant or susceptible isolates are tested, correlations that do not reflect system performance may result. When reviewing percentages of major and very major errors, note the method of calculation. Major and very major errors calculated from the total test set may be misleading as opposed to errors calculated by using the numbers of susceptible and resistant isolates, respectively. The following represents an example.

a. Twenty-five ciprofloxacin-susceptible *E. coli* isolates are tested. No resistant isolates are tested.

b. Category correlation is 100% (0% very major errors, 0% major errors).

c. There are no very major errors, but the possibility for very major errors does not exist since no resistant isolates were included among the 25 test isolates.

V. PROCEDURE NOTES

A. When reviewing journal articles

1. Verify that the same category breakpoints are used for both the test and reference systems.

2. Note the software version (if applicable) used on the test system. Manufacturers continually make data analysis modifications, and the software version used in the published study may not be the most current version available.

B. During the evaluation, determine whether the system fits into the work flow. Changes to the work flow may be necessary if the new system is adopted by the laboratory.

C. Personnel performing testing should become familiar with methods (both reference and test systems) to be used prior to the evaluation in order to limit errors due to technique.

D. If feasible, use as many fresh, clinical, single-patient isolates as possible (up to 300) (Use single-patient isolates to avoid duplicate testing of the same organism.) Fewer isolates may be used, but this may result in less statistically significant correlations, especially when correlations are analyzed by organism group. In addition, two-thirds of the organisms tested for each antimicrobial agent should be representative of the drug's spectrum of activity. If a large-scale comparison to a reference method is not possible, at a minimum consider a comparison to the current testing system. Any differences between the two systems can be evaluated, and potential changes in the annual cumulative susceptibility report may be anticipated.

E. It is important to verify that the system can detect clinically significant resistant isolates. This is especially relevant with rapid systems. If resistant isolates cannot be obtained for the evaluation, available literature should be reviewed to determine the system's capability of detecting resistance. As new resistance patterns emerge, it is important to further verify the system's performance.

F. Isolates with insufficient growth should be retested to determine if the lack of growth is due to technique or to a limitation of the system.

G. Contact the manufacturer for possible explanations of discrepant results. Be aware of factors that affect susceptibility results in both the reference and the test systems. For example, inoculum, time of incubation, pH, temperature, age of culture, etc., can affect results.

H. If comparative data for specific organism-antimicrobial agent combinations fall significantly below acceptable limits, determine whether failure is due to system performance or low sample numbers. If low correlations are attributed to system performance, use an alternate method with proven performance.

I. Do not confirm MIC discrepancies on nonfermenters other than *P. aeruginosa* and *Acinetobacter* spp. by disk diffusion. Use an alternate method (i.e., broth microdilution, broth macrodilution, or agar dilution).

V. PROCEDURE NOTES
(continued)

J. It may be difficult to obtain challenge organisms from within your institution. In that case, contact larger institutions or university hospitals for these isolates. A gram-positive challenge set composed of 100 organisms (catalog no. GP0100) and a gram-negative challenge set composed of 200 organisms (catalog no. GN0250) can be purchased through the CDC.

K. Run QC strains daily for verification of precision and accuracy of the test and the reference procedure. Do not use results from antimicrobial agents that do not meet established QC ranges.

VI. LIMITATIONS OF THE PROCEDURE

A. No system can be better than the system to which it is being compared, since it is assumed that the reference system is correct. Some discrepancies may require additional testing when standard methods are known to have limitations. For example, methicillin-resistant *S. aureus* may occasionally not be detected with disk diffusion and broth dilution. A screen plate containing Mueller-Hinton agar supplemented with 4% NaCl and 6 μg of oxacillin per ml has been suggested as an alternative method for confirming oxacillin resistance in *S. aureus* (2).

B. If a large number of the resistant isolates tested are from your institution, they may be multiple isolates of the same nosocomial strain.

REFERENCES

1. **Food and Drug Administration.** 1991. *Review Criteria for Assessment of Antimicrobial Susceptibility Devices*, revised version. Food and Drug Administration, Washington, D.C. (2000 draft document available for review and comment at http://www.fda.gov/cdrh/ode/631.html.)
2. **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.

SUPPLEMENTAL READING

Ferraro, M. J., and J. H. Jorgensen. 2003. Susceptibility testing instrumentation and computerized expert systems for data analysis and interpretation, p. 208–217. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.

Jorgensen, J. H. 1993. Selection criteria for an antimicrobial susceptibility testing system. *J. Clin. Microbiol.* **31:**2841–2844.

Jorgensen, J. H., and M. J. Ferraro. 1998. Antimicrobial susceptibility testing: general principles and contemporary practices. *Clin. Infect. Dis.* **26:**973–980.

Jorgensen, J. H., and M. J. Ferraro. 2000. Antimicrobial susceptibility testing: special needs for fastidious organisms and difficult-to-detect resistance mechanisms. *Clin. Infect. Dis.* **30:**799–808.

Livermore, D. M., T. G. Winstanley, and K. P. Shannon. 2001. Interpretive reading: recognizing the unusual and inferring resistance mechanisms from resistance phenotypes. *J. Antimicrob. Chemother.* **48**(S1):87–102.

APPENDIX 5.17–1

Susceptibility category errors

Type of error	Category result by method[a]	
	Reference	Test
Very major	R	S
Major	S	R
Minor	I	R
	I	S
	S	I
	R	I

[a] Abbreviations: R, resistant; S, susceptible; I, intermediate.

APPENDIX 5.17–2 Example of a Correlation Table for One Antimicrobial Agent

Cefazolin: XYZ system compared with NCCLS reference microdilution MIC method

Organism(s)	No. of isolates[a]				No. (%) of category errors in XYZ system				No. (%) of essential agreements
	Total	S	I	R	Minor	Major[b]	Very Major[c]	No error	
Citrobacter spp.	28	11	0	17	2 (7.1)	0 (0.0)	0 (0.0)	26 (92.8)	26 (92.9)
Enterobacter spp.	31	0	2	29	2 (6.5)	NA[d]	2 (6.9)	27 (87.1)	28 (90.3)
Escherichia coli	39	17	7	15	3 (7.7)	1 (5.9)	0 (0.0)	35 (97.4)	37 (94.9)
Klebsiella spp.	30	18	3	9	1 (3.3)	1 (5.6)	0 (0.0)	28 (93.3)	27 (90.0)
Proteus mirabilis	31	30	1	0	1 (3.2)	0 (0.0)	NA	30 (96.8)	31 (100.0)
Overall results	159	76	13	70	9 (5.7)	2 (2.6)	2 (2.9)	146 (91.8)	149 (93.7)

[a] As determined by reference method. Abbreviations: S, susceptible; I, intermediate; R, resistant.

[b] Calculations include only isolates susceptible by reference method.

[c] Calculations include only isolates resistant by reference method.

[d] NA, not applicable.

APPENDIX 5.17–3 Example of an Overall Correlation Table

Correlation table for XYZ system compared with NCCLS reference microdilution MIC method

Organism(s)	No. of isolates	% Agreement for[a]:													
		Ampicillin		Cefazolin		Ceftazidime		Ciprofloxacin		Gentamicin		Piperacillin		Overall	
		EA	CA	EA	CA	EA	CA	EA	CA	EA	CA	EA	CA	EA	CA
Citrobacter spp.	28	93	93	93	93	97	94	90	92	94	94	85	83	92	92
Enterobacter spp.	31	84	85	90	87	89	89	95	99	96	96	89	88	91	91
Escherichia coli	39	91	92	95	90	94	93	99	99	95	93	91	91	94	93
Klebsiella spp.	30	89	93	90	93	90	91	94	92	93	93	89	87	91	92
Proteus spp.	42	85	83	98	95	92	93	98	97	91	91	89	89	92	91
Pseudomonas aeruginosa	23					90	89	98	99	94	94	89	87	93	92
Overall	193	88	89	93	92	92	92	96	96	94	94	89	88	92	92

[a] EA, essential agreement; CA, category agreement.

5.18.1 Appendix 5.18.1–1—Sources of Supplies for Antimicrobial Susceptibility Tests

The list below is intended to provide guidance in acquiring materials for performing antimicrobial susceptibility tests as described in section 5. See procedure 3.1 for a listing of companies that supply media, reagents, QC strains, and other products necessary to perform the procedures.

Antimicrobial powders

Sigma Diagnostics
545 South Ewing Ave.
St. Louis, MO 63103
(314) 286-7880
http://www.sigma-aldrich.co

U.S. Pharmacopeia
12601 Twinbrook Parkway
Rockville, MD 20852
(800) 227-8772
http://www.usp.org

In addition, some pharmaceutical companies (generally in the medical department) provide reference-grade powders for antimicrobial agents that they manufacture. To determine the manufacturer, contact your pharmacy.

Antimicrobial disks

BD Diagnostic Systems
7 Loveton Circle
Sparks, MD 21152
(800) 638-8663
http://www.bd.com

Remel, Inc.
12076 Santa Fe Dr.
Lenexa, KS 66215
(800) 255-6730
http://www.remelinc.com

Hardy Diagnostics
1430 W. McCoy Ln.
Santa Maria, CA 93455
(800) 266-2222
http://www.hardydiagnostics.com

Microdilution dispensing system

Sandy Spring Technologies (Autospense IIe dispensing system)
21131 Georgia Ave.
Brookeville, MD 20833
(301) 260-1220

Microdilution testing supplies (disposable plastic trays)

Dynex
14340 Sullyfield Circle
Chantilly, VA 20151
(800) 336-4543
http://www.dynextechnologies.com

Evergreen Scientific
2300 East 49th St.
P.O. Box 58248
Los Angeles, CA 90058
(800) 421-6261
http://www.evergreensci.com

Nunc
75 Panorama Creek Dr.
Rochester, NY 14625-2385
(585) 586-8800
http://www.nuncbrand.com

Petri plates, special (quadrant plates and square plates)
BD Falcon
1 Becton Dr.
Franklin Lakes, NJ 07417
(201) 847-6800
http://www.bd.com

Suppliers of antimicrobial susceptibility test systems (if used for diagnostic testing, make certain the product is Food and Drug Administration cleared)

AB Biodisk North America, Inc. (Etest)
200 Centennial Ave.
Piscataway, NJ 08854
(732) 457-0408
http://www.abbiodisk.com

BD Diagnostic Systems (Pasco, Phoenix)
7 Loveton Circle
Sparks, MD 21152
(800) 638-8663
http://www.bd.com

bioMérieux, Inc. (Vitek and Vitek 2)
100 Rodolphe St.
Durham, NC 27717
(800) 682-2666
http://www.biomerieux-vitek.com

Dade Behring (MicroScan)
MicroScan Microbiology Systems
1584 Enterprise Blvd.
West Sacramento, CA 95691
(800) 242-3233
http://www.dadebehring.com

PML Microbiologicals, Inc.
27120 S. W. 95th Ave.
Wilsonville, OR 97071
(800) 547-0659
http://www.pmlmicro.com

Remel
12076 Santa Fe Dr.
Lenexa, KS 66215
(800) 255-6730
http://www.remelinc.com

Trek Diagnostic Systems (Sensititre)
982 Keynote Circle, Suite 6
Cleveland, OH 44131
(800) 871-8909
http://www.trekds.com

5.18.2

Appendix 5.18.2–1—General References for Antimicrobial Susceptibility Testing

Bryskier, A. *Antibiotics, Antibacterial Agents, and Antifungals*, 2nd ed., in press. ASM Press, Washington, D.C.

Forbes, B. A., D. F. Sahm, and A. S. Weissfeld. 2002. *Bailey & Scott's Diagnostic Microbiology*, 11th ed., p. 214–258. Mosby, St. Louis, Mo.

Gilbert, D. N., R. C. Moellering, and M. A. Sande (ed.). 2003. *The Sanford Guide to Antimicrobial Therapy, 2003*, 33rd ed. Antimicrobial Therapy, Inc., Hyde Park, Vt.

Koneman, E. W., S. D. Allen, W. M. Janda, P. C. Schreckenberger, and W. C. Winn, Jr. (ed.). 1997. *Color Atlas and Textbook of Diagnostic Microbiology*, p. 785–856. Lippincott, Philadelphia, Pa.

Lorian, V. (ed.). 1996. *Antibiotics in Laboratory Medicine*, 4th ed. The Williams & Wilkins Co., Baltimore, Md.

Mandell, G. L., J. E. Bennett, and R. Dolin. 2000. *Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases*, 5th ed. Churchill Livingstone, Philadelphia, Pa.

Murray, P. R., E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.). 2003. *Manual of Clinical Microbiology*, 8th ed., p. 1037–1212. ASM Press, Washington, D.C.

NCCLS. 2003. *Performance Standards for Antimicrobial Disk Susceptibility Tests*, 8th ed. Approved standard M2-A8. NCCLS, Wayne, Pa.

NCCLS. 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 6th ed. Approved standard M7-A6. NCCLS, Wayne, Pa.

NCCLS. 2002. *Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data.* Approved guideline M39-A. NCCLS, Wayne, Pa.

NCCLS. 2001. *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria*, 5th ed. Approved standard M11-A5. NCCLS, Wayne, Pa.

Truant, A. L. (ed.). 2002. *Manual of Commercial Methods in Clinical Microbiology*. ASM Press, Washington, D.C.

SECTION 6 Aerobic Actinomycetes

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6.1 Specimen Examination and Primary Isolation

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Aerobic actinomycetes cause a variety of infections in humans: respiratory, cutaneous (such as mycetoma, lymphocutaneous and superficial skin [abscess or cellulitis] infections, or secondary cutaneous involvement with disseminated disease), and disseminated, with a marked tropism to the central nervous system, from a primary pulmonary infection. See Appendix 6.3.1–1 at the end of this section for a list of etiologic agents and the sites most commonly associated with these infections.

Aerobic actinomycetes are often overlooked or missed unless there is a suspicion of these infections. Knowing which aerobic actinomycete has been isolated assists the physician in diagnosing the disease, in determining appropriate therapy for patients with these infections, and in determining the clinical significance.

This procedure outlines practical procedures that can be used to establish the presence of clinically important aerobic actinomycetes. These include members of several genera: *Actinomadura, Dermatophilus, Gordonia, Nocardia, Nocardiopsis, Rhodococcus, Streptomyces, Tsukamurella,* and, to a lesser extent, *Amycolata, Amycolatopsis, Micromonospora, Saccharopolyspora, Saccharomonospora,* and *Thermoactinomyces.* The genera *Gordonia, Nocardia, Rhodococcus,* and *Tsukamurella,* and the microbiologically closely related genera *Mycobacterium* and *Corynebacterium,* are collectively known as the mycolic acid-containing genera, or "mycolata," and they have a set of chemical markers in common, i.e., the cell wall components *meso*-diaminopimelic acid (DAP), arabinose and galactose, mycolic acids, and cellular fatty acid. This combination of chemical markers is diagnostic for all members of the mycolic acid-containing genera. The major characteristics used for the identification of the mycolata and the amycolata, the non-mycolic acid-containing genera, to the genus level and their separation from *Mycobacterium* and *Corynebacterium* are given in Table 6.1–1 and Fig. 6.2–1.

II. LEVELS OF LABORATORY SERVICE FOR AEROBIC ACTINOMYCETES

The concept of levels of laboratory service is based on the infrequency of isolation of the aerobic actinomycetes and the lack of capabilities in small and medium-size hospitals (Table 6.1–2). Laboratory service is classified into two levels (I and II).

III. MATERIALS

A. Stain reagents

See individual stain procedures below.

B. Media

See Appendix 6.3.2–1 at the end of this section for suppliers.

1. Enriched medium at 35°C
   - **a.** Heart infusion agar with rabbit blood (BAP)
   - **b.** Lowenstein-Jensen slant
2. Minimal medium at 25°C
   Sabouraud dextrose agar (SDA)
3. Liquid medium at 35°C
   - **a.** Thioglycolate (incubated under microaerobic and anaerobic conditions)
   - **b.** Tryptic soy broth (TSB)
   - **c.** Radiometric (BACTEC 12B) blood culture system
4. Selective enrichment broth at 35°C
   - **a.** TSB with and without inhibitor 5% NaCl
   - **b.** TSB without dextrose with and without pretreatment (HCl-KCl [pH 2.2 ± 0.1])

Table 6.1–1 Results of tests used for presumptive identification of aerobic actinomycetes to genus level[a,b]

Genus	Test results										
	Vegetative filaments		Conidia	Acid-fast nature	Presence[c] in whole cells of:		Mycolic acids	Metabolism of glucose	Growth at 50°C	Arylsulfatase	Growth in lysozyme
	Substrate	Aerial			DAP	Sugars					
Actinomadura	+	V	V	−	*meso*	Mad	−	O	−	−	−
Amycolata	+	+	V	−	*meso*	Arab, gal	−	O	−	−	−
Amycolatopsis	+	+	V	−	*meso*	Arab, gal	−	O	−	−	−
Corynebacterium	+	−	−	−	*meso*	Arab, gal	+	O/F	−	−	−
Dermatophilus[d]	+	−	−	−	*meso*	Mad	−	F	−	−	NT
Gordonia	+	−	−	W	*meso*	Arab, gal	+	O	−	−	V
Micromonospora	+	−	+	−	*meso*	Arab, xyl	−	O	−	−	−
Mycobacterium	+	−	−	+	*meso*	Arab, gal	+	O	−	+	−
Nocardia	+	+	V	W	*meso*	Arab, gal	+	O	−	−	+
Nocardiopsis	+	+	+	−	*meso*	None	−	O	−	−	−
Rhodococcus	+	−	−	W	*meso*	Arab, gal	+	O	−	−	V
Saccharomonospora	+	+	+	−	*meso*	Arab, gal	−	O	+	−	−
Saccharopolyspora	+	+	+	−	*meso*	Arab, gal	−	O	+	−	V
Streptomyces	+	+	+	−	L	None	−	O	−	−	−
Thermoactinomyces	+	+	+	−	*meso*	Arab, gal	−	O	+	−	+
Tsukamurella	+	−	−	W	*meso*	Arab, gal	+	O	−	−	+

[a] Data adapted from McNeil and Brown (4).

[b] +, 90% or more of strains are positive; −, 90% or more of strains are negative; V, 11 to 89% of strains are positive; O, oxidative; F, fermentative; NT, not tested; DAP, diaminopimelic acid; Mad, madurose; Arab, arabinose; gal, galactose; xyl, xylose; W, weakly or partially acid-fast.

[c] As determined by the methodology of Becker et al. (1) for whole-cell DAP and sugars.

[d] Genus is motile.

III. MATERIALS *(continued)*

5. Selective enrichment agar at 35°C
- **a.** Buffered charcoal yeast extract (BCYE)
  - (1) BCYE with anisomycin (40 U/ml), polymyxin B (80 µg/ml), and vancomycin (0.5 µg/ml)
  - (2) BCYE without antibiotics
- **b.** Modified Thayer-Martin agar with colistin (7.5 µg/ml), nystatin (12,500 µg/ml), and vancomycin (4 µg/ml)

C. Other supplies
Petri dishes, pipettes, loops, inoculating needles, slides, coverslips, scissors, mortar and pestle, filtration equipment with 0.45-µm-pore-size disposable filters, lysis-centrifugation system, centrifuge and centrifuge tubes, microscope, methanol, sulfuric acid, and potassium hydroxide solution.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. All media and reagents should have recorded arrival date, date put into use, and expiration date, the last appearing on the label.

B. Reagents should be examined regularly for signs of contamination or extraneous material.

C. Organisms with known staining reaction
- **1.** For Gram stain, use QC smears as given in section 3 of this handbook.
- **2.** For acid-fast stain, use a smear containing an acid-fast-positive microorganism (*Mycobacterium fortuitum* ATCC 6841), which stains red (retains the carbol fuchsin), and an acid-fast-negative microorganism (*Staphylococcus aureus* ATCC 25923), which stains blue.

V. SPECIMEN EXAMINATION

It is imperative that these cultures be handled in a biological safety cabinet.

Observe standard precautions.

A. Principle

In addition to the clinical picture, a definitive diagnosis depends upon demonstration of the organism in smears or sections from the original clinical material, along with the isolation and identification of the agent.

B. Preparation of specimen

1. Sputum, bronchial washings, and exudates (nonsterile sites)
   - **a.** Material should be spread out in a petri dish.
   - **b.** Selectively remove clumps or granules and crush between two glass slides in preparation for KOH mount.
2. Body fluids (e.g., blood or CSF)
   - **a.** Centrifuge at 3,000 $\times$ *g* for 20 min.
   - **b.** Make smears with a thin suspension of sediment on clean glass slide.
   - **c.** Heat fix gently.
3. **Tissue**
   - **a.** Cut small portion of tissue with sterile scissors and grind with mortar and pestle or tissue homogenizer.
   - **b.** Add small amount of sterile water or broth, and make smears with a small portion of the homogenate on clean glass slide.
   - **c.** Heat fix gently.

Observe standard precautions.

C. Direct macroscopic examination of specimen

Observe for clumps of microorganisms or granules with a stereomicroscope (magnification, $\times$100).

Observe standard precautions.

D. Microscopic examination of culture material

1. Gram stain (*see* Gram stain procedure in section 3 of this handbook)
2. Acid-fast stain for aerobic actinomycetes (modified Kinyoun method) (2)
   Modified Kinyoun acid-fast stain is a cold method of staining for acid fastness. Acid-fast bacilli are difficult to stain because of the long-chain fatty acids (mycolic acids) in the cell wall. The exact nature of this unique staining reaction is not completely understood, but it is believed that the phenol dissolves the lipid sufficiently to allow penetration of the primary stain. The cell wall retains the primary stain even after exposure to the decolorizing agent, aqueous 1% sulfuric acid. Resistance to 1% sulfuric acid is required for the organism to be termed acid-fast. A counterstain is employed to highlight the stained organisms. This method of staining for acid fastness is preferred because it is more sensitive in the staining of *Gordonia* species, *Nocardia* species, *Rhodococcus* species, and *Tsukamurella* species that are usually negative with the classical Ziehl-Neelsen stain.
   - **a.** Materials and preparation method are given in Appendix 6.3.3–1 item II at the end of this section.

Table 6.1–2 Laboratory level I and II qualifying procedures

Procedure[a]	Level	
	I	II
Microscopic examination of clinical material		
Gram stain	Yes	Yes
Acid-fast stain (modified Kinyoun)	Yes	Yes
Isolation of aerobic actinomycetes	Yes	Yes
Identification of aerobic actinomycetes	No	Yes
Antimicrobial susceptibility tests	No	Yes

[a] Procedures not performed by level I laboratories are referred to a level II laboratory or a recognized reference facility.

V. SPECIMEN EXAMINATION *(continued)*

b. Procedure
(1) Prepare and heat fix the smear on a clean glass slide.
(2) Flood the smear with ~5 drops of Kinyoun fuchsin-phenol and allow to stain for 5 min. *Do not heat.*
(3) Rinse with tap water and drain.
(4) Flood the smear with aqueous 1% H_2SO_4 and destain for 3 min. Then, rinse with tap water and drain.
(5) Flood the smear with methylene blue and then rinse, drain, and air dry.
(6) Examine the smear under oil immersion at ×1,000 magnification.

3. KOH mount
a. KOH reagent (10%) allows the rapid observation of sulfur granules present in tissue by partially digesting the proteinaceous host cell components. *Actinomadura* species, *Nocardia* species, and *Streptomyces* species, when seen as etiologic agents of actinomycotic mycetoma, grow in the tissue as small, hard colonies known as granules.
b. Procedure
(1) To an aliquot of specimen, add a drop of 10% KOH and place a coverslip over the preparation.
(2) Hold the slide at room temperature for 5 to 30 min after the addition of 10% KOH to allow digestion to occur. If necessary, heat gently to enhance digestion.
(3) Observe at ×1,000 magnification.

E. Histopathologic stains for microscopic examination of tissue
Histopathologic stains are useful in demonstrating host response and the microscopic structures of etiologic agents in tissue.

1. Hematoxylin and eosin (H&E)
H&E is the best stain for demonstrating host reaction in tissue and for studying the microscopic features of granules.
a. Gram-positive filaments are not seen.
b. Granules stain blue with hematoxylin, and the surface shows clavate eosinophilia.
c. Shows an infiltration of PMNs surrounding the granule but does not penetrate the interior.

2. Modified Gram stain (Brown-Brenn)
a. Demonstrates the presence of gram-positive branched filaments (≤1.0 μm wide).
b. Demonstrates the gram positivity of filaments of the agents of nocardiosis and actinomycotic mycetoma.

3. Gomori methenamine-silver (GMS) stain
a. Stains aerobic actinomycetes and fungi.
(1) Filaments of aerobic actinomycetes are ≤1.0 μm wide.
(2) Filaments of fungi range from 2 to 6 μm wide.

VI. ISOLATION OF AEROBIC ACTINOMYCETES OF MEDICAL IMPORTANCE

Observe standard precautions.

◩ **NOTE:** Fig. 6.1–1 shows an abbreviated flowchart for the isolation of aerobic actinomycetes.

The selection of media for the isolation of the aerobic actinomycetes depends primarily on the specimen type. Media should support the aerobic actinomycetes while inhibiting the growth of contaminating organisms. Specimens from normally sterile sites may be inoculated into less inhibitory media; specimens from nonsterile sites, such as respiratory specimens, must be inoculated onto selective media that are inhibitory to other bacteria present.

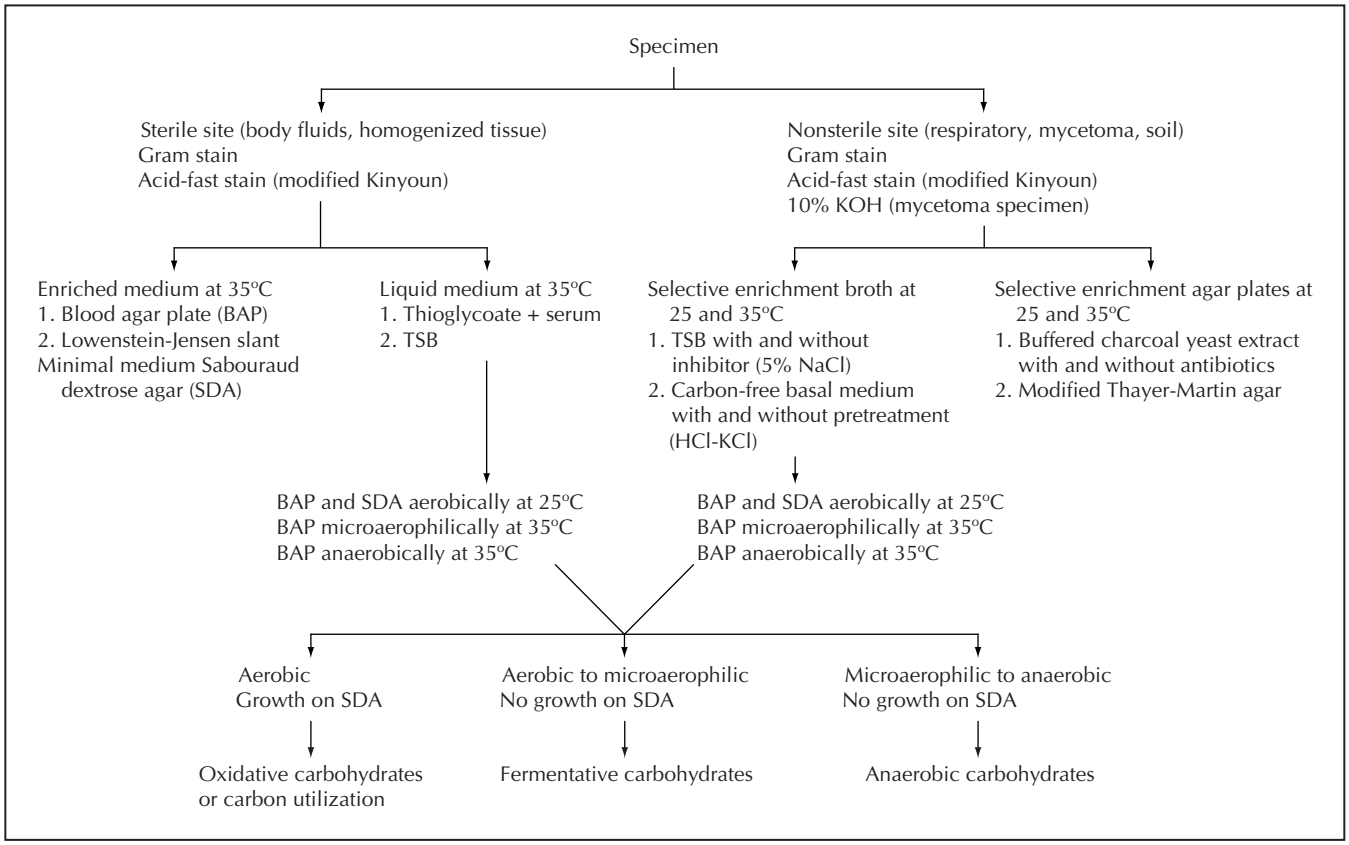
Specimen
Sterile site (body fluids, homogenized tissue)
Gram stain
Acid-fast stain (modified Kinyoun)
Nonsterile site (respiratory, mycetoma, soil)
Gram stain
Acid-fast stain (modified Kinyoun)
10% KOH (mycetoma specimen)
Enriched medium at 35ºC
1. Blood agar plate (BAP)
2. Lowenstein-Jensen slant
Minimal medium Sabouraud dextrose agar (SDA)
Liquid medium at 35ºC
1. Thioglycoate + serum
2. TSB
Selective enrichment broth at 25 and 35ºC
1. TSB with and without inhibitor (5% NaCl)
2. Carbon-free basal medium with and without pretreatment (HCl-KCl)
Selective enrichment agar plates at 25 and 35ºC
1. Buffered charcoal yeast extract with and without antibiotics
2. Modified Thayer-Martin agar
BAP and SDA aerobically at 25ºC
BAP microaerophilically at 35ºC
BAP anaerobically at 35ºC
BAP and SDA aerobically at 25ºC
BAP microaerophilically at 35ºC
BAP anaerobically at 35ºC
Aerobic
Growth on SDA
Aerobic to microaerophilic
No growth on SDA
Microaerophilic to anaerobic
No growth on SDA
Oxidative carbohydrates or carbon utilization
Fermentative carbohydrates
Anaerobic carbohydrates

Figure 6.1–1 Abbreviated flowchart for isolation of aerobic actinomycetes.

VI. ISOLATION OF AEROBIC ACTINOMYCETES OF MEDICAL IMPORTANCE *(continued)*

A. Preparation of inoculum

1. Sterile sites

a. Blood specimens concentrated by lysis-centrifugation system (Isolator) (*see* Appendix 6.3.2–1)

b. Body fluids (CSF, joint fluid, and urine specimens processed by filtration) (3)

(1) Filter using a disposable plastic system with a 0.45-µm-pore-size membrane.

(2) Use sterile forceps to remove membrane filter from apparatus and to place it onto an agar plate.

2. Nonsterile site (respiratory and mycetoma)

Pretreat with 1 ml of a low-pH (2.2) HCl-KCl solution (46.8 ml of 0.2 M HCl and 300 ml of 0.2 M KCl) per 10 ml of sample for 4 min prior to inoculation (5).

B. Isolation

1. Sterile sites (body fluids)

a. Blood specimen—incubate up to 3 weeks aerobically and microaerobically at 35°C.

(1) Use automated radiometric (BACTEC) or nonradiometric culture method.

(2) Inoculate 10 to 50 µl of specimen directly into THIO (supports aerobic growth, as well as growth of anaerobic actinomycetes) and TSB.

VI. ISOLATION OF AEROBIC ACTINOMYCETES OF MEDICAL IMPORTANCE *(continued)*

b. Body fluids (CSF and urine) and homogenized tissue
(1) Inoculate into liquid broth at 35°C.
(2) CSF must be incubated for 2 weeks with frequent subcultures.
(3) Tissue specimens must be incubated for 3 weeks with frequent and terminal subcultures.

◩ **NOTE:** Sediment from lysis-centrifugation of blood, other body fluids, and homogenized tissue may be inoculated initially onto solid media; however, plates must be sealed with parafilm (Parafilm M; American Can Company, Greenwich, CT) to prevent dehydration during prolonged incubation.

2. Nonsterile sites
a. Respiratory-sample problems
It has been demonstrated that digestion-decontamination of respiratory-tract specimens with *N*-acetyl-L-cysteine, sodium hydroxide, and benzalkonium used for mycobacterial isolation is toxic to *Nocardia* species, and it has been shown that antibiotics in fungal culture media inhibit *Nocardia* species.
b. Respiratory, mycetoma, and soil samples
(1) Selective enrichment broth at 25 and 35°C
(a) TSB with and without inhibitor 5% NaCl
(b) Carbon-free basal medium with and without inhibitor
(2) Selective enrichment agar at 25 and 35°C
(a) BCYE with and without antibiotics
(b) Modified Thayer-Martin agar with and without antibiotics

3. After growth, all isolates must be subcultured to determine temperature, oxygen, and nutritional requirements.
a. Enriched medium (BAP) (aerobically and anaerobically at 25 and 35°C)
b. Minimal medium (SDA) at 25°C

POSTANALYTICAL CONSIDERATIONS

VII. INTERPRETATION OF TESTS

A. Gram stain

1. Interpretation of controls
a. Gram-positive organisms appear blue.
b. Gram-negative organisms appear red.

2. Interpretation of structure and Gram reaction of aerobic actinomycetes
a. *Actinomadura* species are gram-positive, short bacilli (1 to 2 μm wide) that are rarely branched.
b. *Gordonia* species are gram-positive, thin, beaded coccobacilli (≤1 μm wide)
c. *Nocardia* species are gram-positive, branched filaments (≤1 μm wide) that may stain gram variable and usually fragment into bacillary forms.
d. *Rhodococcus* species may have many growth stages: some have a simple rod-coccus growth cycle similar to that of *R. equi;* others have a more complex hypha-rod-coccus cycle (*R. coprophilus, R. fascians, R. marinonascens,* and *R. ruber*). *R. coprophilus, R. erythropolis, R. globerulus, R. rhodnii,* and *R. rhodochrous* are differentiated further into elementary branched hypha-rod-coccus cyclic forms. The branched hyphae (≤1 μm wide) may be rudimentary, highly transitory, or extensive.
e. *Streptomyces* species are gram-variable branched filaments (1 to 2 μm wide). May produce chains of spores.
f. *Tsukamurella* species are gram-positive short-to-long bacilli. No branched forms are seen unless on minimal medium, such as tap water.

VII. INTERPRETATION OF TESTS *(continued)*

B. Modified Kinyoun stain

1. Most *Nocardia* species are weakly or partially acid-fast (varies from 10 to 20% per oil immersion field).

2. *Streptomyces* species are usually negative. Occasionally the spores of some *Streptomyces* species are acid-fast.

3. *Tsukamurella* species vary from 20 to 100% per oil immersion field.

4. Both *Gordonia* and *Rhodococcus* species vary from no acid fastness to 10 to 20% per oil immersion field.

5. All *Mycobacterium* species tested in our laboratory have been 100% acid-fast by the modified Kinyoun method.

C. KOH preparation

1. Interpretation of results

a. Actinomycotic granule has beaded branched filaments (1.5 to 2.0 μm wide).

b. Eumycotic granule has hyphae from 4 to 7 μm wide (may be branched).

c. Granules seen in botryomycosis may have filaments (often in association with cocci).

2. Limitations

Observation of granules must be followed by isolation and identification of etiologic agent.

D. Interpretation of growth on different media and under different incubation conditions

1. Aerobic actinomycete

a. Grows aerobically.

b. Grows at 25 or 35°C.

c. Grows on SDA.

2. Fastidious aerobic actinomycete

a. Grows aerobically to microaerobically.

b. Grows at 35°C only.

c. Does not grow on SDA.

3. Anaerobic actinomycete

a. Grows microaerobically to anaerobically.

b. Grows at 35°C only.

c. Does not grow on SDA.

REFERENCES

1. **Becker, B., M. P. Lechevalier, R. E. Gordon, and H. A. Lechevalier.** 1964. Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. *Appl. Microbiol.* **12:**421–423.
2. **Chapin, K. C., and T.-L. Lauderdale.** 2007. Reagents, stains, and media: bacteriology, p. 334–364. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
3. **Haley, L. D., and C. S. Callaway.** 1978. *Laboratory Methods in Medical Mycology.* CDC publication no. 78-8361. U.S. Government Printing Office, Washington, DC.
4. **McNeil, M. M., and J. M. Brown.** 1994. The medically important aerobic actinomycetes: epidemiology and microbiology. *Clin. Microbiol. Rev.* **7:**357–417.
5. **Vickers, R. M., J. D. Rihs, and V. L. Yu.** 1992. Clinical demonstration of isolation of *Nocardia asteroides* on buffered charcoal-yeast extract media. *J. Clin. Microbiol.* **30:**227–228.

SUPPLEMENTAL READING

Bartholomew, J. W. 1962. Variables influencing results, and the precise definition of steps in Gram staining as a means of standardizing the results obtained. *Stain Technol.* **37:**139–155.

Chandler, F. W., W. Kaplan, and L. Ajello. 1980. A color atlas and textbook of histopathology of mycotic diseases. Wolfe Medical Publications, London, United Kingdom.

Conville, P. S., and F. G. Witebsky. 2007. *Nocardia, Rhodococcus, Gordonia, Streptomyces,* and other aerobic actinomycetes, p. 515–542. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.

Garratt, M. A., H. T. Holmes, and F. S. Nolte. 1992. Selective buffered charcoal-yeast-extract medium for isolation of nocardiae from mixed cultures. *J. Clin. Microbiol.* **30:**1891–1892.

Murray, P. R., R. L. Heeren, and A. C. Niles. 1987. Effect of decontamination procedures on recovery of *Nocardia* spp. *J. Clin. Microbiol.* **25:**2010–2011.

Murray, P. R., A. C. Niles, and R. L. Heeren. 1988. Modified Thayer-Martin medium for recovery of *Nocardia* species from contaminated specimens. *J. Clin. Microbiol.* **26:**1219–1220.

Roberts, G. D., N. S. Brewer, and P. E. Hermans. 1974. Diagnosis of nocardiosis by blood cultures. *Mayo Clin. Proc.* **49:**293–296.

6.2 Media and Methods Used for the Identification of Aerobic Actinomycetes

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

After initial isolation, subcultures must be incubated at 25, 35, and 45°C to determine the optimal temperature for growth of the microorganisms. Most isolates of *Nocardia asteroides* grow best at 35°C, while most isolates of *Streptomyces* grow best at 25°C. Any specimen suspected of containing a thermophilic actinomycete must also be incubated at 50°C, since all thermophiles grow at this elevated temperature. The actinomycetes can be identified to the genus level on the basis of a variety of conventional phenotypic characteristics (microscopic [Gram and acid-fast staining] and macroscopic morphologies, growth requirements, metabolism of glucose, arylsulfatase production, and growth in lysozyme) and chemotaxonomic characteristics (whole-cell isomers of diaminopimelic acid and mycolic acid composition) (Table 6.1–1, above). Some of these tests are beyond the capabilities of most clinical laboratories. However, both microscopic and macroscopic morphologies combined with a few physiologic tests can give a presumptive identification to the genus or species level. A schematic flowchart for the tentative identification of medically important aerobic actinomycetes and related genera is given in Fig. 6.2–1.

II. MATERIALS

Include QC information on reagent container and in QC records.

A. Refer to each procedure for the media to be used, and refer to the appendixes at the end of this section for composition and preparation and for commercial sources.
B. Acetamide hydrolysis medium (*see* Appendix 6.3.3–1 item III)
C. Arylsulfatase liquid medium (*see* Appendix 6.3.3–1 item IV)
D. Carbohydrate basal medium—Gordon's oxidative agar slants (*see* Appendix 6.3.3–1 item V)
E. Carbon utilization of carbohydrates—these tests use commercially available kits, which are listed in item IV.D below.
F. Cell wall determination of diaminopimelic acid isomers—isolates are grown in Trypticase (or tryptic) soy broth (*see* Appendix 6.3.2–1). This procedure is described in Appendix 6.3.4–1.
G. Citrate agar (*see* Appendix 6.3.3–1 item VI)
H. Decomposition of substrates (*see* Appendix 6.3.3–1 item VII)
I. Esculin hydrolysis medium (*see* Appendix 6.3.3–1 item VIII)
J. Lysozyme test (*see* Appendix 6.3.3–1 item IX)
K. Nitrate medium (*see* Appendix 6.3.3–1 item X)
L. Urea agar slant (*see* Appendix 6.3.3–1 item XI)

ANALYTICAL CONSIDERATIONS

III. QUALITY CONTROL

A. Controls for acetamide hydrolysis

1. A tube of uninoculated substrate medium (sterility)
2. A tube of substrate medium inoculated with *Nocardia farcinica* (ATCC 3318) (positive)

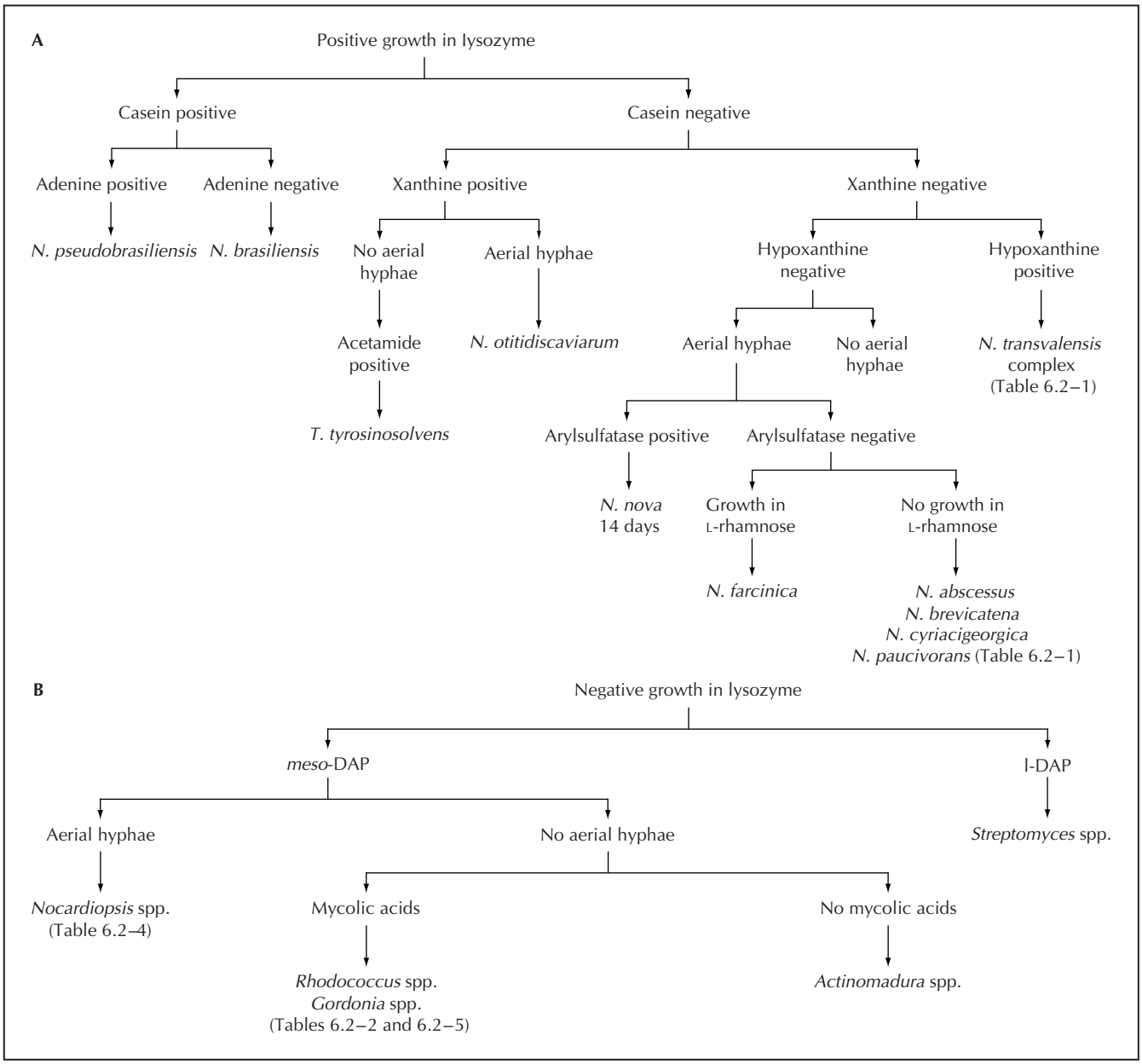

Figure 6.2–1 Schematic flowchart for tentative identification of medically important aerobic actinomycetes and related genera based on positive growth (A) or negative growth (B) in lysozyme.

III. QUALITY CONTROL *(continued)*

3. A tube of substrate medium inoculated with *Nocardia nova* (ATCC 33726) (negative)

B. Controls for arylsulfatase liquid medium

1. A tube of uninoculated substrate medium (negative)
2. A tube of each substrate inoculated with *Mycobacterium fortuitum* (positive; 3 days) and *N. nova* (positive; 2 weeks)

C. Controls for carbohydrate basal medium—Gordon's slants

1. *N. nova* (ATCC 33726) produces oxidative acid from all carbohydrates except dulcitol, i-erythritol, and i-*myo*-inositol.

III. QUALITY CONTROL
(continued)

2. *N. farcinica* (ATCC 3318) produces oxidative acid from i-erythritol.
3. *Nocardia carnea* (ATCC 6847) produces oxidative acid from i-*myo*-inositol.
4. *Tsukamurella inchonensis* (ATCC 700082) produces oxidative acid from dulcitol.

D. Controls for citrate agar
Nocardia brasiliensis (ATCC 19296) (positive) and *N. nova* (ATCC 33726) (negative)

E. Controls for decomposition of substrates
1. *N. farcinica* ATCC 3318 does not hydrolyze adenine, casein, hypoxanthine, tyrosine, or xanthine-negative control.
2. *Nocardia pseudobrasiliensis* (ATCC 51512) hydrolyzes adenine.
3. *Nocardiopsis dassonvillei* (ATCC 23218) hydrolyzes casein, hypoxanthine, tyrosine, or xanthine-positive control.

F. Controls for esculin hydrolysis medium
N. farcinica ATCC 3318 (positive) and *N. nova* ATCC 33726 (negative)

G. Controls for lysozyme test
N. farcinica ATCC 3818 (positive) and *Streptomyces* species (negative)

H. Controls for nitrate reductase
N. abscessus ATCC BAA-279^T (positive) and *N. africana* ATCC BAA-280^T (negative)

I. Controls for urease
N. abscessus ATCC BAA-279^T (positive) and *N. africana* ATCC BAA-280^T (negative)

IV. METHODS OF IDENTIFICATION OF ACTINOMYCETES

It is imperative that these cultures be handled in a biological safety cabinet.

A. Acetamide hydrolysis medium
1. Acylamidase is an enzyme that is capable of deamidating acetamide. This produces ammonia, which increases the pH of the medium, causing a corresponding change from yellow-orange to deep pink or magenta.
2. Procedure
   a. Inoculate the acetamide agar slant with a loopful of culture emulsified in a broth or ~100 µl per slant from a culture grown in a broth.
   b. Incubate inoculated slant for 21 days at 35°C before discarding as negative.
   c. Observe weekly for a color change to deep pink or magenta.

B. Arylsulfatase liquid medium
1. Arylsulfatase is an enzyme capable of hydrolyzing the bond between sulfate and the aromatic ring in a compound such as potassium phenolphthalein disulfate. Free phenolphthalein can then be recognized by the formation of a deep pink color when an alkali is added. This medium is used in the differential identification of rapidly growing mycobacteria and of *N. asteroides* complex and *N. nova.*
2. Materials
   Arylsulfatase broth and the reagent 1.2 N sodium carbonate (10.6 of anhydrous Na_2CO_3 in 100 ml of H_2O)
3. Procedure
   a. Label tubes of substrate with specimen numbers.
   b. Inoculate the tubes with 0.1 ml of a 1-day liquid culture of the organism to be tested.
   c. Incubate at 35°C.
   d. After 3 days of incubation of the 0.001 M substrate, remove test tubes from the incubator and add not more than 6 drops of 2 N sodium carbonate solution.
   e. If negative at 3 days, test again in 2 weeks.

IV. METHODS OF IDENTIFICATION OF ACTINOMYCETES *(continued)*

C. Carbohydrate basal medium—Gordon's oxidative agar slants

1. Oxidative acid production of carbohydrates is used as a differential characteristic to separate all members of the aerobic actinomycetes.

2. Materials

Slants containing basal medium and one of the following: D-glucose, D-mannose, D-mannitol, D-sorbitol, salicin, dulcitol, glycerol, raffinose, i-*myo*-inositol, adonitol, trehalose, i-erythritol, D-galactose, melibiose, lactose, starch, sucrose, D-fructose, maltose, cellobiose, xylose, L-arabinose, L-rhamnose

3. Procedure

a. Inoculate ~10 μl of growth from a broth medium and incubate for 21 days at optimal growth temperature.

b. Read change in color from purple to yellow as acid production.

c. Read no change in color as negative for acid production.

D. Carbon utilization of carbohydrates

1. Carbon utilization of carbohydrates is used as a differential characteristic to separate all members of the aerobic actinomycetes. Growth of an organism occurs in each of the different carbohydrates in which the organism is able to utilize the carbohydrate as a sole carbon source.

2. Commercial tests that have carbon utilization of carbohydrates included in their panels (*see* Appendix 6.3.2–1 at the end of this section)

a. API 20C AUX system

b. API 50 CH system

c. Biotype-100

E. Citrate agar (Simmons)

1. Citrate agar is a medium consisting of inorganic salts in which ammonium salts are the only source of nitrogen and citrate is the carbon source. Organisms capable of utilizing ammonium dihydrogen phosphate and sodium citrate as the sole source of nitrogen and carbon, respectively, grow on this medium and produce an alkaline reaction, as evidenced by a change in the color of bromthymol blue indicator from green (neutral) to blue (alkaline).

2. Procedure

Inoculate the agar slant lightly with a loop by using growth from an 18- to 24-h heart infusion agar slant, and incubate at 35°C for up to 7 days.

F. Decomposition of substrates

1. The ability or inability of the aerobic actinomycetes to hydrolyze adenine, casein, hypoxanthine, tyrosine, and xanthine continues to be a major and generally accepted criterion for the tentative identification of *N. brasiliensis, N. pseudobrasiliensis, Nocardia otitidiscaviarum, Nocardia transvalensis, Actinomadura* species, *Nocardiopsis* species, *Streptomyces* species, and *Tsukamurella* species.

2. Materials

Casein medium and basal medium with adenine, hypoxanthine, tyrosine, and xanthine

3. Procedure

Inoculate the unknown culture onto the surfaces of the casein, xanthine, hypoxanthine, and adenine decomposition test media using a quadrant of a plate for each culture. Incubate at optimal temperature (25 or 35°C). Observe xanthine, hypoxanthine, adenine, and tyrosine plates for clearing of the crystals at 7, 14, and 21 days.

G. Esculin hydrolysis medium

1. Esculin hydrolysis medium is used to determine whether an organism will hydrolyze the glycoside esculin, which produces esculetin and dextrose. The

IV. METHODS OF IDENTIFICATION OF ACTINOMYCETES *(continued)*

Include QC information on reagent container and in QC records.

esculetin reacts with the iron salt (ferric ammonium citrate) contained in the medium to produce a dark-brown-to-black complex.

2. Procedure

a. Inoculate esculin agar slant with a loopful of culture emulsified in a broth or ~10 μl per slant from a culture grown in a broth.

b. Incubate inoculated slant for 14 days at 35°C before discarding as negative.

H. Lysozyme test

1. To determine an organism's ability to grow in the presence of lysozyme

2. Procedure

Inoculate tubes of the lysozyme-glycerol mixture and basal glycerol broth (control) with the unknown culture. After 2 to 4 weeks of incubation at 25°C, observe the unknown culture for signs of growth.

I. Identification using commercial biochemical or miniaturized test systems

In contrast to the use of conventional growth-dependent procedures for identifying bacteria, the use of chromogenic enzyme substrates can rapidly detect constitutive enzymes produced by microorganisms.

Kilian (33), in 1978, evaluated the API ZYM system (Societé Analytab Products Inc., La Balme Les Grottes, France) for the rapid identification of members of the family *Actinomycetaceae* and related bacteria. In combination with the β-xylosidase test, catalase activity, and oxygen requirements, 12 of 19 enzymes screened proved to be of value for differentiating species of the family *Actinomycetaceae* and some related species within 4 h. In 1990, Boiron and Provost (5) further evaluated the API ZYM system for detection of constitutive enzymes by chromogenic substrates.

A related application of rapid chromogenic substrate tests is the fluorogenic enzyme method, described by Goodfellow and colleagues (12, 14), to study the numerical taxonomy of both *Rhodococcus* and *Streptococcus* species. Other miniaturized studies include two Dade MicroScan Inc. (West Sacramento, CA) products, Rapid Anaerobe Identification and *Haemophilus-Neisseria* panels that test for preformed enzymes; the ID 32C Yeast Identification System (bioMérieux Vitek, Inc., Marcy L'Etoile, France); and another system based on 273 phenotypic tests (3, 4, 44). However, the last approach, which uses numerous tests, is not applicable in the routine clinical laboratory and may be more suitable to specialized reference or research laboratories.

J. Nitrate reductase

Nitrate broth is a broth used to determine an organism's ability to reduce nitrate to nitrite, free nitrogen gas, or other reduced nitrogen compounds.

1. Materials

Routine nitrate broth (Bacto peptone, 20 g; potassium nitrate, 2 g; distilled water, 1,000 ml)

Reagents

Solution 1 (sulfanilic acid, 2.8 g; distilled water, 250 ml; glacial acetic acid, 100 ml)

Solution 2 (dimethyl-α-naphthylamine, 2.1 ml; distilled water, 250 ml; glacial acetic acid, 100 ml)

2. Procedure

a. Inoculate tubes of nitrate broth (control) with unknown culture. After 2 days of incubation at 35°C, add 2 drops of solution 1 followed by 2 drops of solution 2.

b. Observe tubes for a change in color; if nitrate is reduced, broth takes on a pink/red color.

IV. METHODS OF IDENTIFICATION OF ACTINOMYCETES *(continued)*

K. Urease

Urease medium is used to determine an organism's ability to hydrolyze urea to form ammonia through the utilization of the enzyme urease.

1. Materials

Bacto urea agar base (Difco), 29 g (Bacto peptone, 1 g; Bacto dextrose, 1 g; sodium chloride, 5 g; monopotassium phosphate, 2 g; urea [Difco], 20 g; Bacto phenol red, 0.012 g); distilled water, 100 ml

2. Procedure

a. Inoculate slant with 1 drop of a 24-h culture.

b. Incubate at 35°C for 7 days. A positive test is indicated by a pronounced pink color in the agar.

POSTANALYTICAL CONSIDERATIONS

V. INTERPRETATION OF RESULTS

A. Acetamide hydrolysis

Acetamide reaction is used as one way to differentiate among members of the genus *Nocardia. N. farcinica* is positive, and the other *Nocardia* spp. are negative.

B. Arylsulfatase detection

Positive is a color change to pink or red, and negative shows no color change.

◩ **NOTE:** Most *N. nova* isolates are positive in 14 days, and most *M. fortuitum* isolates are positive in 3 days.

C. Citrate agar

A positive reaction, denoting alkalinization, is indicated by the development of a dark blue color. Growth without color change in the medium is considered a negative reaction.

D. Decomposition of substrates

1. Adenine, casein, hypoxanthine, tyrosine, and xanthine are useful differential characteristics to separate members of the genus *Nocardia* (Table 6.2–1).

2. Adenine and tyrosine are useful differential characteristics to separate members of the genus *Rhodococcus* (Table 6.2–2).

3. Hypoxanthine, tyrosine, and xanthine are useful differential characteristics to separate members of the genus *Tsukamurella* (Table 6.2–3).

4. Adenine, hypoxanthine, tyrosine, and xanthine are useful differential characteristics to separate members of the genus *Nocardiopsis* (Table 6.2–4).

◩ **NOTE:** Isolates of *Dermatophilus congolensis* may take up to 7 days to decompose casein. Hypoxanthine, tyrosine, and xanthine are not decomposed. The thermophilic actinomycetes are separated by decomposition of the following substrates: adenine, casein, hypoxanthine, tyrosine, and xanthine. Most isolates of *Micromonospora* are strongly proteolytic; all isolates from human clinical specimens have decomposed casein, and 75% have decomposed tyrosine. No isolates decompose hypoxanthine or xanthine.

E. Esculin hydrolysis

Esculin fluoresces under the UV rays of a Wood's lamp; when esculin is hydrolyzed (a positive test), the medium turns reddish black and the esculin no longer fluoresces. An inoculated control tube of the medium without esculin may be useful in interpreting weak results. Esculin is useful in separating *Actinomadura madurae* (positive) from *Actinomadura pelletieri* (negative).

F. Lysozyme

1. The lysozyme test is considered positive if good growth is noted in both tubes, and negative if growth is good in the control tube but poor or absent in the tube containing the lysozyme.

Table 6.2–1 Characteristics differentiating the medically important species of the genus *Nocardia*[a,b]

Species	Growth at 45°C	Production of:			Hydrolysis of:						Utilization of:			
		Arylsulfatase (14 days)	Nitrate reductase	Urease	Adenine	Casein	Esculin	Hypoxanthine	Tyrosine	Xanthine	Acetamide[c]	Citrate[d]	L-Rhamnose[e]	D-Sorbitol[e]
N. abscessus	−	−	+	+	−	−	−	−	−	−	−	+	−	−
N. africana	+	+	−	−	−	+	−	−	−	−	−	−	−	−
N. anaemiae	−	−	+	+	−	−	−	+	−	−	−	+	−	+
N. aobensis	−	−	NA	+	−	−	+	−	−	−	+	−	−	−
N. araoensis	+	−	−	+	−	−	−	−	−	−	−	+	−	−
N. arthritidis	−	−	+	+	−	−	W	−	−	−	−	+	−	−
N. asiatica	−	−	+	V	−	−	−	−	−	−	−	+	+	−
N. asteroides ATCC 19247[T]	−	−	NA	+	−	−	−	−	−	−	−	−	−	−
N. beijingensis	−	−	+	+	−	−	−	−	−	+	+	+	+	+
N. brasiliensis	−	−	+	+	−	+	+	+	+	−	−	+	−	−
N. brevicatena	−	−	−	−	−	−	+	−	−	−	−	−	−	−
N. carnea	−	−	+	−	−	−	+	−	−	−	+	−	−	+
N. concava	+	−	+	+	+	−	+	+	−	+	−	+	−	−
N. cyriacigeorgica[f]	+	−	+	−	−	−	−	−	−	−	+	−	−	−
N. elegans	+	+	−	−	−	−	−	−	−	−	−	+	−	−
N. exalbida	+	−	−	+	−	−	+	−	−	−	+	+	+	−
N. farcinica	+	−	−	+	−	−	+	−	−	−	+	−	+	−
N. higoensis	+	NA	NA	+	−	−	NA	−	−	−	NA	−	−	−
N. inohanensis	−	+	+	+	−	−	+	+	−	−	−	+	−	−
N. kruczakiae	+[g]	+	+	−	−	−	+	−	−	−	−	+	−	−
N. mexicana	−	−	NA	+	+	−	NA	+	−/W	−	NA	NA	+	+
N. niigatensis	−	−	NA	V	−	−	+	W	−	−	−	−	−	−
N. ninae	−	NA	NA	+	+	−	NA	+	−	NA	NA	NA	−	−
N. nova	−	+	+	+	−	−	−	−	−	−	−	−	−	−
N. otitidiscaviarum	V	−	+H	+H	−	−	+	+	−	+	−	−	−	−
N. paucivorans	+	−	−	+	−	−	+	−	−	−	−	−	−	−
N. pneumoniae	−	W	+	−	NA	+	+	NA	NA	−	+	+	NA	NA
N. pseudobrasiliensis	−	−	−	+	+	+	+	+	+	−	NA	+	−	−
N. puris	−	−	+	+	−	−	+	−	−	−	−	+	−	+
N. sienata[f]	−	NA	NA	−	−	NA	−	−	−	−	NA	−	−	−
N. testacea[f]	+	NA	NA	−	−	−	−	−	−	−	NA	−	+	−
N. thailandica	−	NA	NA	+	−	−	NA	−	−	−	NA	−	−	−
N. transvalensis complex	V	−	+	+	−	−	+	+	−	−	−	+	−	V
N. vermiculata	−	NA	NA	−	−	−	NA	−	−	−	NA	−	−	−
N. veterana	+	−	−	+	−	−	−	−	−	−	−	−	−[h]	−
N. vinacea	−	−	+	+	−	−	d	+	−	−	+	d	−	+
N. yamanashiensis	−	−	NA	+	−	−	−	+	−	−	+	+	−	−

[a] Symbols and abbreviations: −, negative; +, positive; NA, not available; V, variable; d, doubtful.
[b] Composite data from references 3, 5, 6, 7, 9, 16, 17, 18, 21–31, 34, 37, 46, 48, 50, 52–58, and 64–67.
[c] Utilization as sole source of carbon and nitrogen.
[d] Utilization as sole source of carbon.
[e] Results vary according to method.
[f] Original spelling has been corrected by LISTS Editor of IJSEM (41, 43).
[g] Optimal growth.
[h] Results differ in different publications (16).

Table 6.2–2 Differential characteristics of the genus *Rhodococcus*[a,b]

Characteristic	Presence in:								
	R. coprophilus	*R. equi*	*R. erythropolis*	*R. fascians*	*R. globerulus*	*R. marinonascens*	*R. rhodnii*	*R. rhodochrous*	*R. ruber*
Species isolated from humans	−	+	−	−	−	−	−	−	−
Species implicated as cause of human infections	−	+	−	−	−	−	−	−	−
Morphogenetic sequence	AH-R-C	R-C	RB-R-C	H-R-C	RB-R-C	H-R-C	RB-R-C	RB-R-C	H-R-C
Pigment	Orange	Pink	Orange to red	Yellow	Pink to red	Cream to pink	Red	Rose	Red
Decomposition of:									
Adenine	−	+	+	+	−	−	−	V	V
Tyrosine	−	−	V	+	−	V	V	V	+
Utilization of:									
D-Galactose	−	+	V	+	−	NT	+	V	V
i-*myo*-Inositol	−	−	+	−	−	+	−	−	V
D-Mannitol	−	−	+	+	V	−	+	+	+
L-Rhamnose	−	V	−	−	−	−	−	−	V
D-Sorbitol	−	−	+	+	V	V	−	+	+
D-Sucrose	−	−	+	+	V	NT	−	V	V
Citrate	−	−	+	V	+	NT	−	V	V

[a] Data adapted from references 12, 13, 35, and 36. Except for *R. equi*, no *Rhodococcus* species has been demonstrated to cause human infections. Many of the new species of *Rhodococcus* that are being identified are from the environment.

[b] AH-R-C, aerial hypha-rod-coccus; R-C, rod-coccus; RB-R-C, rudimentary branching-rod-coccus; H-R-C, hypha-rod-coccus growth cycle; +, positive, −, negative; V, variable; NT, not tested.

Table 6.2–3 Differential characteristics of the genus *Tsukamurella*[a,b]

Characteristic	Presence in:				
	T. inchonensis	*T. paurometabola*[c]	*T. pulmonis*	*T. tyrosinosolvens*	*T. strandjordii*[d]
Decomposition of:					
Hypoxanthine	+	−	−	+	−
Xanthine	−	−	−	+	−
Tyrosine	−	−	−	+	−
Utilization as sole carbon source:					
Cellobiose	+	−	−	−	−
Maltose	+	−	−	+	+
i-*myo*-Inositol	+	−	−	+	+
D-Mannitol	+	−	+	+	+
D-Sorbitol	+	−	+	+	+
Citrate	+	+	−	V	+
Utilization of acetamide as sole carbon and nitrogen source	+	−	+	+	+
Growth at 45°C	+	−	−	−	−

[a] Data adapted from references 8, 32, and 60–62.

[b] Symbols: +, positive; −, negative; V, variable.

[c] Although Collins et al. named the organism *Tsukamurella parometabolum*, the species name was changed by the LISTS Editor of the IJSEM to *T. paurometabola* that is proposed as the correct etymology.

[d] Although Kattar et al. named the organism *Tsukamurella strandjordae*, the species name was changed by the LISTS Editor of the IJSEM to *T. strandjordii* that is proposed as the correct etymology (42).

Table 6.2–4 Differential characteristics of the genus *Nocardiopsis*[a,b]

Characteristic	Presence in:						
	N. alba	*N. dassonvillei*	*N. halophila*	*N. listeri*	*N. lucentensis*	*N. prasina*	*N. synnemataformans*
Species isolated from humans	−	+	−	−	−	−	+
Species implicated as cause of human disease	−	+	−	−	−	−	+
Color of substrate hyphae	Colorless	Yellow; orange to brown	Yellow to coral red	Colorless	Yellow to brown	Colorless	Deep pimento
Decomposition of:							
Adenine	+	+	+	+	+	−	+
Hypoxanthine	+	+	+	+	+	−	+
Tyrosine	+	+	−	+	+	+	+
Xanthine	+	+	−	+	−	+	+
Utilization as carbon source							
Cellobiose	+	+	NT	−	+	−	+
D-Galactose	+	+	−	−	−	−	+
D-Glucose	+	+	−	−	+	−	+
i-*myo*-Inositol	−	−	+	−	+	−	+
Maltose	+	−	+	−	+	−	+
D-Mannitol	−	+	+	−	+	−	+
Raffinose	−	−	−	−	+	−	−
L-Rhamnose	+	W	+	−	+	−	+
Sucrose	−	+	−	−	+	−	−
Trehalose	+	+	NT	−	+	−	−
D-Xylose	−	+	+	−	−	−	+

[a] Data adapted from references 1, 15, 59, and 63.

[b] +, positive; −, negative; W, weakly positive; NT, not tested.

V. INTERPRETATION OF RESULTS *(continued)*

2. Except for 35% of the isolates of the *Nocardia brevicatena* complex, all *Nocardia* species grow in the presence of lysozyme.
3. *Streptomyces* species are sensitive to lysozyme and are inhibited by its presence.

G. Nitrate reductase test

The nitrate reductase test is considered positive after a color change to red within 1 to 2 min. If red is not observed, either the nitrate has not been reduced or the nitrate has been reduced beyond the oxidative level of nitrite (gas may be elevated in the Durham tube). To determine which has taken place, a little powered zinc is added to the tube. If nitrates are still present, they will be reduced by the zinc and a red color will develop in 5 to 10 min.

H. Urease production

A positive urease test is indicated by the development of a pronounced pink color in the agar.

VI. LIMITATIONS

A. Carbohydrate basal medium—Gordon's oxidative agar slants

Cellobiose is easily reverted. Use 10 ml of 20% solution with a final concentration of 2%.

B. Decomposition of substrates

The tests for decomposition of substrates are not useful for separating *N. asteroides* sensu stricto, *N. farcinica, N. nova,* and *N. brevicatena* from each other or from the related genera *Corynebacterium, Gordonia* (Table 6.2–5), *Mycobacterium,* and *Rhodococcus.*

Table 6.2–5 Differential physiological characteristics of the medically important *Gordonia* species[a]

Characteristic[b]	Presence in:								
	G. aichiensis	*G. araii*	*G. bronchialis*	*G. effusa*	*G. otitidus*	*G. polyisoprenivorans*	*G. rubripertincta*[c]	*G. sputi*	*G. terrae*
Species isolated from humans	+	+	+	+	+	+	+	+	+
Species implicated as cause of human infections	ND	+	+	+	+	+	+	+	+
Presence of synnemata or rare microscopic hyphae	−	−	+	−	−	−	−	−	−
Utilization of:									
D-Galactose	+	−	−	−	−	+	+	+	+
i-*myo*-Inositol	−	−	+	−	−	−	−	−	−
D-Mannitol	−	−	−	+	ND	+	+	+	+
Raffinose	−	−	−	−	ND	−	−	+	+
L-Rhamnose	−	−	−	−	ND	−	−	−	−
D-Sorbitol	−	−	−	−	ND	+	+	+	+
Citrate	+	−	−	+	−	+	+	−	−

[a] Data adapted from references 4, 19, 20, and 40.

[b] Symbols: +, ≥ 90% of the strains are positive; −, ≥ 90% of the strains are negative; ND, not determined.

[c] The original spelling of the specific epithet, *G. rubropertinctus*, has been corrected by Euzèby (11).

VII. IDENTIFICATION OF AEROBIC ACTINOMYCETES

All actinomycetes should be identified to the genus level and, in most cases, to the species level with the media and methods given in the previous section. Tables 6.2–1 through 6.2–5 serve as identification guides. Because of a rapidly evolving taxonomy, these tables may require modification as more new species are described. A schematic identification key is given in Fig. 6.2–1.

REFERENCES

1. **Al-tai, A. M., and J.-S. Ruan.** 1994. *Nocardiopsis halophila* sp. nov., a new halophilic actinomycete isolated from soil. *Int. J. Syst. Bacteriol.* **44:**474–478.
2. **Bendinger, B.** 1995. *Gordona hydrophobica* sp. nov., isolated from biofilters for waste gas treatment. *Int. J. Syst. Bacteriol.* **45:**544–548.
3. **Biehle, J. R., S. J. Cavalieri, T. Felland, and B. L. Zimmer.** 1996. Novel method for rapid identification of *Nocardia* species by detection of preformed enzymes. *J. Clin. Microbiol.* **34:**103–107.
4. **Bizet, C., C. Barreau, C. Harmant, M. Nowakowski, and A. Pietfroid.** 1997. Identification of *Rhodococcus*, *Gordona*, and *Dietzia* species using carbon source utilization tests ("Biotype-100" strips). *Res. Microbiol.* **148:**799–809.
5. **Boiron, P., and F. Provost.** 1990. Enzymatic characterization of *Nocardia* spp. and related bacteria by API ZYM profile. *Mycopathologia* **110:**51–56.
6. **Brown, B. A., R. W. Wilson, V. A. Steingrube, Z. Blacklock, and R. J. Wallace, Jr.** 1997. Characterization of a *Nocardia brevicatena* complex among clinical and reference isolates, abstr. C-65, p. 131. Abstracts of the 97th General Meeting of the American Society for Microbiology. American Society for Microbiology, Washington, DC.
7. **Brown-Elliott, B. A., J. M. Brown, P. S. Conville, and R. J. Wallace, Jr.** 2006. Clinical and laboratory features of the *Nocardia* spp. based on current molecular taxonomy. *Clin. Microbiol. Rev.* **19:**259–282.
8. **Collins, M. D., J. Smida, M. Dorsch, and E. Stackebrandt.** 1988. *Tsukamurella* gen. nov. harboring *Corynebacterium paurometabolum* and *Rhodococcus aurantiacus. Int. J. Syst. Bacteriol.* **38:**385–391.
9. **Conville, P. S., J. M. Brown, A. G. Steigerwalt, J. W. Lee, V. L. Anderson, J. T. Fishbain, S. M. Holland, and F. G. Witebsky.** 2004. *Nocardia kruczakiae* sp. nov., a pathogen in immunocompromised patients and a member of the "*N. nova* complex." *J. Clin. Microbiol.* **42:**5139–5145.
10. **Eppinger, H.** 1891. Uber eine neue pathogene *Cladothrix* und eine durch sie hervorgerufene *Pseudotuberculosis* (*Cladothrichica*). *Beitr. Pathol. Anat. Allg. Pathol.* **9:**287–328.
11. **Euzèby, J. P.** 1998. Taxonomic note: necessary correction of specific and subspecific epithets according to Rules 12c and 13b of the International Code of Nomenclature of Bacteria (1990 Revision). *Int. J. Syst. Bacteriol.* **48:**1073–1075.
12. **Goodfellow, M.** 1987. The taxonomic status of *Rhodococcus equi*. *Vet. Microbiol.* **14:**205–209.

REFERENCES *(continued)*

13. **Goodfellow, M.** 1989. Genus *Rhodococcus* zopt 1891, p. 2362–2371. *In* S. T. Williams, M. E. Sharpe, and J. G. Holt (ed.), *Bergey's Manual of Systematic Bacteriology*, vol. 4. Williams & Wilkins, Baltimore, MD.
14. **Goodfellow, M., E. V. Ferguson, and J. J. Sanglier.** 1992. Numerical classification and identification of *Streptomyces* species. *Gene* **115:**225–233.
15. **Grund, E., and R. M. Kroppenstedt.** 1990. Chemotaxonomy and numerical taxonomy of the genus *Nocardiopsis. Int. J. Syst. Bacteriol.* **40:**5–11.
16. **Gürtler, V., R. Smith, B. C. Mayall, G. Pötter-Reinemann, E. Stackebrandt, and R. M. Kroppenstedt.** 2001. *Nocardia veterana* sp. nov. isolated from human bronchial lavage. *Int. J. Syst. Evol. Microbiol.* **51:**933–936.
17. **Hamid, M. E., L. Maldonado, G. S. Sharaf Eldin, M. F. Mohamed, N. S. Saeed, and M. Goodfellow.** 2001. *Nocardia africana* sp. nov., a new pathogen isolated from patients with pulmonary infections. *J. Clin. Microbiol.* **39:**625–630.
18. **Iida, S., A. Kageyama, K. Yazawa, N. Uchiyama, T. Toyohara, N. Chohnabay, S. Suzuki, R. Nomura, R. M. Kroppenstedt, and Y. Mikami.** 2006. *Nocardia exalbida* sp. nov. isolated from Japanese patients with nocardiosis. *Int. J. Syst. Evol. Microbiol.* **56:**1193–1196.
19. **Iida, S., H. Taniguchi, A. Kageyama, K. Yazawa, H. Chibana, S. Murata, F. Nomura, R. M. Kroppenstedt, and Y. Mikami.** 2005. *Gordonia otitidis* sp. nov., isolated from a patient with external otitis. *Int. J. Syst. Evol. Microbiol.* **55:**1871–1876.
20. **Kageyama, A., S. Iida, K. Kazawa, T. Kudo, S. Suzuki, T. Koga, H. Saito, H. Inagawa, A. Wada, R. M. Kroppenstedt, and Y. Mikami.** 2006. *Gordonia araii* sp. nov. and *Gordonia effusa* sp. nov., isolated from patients in Japan. *Int. J. Syst. Evol. Microbiol.* **56:**1817–1821.
21. **Kageyama, A., N. Poonwan, K. Yazawa, Y. Mikami, and K. Nishimura.** 2004. *Nocardia asiatica* sp. nov., isolated from patients with nocardiosis in Japan and clinical specimens from Thailand. *Int. J. Syst. Evol. Microbiol.* **54:**125–130.
22. **Kageyama, A., N. Poonwan, K. Yazawa, Y. Mikami, and K. Nishimura.** 2004. *Nocardia beijingensis*, is a pathogenic bacterium to humans: the first infectious cases in Thailand and Japan. *Mycopathologia* **157:**155–161.
23. **Kageyama, A., N. Poonwan, K. Yazawa, S.-I. Suzuki, R. M. Kroppenstedt, and Y. Mikami.** 2004. *Nocardia vermiculata* sp. nov. and *Nocardia thailandica* sp. nov. isolated from clinical specimens. *Actinomycetologica* **18:**27–33.
24. **Kageyama, A., S. Suzuki, K. Yazawa, K. Nishimura, and R. M. Kroppenstedt.** 2004. *Nocardia aobensis* sp. nov., isolated from patients in Japan. *Microbiol. Immunol.* **48:**817–822.
25. **Kageyama, A., K. Torikoe, M. Iwamoto, J. Masuyama, Y. Shibuya, H. Okazaki, K. Yazawa, S. Minota, R. M. Kroppenstedt, and Y. Mikami.** 2004. *Nocardia arthritidis* sp. nov., a new pathogen isolated from a patient with rheumatoid arthritis in Japan. *J. Clin. Microbiol.* **42:**2366–2371.
26. **Kageyama, A., K. Yazawa, A. Mukai, M. Kinoshita, N. Takata, K. Nishimura, R. M. Kroppenstedt, and Y. Mikami.** 2004. *Nocardia shimofusensis* sp. nov., isolated from soil, and *Nocardia higoensis* sp. nov., isolated from a patient with lung nocardiosis in Japan. *Int. J. Syst. Evol. Microbiol.* **54:**1927–1931.
27. **Kageyama, A., K. Yazawa, A. Mukai, T. Kohara, K. Nishimura, R. M. Kroppenstedt, and Y. Mikami.** 2004. *Nocardia araoensis* sp. nov. and *Nocardia pneumoniae* sp. nov., isolated from patients in Japan. *Int. J. Syst. Evol. Microbiol.* **54:**2025–2029.
28. **Kageyama, A., K. Yazawa, K. Nishimura, and Y. Mikami.** 2004. *Nocardia inohanensis* sp. nov., *Nocardia yamanashiensis* sp. nov., and *Nocardia niigatensis* sp. nov., isolated from clinical specimens. *Int. J. Syst. Evol. Microbiol.* **54:**563–569.
29. **Kageyama, A., K. Yazawa, K. Nishimura, and Y. Mikami.** 2004. *Nocardia testaceus* sp. nov., and *Nocardia senatus* sp. nov., isolated from patients in Japan. *Microbiol. Immunol.* **48:**271–276.
30. **Kageyama, A., K. Yazawa, K. Nishimura, and Y. Mikami.** 2004. *Nocardia anaemiae* sp. nov. isolated from an immunocompromised patient and the first isolation report of *Nocardia vinacea* from humans. *Jpn. J. Med. Mycol.* **46:**21–26.
31. **Kageyama, A., K. Yazawa, H. Taniguchi, H. Chibana, K. Nishimura, R. M. Kroppenstedt, and Y. Mikami.** 2005. *Nocardia concava* sp. nov., isolated from Japanese patients. *Int. J. Syst. Evol. Microbiol.* **55:**2081–2083.
32. **Kattar, M. M., B. T. Cookson, L. C. Carlson, S. K. Stiglich, M. A. Schwartz, T. T. Nguyen, R. Daza, C. K. Wallis, S. L. Yarfitz, and M. B. Coyle.** 2001. *Tsukamurella strandjordae* sp. nov., a proposed new species causing sepsis. *J. Clin. Microbiol.* **39:**1467–1476.
33. **Kilian, M.** 1978. Rapid identification of *Actinomycetaceae* and related bacteria. *J. Clin. Microbiol.* **7:**127–133.
34. **Kinoshita, N., Y. Homma, M. Igarashi, S. Ikeno, M. Hori, and M. Hamada.** 2001. *Nocardia vinacea* sp. nov. *Actinomycetologica* **15:**1–5.
35. **Klatte, S., K. Jahnke, R. M. Kroppenstedt, F. Rainey, and E. Stackebrandt.** 1994. *Rhodococcus luteus* is a later subjective synonym of *Rhodococcus fascians. Int. J. Syst. Bacteriol.* **44:**627–630.
36. **Klatte, S., R. M. Kroppenstedt, and F. A. Rainey.** 1994. *Rhodococcus opacus* sp. nov. an unusual nutritionally versatile *Rhodococcus* species. *Syst. Appl. Microbiol.* **17:**355–360.

REFERENCES *(continued)*

37. **Laurent, F., V. Rodréguez-Nava, L. Noussair, A. Couble, M. H. Nicolas-Chanoine, and P. Boiron.** 2007. *Nocardia ninae* sp. nov., isolated from a bronchial aspirate. *Int. J. Syst. Evol. Microbiol.* **57:**661–665.
38. **Lechevalier, H. A., and M. Solotorovsky.** 1961. A new genus of Actinomycetales: *Micropolyspora* gen. nov. *J. Gen. Microbiol.* **26:**11–18.
39. **Lindenberg, L. A.** 1908–1909. Un nouveau mycétome. *Arch. Parasitol.* **13:**265–282.
40. **Linos, A., A. Steinbüchel, C. Spröer, and R. M. Kroppenstedt.** 1999. *Gordonia polyisoprenivorans* sp. nov., a rubber-degrading actinomycete isolated from an automobile tyre. *Int. J. Syst. Bacteriol.* **49:**1785–1791.
41. **LISTS Editor, IJSEM.** 2001. Notification that new names and new combinations have appeared in volume 51, part 4, of the IJSEM. *Int. J. Syst. Evol. Microbiol.* **51:**1621–1623.
42. **LISTS Editor, IJSEM.** 2002. The original spelling has been corrected to *Tsukamurella strandjordii* by associate editor. *Int. J. Syst. Evol. Microbiol.* **52:**1075–1076.
43. **LISTS Editor, IJSEM.** 2004. The original spelling has been corrected. *Int. J. Syst. Evol. Microbiol.* **54:**1005–1006**.**
44. **Muir, D. B., and R. C. Pritchard.** 1997. Use of bioMérieux ID 32C yeast identification system for identification of aerobic actinomycetes of medical importance. *J. Clin. Microbiol.* **35:**3240–3243.
45. **Pijper, A., and B. D. Pullinger.** 1927. South African nocardioses. *J. Trop. Med. Hyg.* **30:**153–156.
46. **Rodréguez-Nava, V., A. Couble, C. Molinard, H. Sandoval, P. Boiron, and F. Laurent.** 2004. *Nocardia mexicana* sp. nov., a new pathogen isolated from human mycetomas. *J. Clin. Microbiol.* **42:**4530–4535.
47. **Rossi-Doria, T.** 1891. Su di alcune specie di 'Streptothrix' trovate nellaria studiate in rapporto a quelle già note e specialmente all' 'Actinomyces'. *Ann. Isti. Igi. Speri. Univ. Roma* **1:**399–438.
48. **Ruimy, R., P. Riegel, A. Carlotti, P. Boiron, G. Bernardin, H. Monteil, R. J. Wallace, Jr., and R. Christen.** 1996. *Nocardia pseudobrasiliensis* sp. nov., a new species of *Nocardia* which groups bacterial strains previously identified as *Nocardia brasiliensis* and associated with invasive diseases. *Int. J. Syst. Bacteriol.* **46:**259–264.
49. **Snijders, E. P.** 1924. Cavia-scheefkopperij, een nocardiose. *Geneesku. Tijdschr. Ned.-Indie* **64:**85–87.
50. **Steingrube, V. A., R. J. J. Wallace, B. A. Brown, Y. Pang, B. Zeluff, L. C. Steele, and Y. Zhang.** 1991. Acquired resistance of *Nocardia brasiliensis* to clavulanic acid related to a change in beta-lactamase following therapy with amoxicillin-clavulanic acid. *Antimicrob. Agents Chemother.* **35:**524–528.
51. **Trevisan, V.** 1889. *Generi e le Specie delle Batteriacee.* Zanaboni and Gabuzzi, Milan, Italy.
52. **Tsukamura, M.** 1982. Numerical analysis of the taxonomy of nocardiae and rhodococci. Division of *Nocardia asteroides* sensu stricto into two species and descriptions of *Nocardia paratuberculosis* sp. nov. Tsukamura (formerly the Kyoto-I group of Tsukamura), *Nocardia nova* sp. nov. Tsukamura, *Rhodococcus aichiensis* sp. nov., Tsukamura, *Rhodococcus chubuensis* sp. nov., Tsukamura, and *Rhodococcus obuensis* sp. nov., Tsukamura. *Microbiol. Immunol.* **26:**1101–1119.
53. **Wallace, R. J., Jr., B. A. Brown, Z. Blacklock, R. Ulrich, K. Jost, J. M. Brown, M. M. McNeil, G. Onyi, V. A. Steingrube, and J. Gibson.** 1995. New *Nocardia* taxon among isolates of *Nocardia brasiliensis* associated with invasive disease. *J. Clin. Microbiol.* **33:**1528–1533.
54. **Wallace, R. J., Jr., B. A. Brown, M. Tsukamura, J. M. Brown, and G. O. Onyi.** 1991. Clinical and laboratory features of *Nocardia nova. J. Clin. Microbiol.* **29:**2407–2411.
55. **Wallace, R. J., Jr., L. C. Steele, G. Sumter, and J. M. Smith.** 1988. Antimicrobial susceptibility patterns of *Nocardia asteroides. Antimicrob. Agents Chemother.* **32:**1776–1779.
56. **Wallace, R. J., Jr., M. Tsukamura, B. A. Brown, J. Brown, V. A. Steingrube, Y. S. Zhang, and D. R. Nash.** 1990. Cefotaxime-resistant *Nocardia asteroides* strains are isolates of the controversial species *Nocardia farcinica. J. Clin. Microbiol.* **28:**2726–2732.
57. **Wilson, R. W., V. A. Steingrube, B. A. Brown, Z. Blacklock, K. C. Jost, A. McNabb, W. D. Colby, J. R. Biehle, J. L. Gibson, and R. W. Wallace, Jr.** 1997. Recognition of a *Nocardia transvalensis* complex by resistance to aminoglycosides, including amikacin, and PCR-restriction fragment length polymorphism analysis. *J. Clin. Microbiol.* **35:**2235–2242.
58. **Yassin, A. F., and S. Brennner.** 2005. *Nocardia elegans* sp. nov., a member of the *Nocardia vaccinii* clade isolated from sputum. *Int. J. Syst. Evol. Microbiol.* **55:**1505–1509.
59. **Yassin, A. F., E. A. Galinski, A. Wohlfarth, K. Jahnke, K. P. Schaal, and H. G. Truper.** 1993. A new actinomycete species, *Nocardiopsis lucentensis* sp. nov. *Int. J. Syst. Bacteriol.* **43:**266–271.
60. **Yassin, A. F., F. A. Rainey, H. Brzezinka, J. Burghardt, H. J. Lee, and K. P. Schaal.** 1995. *Tsukamurella inchonensis* sp. nov. *Int. J. Syst. Bacteriol.* **45:**522–527.
61. **Yassin, A. F., F. A. Rainey, H. Brzezinka, J. Burghardt, M. Rifai, P. Seifert, K. Feldmann, and K. P. Schaal.** 1996. *Tsukamurella pulmonis* sp. nov. *Int. J. Syst. Bacteriol.* **46:**429–436.
62. **Yassin, A. F., F. A. Rainey, J. Burghardt, H. Brzezinka, S. Schmitt, P. Seifert, O. Zimmermann, H. Mauch, D. Gierth, I. Lux, and K. P. Schaal.** 1997. *Tsukamurella tyrosinosolvens* sp. nov. *Int. J. Syst. Bacteriol.* **47:**607–614.

REFERENCES *(continued)*

63. **Yassin, A. F., F. A. Rainey, J. Burghardt, D. Gierth, J. Ungerechts, I. Lux, P. Seifert, C. Bal, and K. P. Schaal.** 1997. Description of *Nocardiopsis synnemataformans* sp. nov., elevation of *Nocardiopsis alba* subsp. *prasina* to *Nocardiopsis prasina* comb. nov., and designation of *Nocardiopsis antarctica* and *Nocardiopsis alborubida* as later subjective synonyms of *Nocardiopsis dassonvillei. Int. J. Syst. Bacteriol.* **47:**983–988.
64. **Yassin, A. F., F. A. Rainey, J. Brzezinka, H. Burghardt, and M. Mauch.** 2000. *Nocardia paucivorans* sp. nov. *Int. J. Syst. Evol. Microbiol.* **50:**803–809.
65. **Yassin, A. F., F. A. Rainey, U. Mendrock, H. Brzezinka, and K. P. Schaal.** 2000. *Nocardia abscessus* sp. nov. *Int. J. Syst. Evol. Microbiol.* **50:**1487–1493.
66. **Yassin, A. F., F. A. Rainey, and U. Steiner.** 2001. *Nocardia cyriacigeorgici* sp. nov. *Int. J. Syst. Evol. Microbiol.* **51:**1419–1423.
67. **Yassin, A. F., B. Straubler, P. Schumann, and P. Schaal.** 2003. *Nocardia puris* sp. nov. *Int. J. Syst. Evol. Microbiol.* **53:**1595–1599.

SUPPLEMENTAL READING

Becker, B., M. P. Lechevalier, R. E. Gordon, and H. A. Lechevalier. 1964. Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. *Appl. Microbiol.* **12:**421–423.

Berd, D. 1973. Laboratory identification of clinically important aerobic actinomycetes. *Appl. Microbiol.* **25:**665–681.

Gilardi, G. L. 1973. Nonfermentative gram-negative bacteria encountered in clinical specimens. *Antonie Leeuwenhoek* **39:**229–242.

Gordon, R., and D. Barnett. 1977. Resistance to rifampin and lysozyme of strains of some species of *Nocardia* and *Mycobacterium* as a taxonomic tool. *Int. J. Syst. Bacteriol.* **27:**126.

Gordon, R., and D. Barnett. 1977. Resistance to rifampin and lysozyme of strains of some species of *Mycobacterium* and *Nocardia* as a taxonomic tool. *Int. J. Syst. Bacteriol.* **27:**176.

Gordon, R. E. 1973. *The Genus Bacillus.* Agriculture handbook no. 427. Agriculture Research Service. U.S. Department of Agriculture, Washington, DC.

Hoare, D. S., and E. Work. 1957. The stereoisomers of diaminopimelic acid. *J. Biochem.* **65:**441–447.

Kubica, G. P., and A. L. Ridgeon. 1961. The arylsulfatase activity of acid-fast bacilli. III. Preliminary investigation of rapidly growing acid-fast bacilli. *Am. Rev. Respir. Dis.* **83:**737–740.

Mishra, S. K., R. E. Gordon, and D. A. Barnett. 1980. Identification of nocardiae and streptomycetes of medical importance. *J. Clin. Microbiol.* **11:**728–736.

Simmons, J. S. 1926. A culture medium for differentiating organisms of typhoid-colon-aerogenes group and for isolation of certain fungi. *J. Infect. Dis.* **39:**201–214.

Sneath, P. H. A. 1956. Cultural and biochemical characteristics of the genus *Chromobacterium. J. Gen. Microbiol.* **15:**70–98.

Vickers, R. M., J. D. Rihs, and V. L. Yu. 1992. Clinical demonstration of isolation of *Nocardia asteroides* on buffered charcoal-yeast extract media. *J. Clin. Microbiol.* **30:**227–228.

6.3.1 Appendix 6.3.1–1—Human Clinical Diseases Associated with Aerobic Actinomycetes

Actinomadura madurae—causative agent of mycetoma (rare in the United States but more common in tropical and subtropical countries) and nonmycetomic infections (very rare, but some reports of postoperative wound infections, pneumonia, and bacteremia)

Actinomadura pelletieri—causative agent of mycetoma (rare)

Dermatophilus congolensis—causative agent of dermatophilosis, particularly affecting workers in contact with infected animals (rare)

Gordonia araii—causative agent of pulmonary disease (rare) and medical device-associated infection

Gordonia bronchialis, Gordonia sputi, Gordonia terrae, and *Gordonia* spp.—causative agents of pulmonary disease (rare), bacteremia (rare), and postoperative infections (*Gordonia bronchialis*; rare)

Gordonia effusa—causative agent of pulmonary disease (rare)

Gordonia polyisoprenivorans—causative agent of bacteremia (rare)

Nocardia abscessus—causative agent of nocardiosis (pulmonary and disseminated)

Nocardia africana—causative agent of nocardiosis (pulmonary)

Nocardia anaemiae—causative agent of nocardiosis (source not known)

Nocardia aobensis—causative agent of nocardiosis (pulmonary)

Nocardia araoensis—causative agent of nocardiosis (pulmonary)

Nocardia arthritidis—causative agent of nocardiosis (pulmonary)

Nocardia asiatica—causative agent of nocardiosis (pulmonary)

Nocardia asteroides—causative agent of nocardiosis (pulmonary, cutaneous, and disseminated)

Nocardia beijingensis—causative agent of nocardiosis (pulmonary and disseminated)

Nocardia brasiliensis—causative agent of nocardiosis (cutaneous and lymphocutaneous [rare] and mycetoma [rare in the United States but more common in South America, Mexico, and Australia])

Nocardia brevicatena—causative agent of nocardiosis (pulmonary)

Nocardia carnea—causative agent of nocardiosis (source not known)

Nocardia concava—causative agent of nocardiosis (source not known)

Nocardia cyriacigeorgica—causative agent of nocardiosis (pulmonary and disseminated)

Nocardia elegans—causative agent of nocardiosis (pulmonary)

Nocardia exalbida—causative agent of nocardiosis (pulmonary and cutaneous)

Nocardia farcinica—causative agent of nocardiosis (pulmonary and/or CNS; postoperative infections [rare])

Nocardia higoensis—causative agent of nocardiosis (pulmonary and disseminated)

Nocardia inohanensis—causative agent of nocardiosis (cutaneous)

Nocardia kruczakiae—causative agent of nocardiosis (pulmonary)

Nocardia mexicana—causative agent of nocardiosis (mycetoma)

Nocardia niigatensis—causative agent of nocardiosis (cutaneous)

Nocardia ninae—causative agent of nocardiosis (pulmonary)

Nocardia nova—causative agent of nocardiosis (pulmonary and disseminated)

Nocardia otitidiscaviarum—causative agent of nocardiosis (pulmonary and/or cutaneous) (rare)

Nocardia paucivorans—causative agent of nocardiosis (pulmonary)

Nocardia pneumoniae—causative agent of nocardiosis (pulmonary)

Nocardia pseudobrasiliensis—causative agent of nocardiosis (pulmonary) (rare)

Nocardia puris—causative agent of nocardiosis (cutaneous)

Nocardia sienata—causative agent of nocardiosis (pulmonary)

Nocardia testacea—causative agent of nocardiosis (pulmonary)

Nocardia thailandica—causative agent of nocardiosis (source not known)

Nocardia transvalensis complex—causative agent of nocardiosis (pulmonary, disseminated, and myetoma [very rare])

Nocardia vermiculata—causative agent of nocardiosis (source not known)

Nocardia veterana—causative agent of nocardiosis (pulmonary)

Nocardia vinacea—causative agent of nocardiosis (source not known)

Nocardia yamanashiensis—causative agent of nocardiosis (cutaneous)

Rhodococcus equi—causative agent of pulmonary disease (rare) and bacteremia (rare), particularly in human immunodeficiency virus-infected persons

Rhodococcus spp.—causative agents of invasive disease (very rare)

Streptomyces somaliensis—causative agent of mycetoma (very rare)

Tsukamurella inchonensis, *Tsukamurella pulmonis*, and *Tsukamurella tyrosinosolvens*—causative agents of pulmonary disease (rare), cutaneous disease (rare), and bacteremia (rare)

Tsukamurella strandjordii—causative agent of bacteremia (rare)

6.3.2 Appendix 6.3.2–1—Suppliers

Remel
P.O. Box 14428
Lenexa, KS 66285
- Acetamide agar slant
- Arylsulfatase broth (3-day test)
- Arylsulfatase broth (2-week test)
- Bile esculin agar slant
- Buffered charcoal yeast extract selective agar (with polymyxin B, anisomycin, and vancomycin)
- Citrate agar slant
- Gram stain
- Lowenstein-Jensen medium slant
- Lysozyme broth
- Lysozyme control broth
- Sabouraud dextrose agar slant
- Thayer-Martin agar, modified
- Thioglycolate medium without indicator, with dextrose
- Tryptic soy broth

BBL, Becton Dickinson Microbiology Systems
P.O. Box 243
Cockeysville, MD 21030
- Acetamide agar slants
- Arylsulfatase broth (0.001 M)
- Arylsulfatase broth (0.003 M)
- Bile esculin agar slants
- Gram stain
- Lowenstein-Jensen medium slants
- Modified Thayer-Martin agar plates with vancomycin (300 µg/ml), colistin (750 µg/ml), and nystatin (1,250 U/ml)
- Sabouraud dextrose agar slant

Becton Dickinson Diagnostic Instruments Systems
7 Loveton Circle
Sparks, MD 21152
- BACTEC 12B medium
- BACTEC reconstituting fluid
- BACTEC 460 instrument

BBL, BD Biosciences
Becton Dickinson and Company
Sparks, MD 21152
Heart infusion agar with rabbit blood

bioMérieux
La Balme les Grottes, France
Biotype-100 strips

Merck & Co., Inc.
West Point, PA 19486
Isolator

bioMérieux Vitek, Inc.
Hazelwood, MO 63042
API 20C AUX system
API 50 CH system

Fisher Scientific Worldwide Projects Division
50 Fadem Rd.
Springfield, NJ 07081
Gram stain

6.3.3

Appendix 6.3.3–1—Medium Composition and Preparation

I. GRAM STAIN

A. Crystal violet

1. Crystal violet powder (99% dye content)
Use 13.87 g (the amount of dye used is adjusted in proportion to the dye content of the powder, stated on the label) and add 200 ml of 95% ethanol.

2. Ammonium oxalate (8 g in 800 ml of distilled water)

3. Mix solutions 1 and 2 and allow to stand overnight or until the dye dissolves.

4. Filter through no. 2 Whatman filter paper.

B. Gram's iodine

iodine crystals ……1.00 g
potassium iodide …..2.00 g
distilled water ……300 ml
Mix reagents 1 and 2 in a mortar and gradually add distilled water while emulsifying with a pestle. Store in a brown bottle at room temperature.

C. Decolorizing solution: 95% ethanol

D. Safranin-O counterstain

safranin-O ……3.41 g
95% ethanol ……100 ml
distilled water ……900 ml
Safranin-O is dissolved in 100 ml of 95% ethanol, and the mixture is then combined with 900 ml of distilled water.

II. MODIFIED KINYOUN STAIN

A. Modified Kinyoun carbol fuchsin

1. Dissolve 4 g of basic fuchsin in 20 ml of 90 to 95% ethanol.

2. Dissolve 8 g of phenol crystals in 100 ml of distilled water.

3. Mix solutions 1 and 2.

B. Decolorizer (1% [vol/vol] H_2SO_4)

Add 1 ml of concentrated sulfuric acid to 99 ml of distilled water.

C. Methylene blue

Dissolve 2.5 g of methylene blue chloride in 100 ml of 95% ethyl alcohol.

III. ACETAMIDE HYDROLYSIS MEDIUM

Reagents

acetamide	10.000 g
sodium chloride	5.000 g
dipotassium phosphate	1.390 g
monopotassium phosphate	0.730 g
magnesium sulfate	0.500 g
phenol red	0.0120 g
agar	15.000 g
distilled water	1,000 ml

Prepare according to the manufacturer's instructions and place in 5-ml amounts into 12- by 125-mm tubes; autoclave at 121°C for 15 min and slant for cooling.

IV. ARYLSULFATASE LIQUID MEDIUM

A. Preparation of stock substrate

1. Dissolve 2.6 g of phenolphthalein disulfate, tripotassium salt, in 50 ml of distilled water; this is a 0.08 M solution.

2. Sterilize by membrane filtration (0.22-μm-pore-size filter).

3. Store in refrigerator.

◩ **NOTE:** Before use, the phenolphthalein disulfate should be tested for free phenolphthalein. Dissolve a small portion in a minimal amount of water, and add a few drops of 2 N sodium carbonate. If a pink color forms, this indicates free phenolphthalein, which will give false-positive reactions under actual test conditions. Sufficient purification of the product may be attained by dissolving 3 to 5 g of phenolphthalein disulfate in a minimal amount of water; this salt may be precipitated from solution (free of most phenolphthalein) by the addition of excess ethanol. The precipitated disulfate salt may be filtered off on a Buchner funnel, washed several times with fresh ethanol, allowed to dry, and bottled for use in preparation of stock solution as described above.

B. Arylsulfatase broth

1. Aseptically add 2.5 ml of 0.08 M stock substrate to 200 ml of sterile Middlebrook 7H-9 broth. This provides 0.008 M substrate for the 3-day and 2-week tests.

2. Prepare two flasks, each with 200 ml of the sterile liquid medium.

3. Aseptically dispense each substrate in 2-ml amounts into sterile 16- by 125-mm screw cap test tubes.

4. The ready-to-use substrates may be stored at room temperature. As a QC check, add to a tube of each uninoculated substrate a few drops of sodium carbonate. The substrates should remain colorless. If the solution turns pink, discard the substrate and check the quality of the stock powder (as indicated in the note above) before preparing fresh substrate.

V. GORDON'S OXIDATIVE SLANTS

A. Basal medium

$(NH_4)_2HPO_4$	1.00 g
KCl	0.02 g
$MgSO_4 \cdot 7H_2O$	0.20 g
Agar	15.00 g
Distilled H_2O	1,000 ml

1. Add one salt at a time slowly.

2. Adjust the pH to 7.0.

3. Add 15 ml of 0.04% solution of bromcresol purple.

B. Autoclavable sugars

1. To a 100-ml aliquot of basal medium add 1 g of carbohydrate or 10 ml of 10% carbohydrate solution to 90 ml of basal medium. Autoclave for 10 min. Dispense 5 ml into 15- by 125-mm screw cap tubes and slant.

2. Autoclavable sugars

Basal medium (no carbohydrate)—control
D-Glucose
D-Mannitol
Salicin
Glycerol
i-*myo*-Inositol
Trehalose
D-Galactose
D-Mannose
D-Sorbitol
Dulcitol
Raffinose
Adonitol
i-Erythritol
Melibiose

V. GORDON'S OXIDATIVE SLANTS *(continued)*

C. Nonautoclavable sugars

1. Autoclave 90-ml aliquots of basal medium. Allow to cool to 45°C. Add aseptically 10 ml of 10% Seitz-filtered carbohydrate solution. Dispense 5 ml into 15- by 125-mm tubes and slant.

2. Seitz-filtered carbohydrates
Lactose
Sucrose
Maltose
Xylose
L-Arabinose
Starch
D-Fructose
Cellobiose
L-Rhamnose

VI. CITRATE AGAR

magnesium sulfate	0.02 g
monoammonium phosphate	1.00 g
dipotassium phosphate	1.00 g
sodium citrate	2.00 g
sodium chloride	5.00 g
agar	15.00 g
bromthymol blue	0.08 g
distilled water	1,000 ml

Prepare according to the manufacturer's instructions and place in 5-ml amounts into 12- by 125-mm tubes; autoclave at 121°C for 15 min and slant for cooling.

VII. DECOMPOSITION OF SUBSTRATES

A. Media

1. Casein medium
Prepare separately skim milk and agar.
skim milk (dehydrated) ..... 10 g
distilled water ..... 100 ml
Autoclave at 15 lb/in² for 20 min at 121°C.
agar ..... 2.0 g
distilled water ..... 100 ml
Cool both solutions to 45°C. Mix and pour into quadrant petri plates.

2. Basal medium for hypoxanthine, tyrosine, xanthine, and adenine
nutrient agar
peptone ..... 5 g
beef extract ..... 3 g
agar ..... 15 g
distilled water ..... 1,000 ml
Autoclave at 15-lb pressure for 20 min at 121°C. Adjust final pH to 7.0.

B. Addition of crystals

Suspend 0.4 g of xanthine, 0.4 g of tyrosine, 0.4 g of hypoxanthine, and 0.4 g of adenine in individual 10-ml amounts of distilled water and autoclave. When the agar has cooled to 45°C, mix the crystals and the basal medium and pour individual crystal suspensions into quadrant plates.

VIII. ESCULIN HYDROLYSIS MEDIUM

esculin	1 g
ferric citrate	0.5 g
infusion agar	40.0 g
distilled water	1,000 ml

Heat the ingredients to dissolve, and then cool to 55°C. Adjust the pH to 7.0. Pour the medium in 5-ml amounts into 15- by 125-mm tubes; autoclave at 121°C for 15 min and slant for cooling.

IX. LYSOZYME TEST

A. Basal glycerol broth

peptone .....5.0 g
beef extract .....3.0 g
glycerol ..... 70 ml
distilled water ..... 1,000 ml

Autoclave at 15 lb/in^2 for 15 min at 121°C. Dispense 5 ml into test tubes for control.

B. Lysozyme solution

lysozyme .....100 mg
distilled water .....100 ml

Sterilize by filtration. This stock solution should be stored at 4°C. Any stock solution remaining after 2 months should be discarded, since it deteriorates in that time. Mix 5 ml of the stock lysozyme solution with 95 ml of basal glycerol broth, and dispense 5-ml quantities into test tubes.

X. NITRATE REDUCTASE

A. Routine nitrate broth

Bacto peptone ..... 20.0 g
potassium nitrate.....2.0 g
distilled water..... 1,000 ml

B. Reagents

Solution 1

sulfanilic acid.....2.8 g
distilled water.....250 ml
glacial acetic acid....100 ml

Solution 2

dimethyl-α-naphthylamine acid .....2.1 ml
distilled water.....250 ml
glacial acetic acid.....100 ml

Each of the broth media is dispensed in 4-ml amounts into test tubes containing inverted Durham vials and autoclaved at 121°C for 15 min.

XI. UREASE

Bacto agar ..... 15 g
Bacto peptone .....1 g
Bacto dextrose .....1 g
sodium chloride .....5 g
monopotassium phosphate .....2 g
urea (Difco) ..... 20 g
Bacto phenol red ..... 0.012 g
distilled water .....100 ml

6.3.4

Appendix 6.3.4–1—Cell Wall Determination of Diaminopimelic Acid Isomers

The cell wall of actinomycetes, like those of other bacteria, contains a murein layer that can be used as a criterion of taxonomic value. In the case of actinomycetes, it is possible to assign most strains to a cell wall group with a crude whole-cell extraction and without the laborious task of making cell wall preparations (Table 6.3.4–A1). *meso*-Diaminopimelic acid (DAP) is found in *Actinomadura* spp., *Corynebacterium* spp., *Gordonia* spp., *Mycobacterium* spp., *Nocardia* spp., *Nocardiopsis* spp., *Rhodococcus* spp., and *Tsukamurella* spp. as a major constituent of the cell wall; **L-DAP is found in** *Streptomyces* spp.

I. SUPPLIES

Include QC information on reagent container and in QC records.

A. General

1. Glassware—125-mm tubes; 250- and 500-ml flasks

2. Pipettes—10 and 100 μl

3. Membrane filter; 0.22-μm pore size

B. Chromatographic procedures

Ampoules, Pyrex chromatography tank (12 by 12 by 24 in) fitted with a glass rack for paper strips, Whatman chromatography paper no. 1 (46 by 57 mm and 4.24-cm diameter), oven, steam bath, fume cupboard

C. Control for detecting DAP isolates in cell walls

Control DAP (DL-α-ε-diaminopimelic acid [DL-2,6-diaminoheptanedoic acid])

Table 6.3.4–A1 Major constituents of cell walls of actinomycetes[a]

Cell wall type	Species	Major constituents	Whole-cell sugar pattern	
			Type	Diagnostic sugar(s)
I	*Streptomyces* spp.	L-DAP; glycine	None	None
II	*Micromonospora* spp.	*meso*-DAP; glycine; hydroxy DAP may also be present	D	Xylose; arabinose
III	*Actinomadura* spp.	*meso*-DAP	B	Madurose[b]
	Nocardiopsis spp.	*meso*-DAP	C	None
IV	*Nocardia* spp.	*meso*-DAP; arabinose and galactose	A	Arabinose; galactose
	Rhodococcus spp.	*meso*-DAP; arabinose and galactose	A	Arabinose; galactose
	Gordonia spp.	*meso*-DAP; arabinose and galactose	A	Arabinose; galactose
	Tsukamurella spp.	*meso*-DAP; arabinose and galactose	A	Arabinose; galactose

[a] All cell wall preparations contain major amounts of alanine, glutamic acid, glucosamine, and muramic acid.

[b] Madurose, 3-*O*-methyl-D-galactose.

II. METHOD

A. Materials

1. Whole-cell preparations of isolates

2. Reagents

a. 6 N HCl

b. Solvents and reagents

methanol .....400 ml
distilled water ..... 89 ml
12 N HCl ..... 11 ml
pyridine .....100 ml

c. Control DAP

d. Developer—400 mg of ninhydrin (1,2,3-triketohydrinedene hydrate) dissolved in 100 ml of acetone

e. Pyrex tank

CALAB 12- by 12- by 24-in Pyrex brand glass tank, using a Pyrex brand glass rack for paper strip chromatography. The trough support unit, which holds the troughs, is placed in position on the two uprights in such a manner that the solvent troughs will be perpendicular to the two sides of the tank, where the two uprights are located. Thus, the two heavier ends of the rack will be directly above the uprights. The apparatus is so designed that placement in this manner eliminates the possibility of the unit dropping into the bottom of the tank.

f. Chromatography paper—Whatman no. 1 Chr (46 by 57 mm and 4.25 cm in diameter)

B. Procedure

It is imperative that these cultures be handled in a biological safety cabinet.

1. Growth of cultures

a. A pure isolate of the organism is grown in 200 ml TSB broth with a few 3-mm-diameter glass beads in a 500-ml flask incubated at 28 to 35°C aerobically on a rotator.

b. After maximal growth (24 h to 2 weeks), the cells are harvested.

2. Harvesting and drying the cells

a. Autoclave the flask to inactivate the cells (121°C for 15 min).

b. Transfer the cell suspension into a 250-ml screw cap centrifuge bottle, trying not to include the beads.

c. Centrifuge cell suspension for 20 min at 4,000 × *g*. Repeat this step if the cells are still in suspension.

d. Pour off the supernatant, and add 100 ml of ethanol to the bottle containing the cells. Resuspend the cells in the ethanol by shaking the bottle.

e. Incubate the cells at 35°C for 18 to 24 h.

f. Centrifuge the cell suspension for 20 min at 4,000 × *g*. Repeat this step if the cells are still in suspension. Pour off the ethanol supernatant.

g. Using a wooden tongue depressor, scrape the cell mass into a disposable petri dish. Place the cover over the dish, and incubate it at 35°C for 18 to 24 h (or until the cell mass is dry enough to grind with a mortar and pestle).

3. Hydrolysis of the cells

a. Weigh 10 mg of the cell material into a 2-ml prescored Wheaton vacule or ampoule.

b. Using a glass pipette, fill the vacule with 1 ml of 6 N HCl.

c. Seal the vacule with oxygen gas flame.

d. Incubate the sealed vacule in a 100°C drying oven for 18 h.

4. Preparation of cell hydrolysate

a. Remove the Wheaton vacule from the drying oven and open it. Set up a 20-ml beaker with a folded piece of qualitative Whatman no. 1 4.25-cm-

II. METHOD *(continued)*

diameter filter paper held in the mouth of the beaker with a metal paper clip. Transfer the Wheaton vacule contents into the filter paper, and filter the cell suspension into the beaker. Rinse the filter paper with sterile distilled water three times.

b. Remove and dispose of the filter paper and paper clip.

c. Heat the beaker over a pervaporator or steam bath to evaporate off the HCl solution. The filtrate is dried three consecutive times (add 1 ml of sterile distilled water each time).

d. After last evaporation step, reconstitute the residue with 300 μl of sterile distilled water.

e. Transfer cellular hydrolysates into a screw cap tube and store at 4°C until ready for use.

5. Preparation of chromatography paper

Using Whatman chromatography paper (no. 1 Chr) in 46- by 57-cm sheets, cut a piece down the middle to make two 23- by 57-cm sheets.

a. Cut straight across the bottom edge with pinking scissors, which will leave a zigzag cut.

b. On the opposite edge, draw lines with a graphite pencil at 1, 2, and 3 in.

c. On the line, 3 in. from the top, make a 1/4-in. tick every 3/4 in. as a guide for the samples and control DAP.

d. Load each position with 20 μl of sample.

e. Let dry thoroughly before placing in solvent tank.

6. Preparation of chromatography system

Caution: *The tank and solvent preparation should be performed in a chemical fume hood.*

a. Prepare the solvent and mix well. Reserve ~100 ml of the solvent for the trough, and pour the remaining solvent into a 1,000-ml beaker to place in one trough.

b. Place the 1,000-ml beaker of solvent in the center of the tank between the two troughs. When the tank is being set up for the first time, the tank is equilibrated for 24 h with a batch of solvent and is sealed with silicone lubricant. Between uses, the tank is stored sealed with the solvent remaining in it. When the solvent level in the tank reaches about 1 in. below the paper, remove about half of the solvent from the tank.

c. Place paper(s) in the glass trough(s).

d. Pour the 100-ml beaker of solvent into the glass well, and seal the tank. Let the test run for 18 to 20 h. Solvent should be seen dripping from the paper's jagged edges when it has descended the length of the paper.

7. Drying and spraying the paper chromatograph for DAP determination

a. Remove the paper from the tank and air dry completely (10 to 15 min).

b. Prepare the acetonic ninhydrin developer. Store in a dark bottle for reuse (developer is good for 1 month).

c. Pour a layer of developer into a shallow pan (Pyrex or plastic), and wet the paper completely from end to end. Place the paper in a 100°C drying oven for 2 min. Try not to lay the paper flat against anything.

◩ **NOTE:** DAP is more soluble in H_2O than in pyridine. Better separation of the L-DAP and *meso*-DAP can be achieved if the system goes more slowly. To slow down the flow rate, use more pyridine. If the ninhydrin solution is too old, poor development of the paper may result. Make up fresh developer, and redip the paper.

8. Interpretation of results

DAP spots are olive green and will turn yellow with time. *meso*-DAP runs more slowly than L-DAP, so it is closer to the origin of the spots. Other amino acids present appear as purple spots, which travel faster than the DAP, some migrating off the paper during the migration process.

SECTION 7

Mycobacteriology and Antimycobacterial Susceptibility Testing

SECTION EDITOR: *Phyllis Della-Latta*

Observe standard precautions. For nucleic acid amplification tests, see section 12.

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7.1.1 Safety and Levels of Laboratory Service

I. SAFETY

All specimens and cultures handled in the mycobacteriology laboratory must be processed under an appropriate biological safety cabinet (BSC). Personal protective clothing (gowns, gloves, and respirators) is required for processing, smear preparation, and culturing for mycobacteria. Centrifuges must be equipped with aerosol-free safety canisters. Refer to CDC publications for detailed safety recommendations (1, 2, 4).

II. LEVELS OF LABORATORY SERVICE

The levels of laboratory service promulgated by the CDC, American Thoracic Society, and CAP (3, 5) are based on workload, expertise, and cost-effectiveness. A laboratory qualifies for levels I through III by performing the procedures listed below. Procedures not performed by level I or II laboratories are referred to a nationally recognized reference facility or a higher-level laboratory.

Procedure	Level		
	I	II	III
Acid-fast smears	Yes	Yes	Yes
Culture	No	Yes	Yes
Identification of *Mycobacterium tuberculosis* complex	No	Yes	Yes
Identification of all mycobacteria	No	No	Yes
Drug susceptibility	No	Yes	Yes

To maintain proficiency to qualify for level I status, a laboratory must prepare at least 10 to 15 specimens per week and examine them for acid-fast bacilli; to qualify for levels II and III, a laboratory must culture and identify at least 20 specimens per week. Level I laboratories must render the mycobacteria nonviable before acid-fast staining by treating the specimen with an equal volume of 5% sodium hypochlorite solution (undiluted household bleach) and waiting exactly 15 min before pouring this into a 15-ml sterile centrifuge tube. Staining can then be conducted on an open bench after centrifuging and decanting.

Safety precautions for level II laboratories include BSL 2 practices, facilities, and containment equipment (e.g., a class I or class II BSC and centrifuges with safety carriers), and personal protective clothing is required for processing and preparation of smears and culturing for mycobacteria.

REFERENCES

1. **Centers for Disease Control and Prevention.** 1999. *Biosafety in Microbiological and Biomedical Laboratories*, 4th ed. Centers for Disease Control and Prevention, Atlanta, Ga.
2. **Garner, J.** 1996. *CDC Guideline for Isolation Precautions in Hospitals, Hospital Infection Control Advisory Committee.* Centers for Disease Control and Prevention, Atlanta, Ga.
3. **Hawkins, J. E., R. C. Good, G. P. Kubica, P. R. J. Gangadharam, H. M. Guft, D. D. Stottmeier, H. M. Sommers, and L. G. Wayne.** 1983. Levels of laboratory services for mycobacterial diseases: official statement of the American Thoracic Society. *Am. Rev. Respir. Dis.* **128:**213.
4. **Richmond, J. Y. R., C. Knudsen, and R. C. Good.** 1996. Biosafety in the clinical microbiology laboratory. *Clin. Lab. Med.* **16:**527–550.
5. **Salfinger, M.** 1995. Role of the laboratory in evaluating patients with mycobacterial disease. *Clin. Microbiol. Newsl.* **17:**108–109.

7.1.2 Digestion-Decontamination Procedures

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Optimal recovery of mycobacteria from clinical specimens known to be contaminated requires liquefying the organic debris (digestion) and eliminating contaminating organisms (decontamination). Decontaminating procedures are toxic to mycobacteria. Five methods available for digestion and decontamination of specimens for culture of acid-fast bacteria are discussed in this procedure. The most commonly used procedure in the United States is the *N*-acetyl-L-cysteine–sodium hydroxide (NALC-NaOH) method.

A. NACL-NaOH method

NALC is a very effective mucolytic agent that at concentrations of 0.5 to 2.0% can rapidly digest sputa within 2 min. Decontamination is achieved by the addition of sodium hydroxide. One advantage of using the NALC-NaOH method is that a very good mucolytic agent can be used with reduced concentrations of a digestant-decontaminant (the final NaOH concentration in the sputum is 1%). A disadvantage is the short shelf life (24 h) of the final NALC-NaOH working reagent (6, 7, 9).

B. NaOH method

NaOH is a digestant and a decontaminant (6, 9). At a final specimen concentration of 2%, it is toxic for contaminants; however, some mycobacteria are also affected. Timing of the digestion-decontamination step is crucial for effective recovery of mycobacteria. Many laboratories use a combination of NaOH and NALC, which, as stated above, is an effective mucolytic reagent.

C. Zephiran-trisodium phosphate (Z-TSP) method

Zephiran, benzalkonium chloride, is a quaternary ammonium compound. The modification of Wayne et al. utilizes a reduced Zephiran concentration to prevent excessive sediment viscosity while decontaminating the specimen. The Z-TSP method (3, 6, 9) is considered a mild digestion-decontamination procedure. An advantage of Z-TSP is that mycobacteria can withstand prolonged treatment with it, so timing of the digestion-decontamination process is not critical. A disadvantage is that residual Z-TSP is mycobacteriostatic. Inoculation of agar-based media requires a neutralization step. Egg-based media contain lecithin, which neutralizes the bacteriostatic effect.

D. Oxalic acid method

Oxalic acid (ethanedioic acid [$C_2H_2O_4$]) has a reduced tuberculocidal effect (1). This method is not widely used except as an alternative decontamination method when specimens are known to be consistently contaminated with *Pseudomonas aeruginosa* (1, 6, 9).

E. Cetylpyridinium chloride (CPC)-sodium chloride method

CPC (1-hexadecylpyridinium chloride) is a quaternary ammonium compound. It has been demonstrated that a 1% CPC–2% NaCl solution effectively digested and decontaminated specimens that contained viable tubercle bacteria even after 8 days of exposure (10).

II. SPECIMEN COLLECTION, TRANSPORT, AND STORAGE

A. Specimen collection (5)

1. Use a sterile, leakproof, disposable plastic container, preferably a Sage collection kit (Becton Dickinson Microbiology Systems).
2. Collect specimens before antimicrobial therapy is begun.
3. Specimens that are unacceptable include dry swabs, nonsterile containers, leaky specimens, and unlabeled specimens. Request another specimen.

B. Specimen transport

1. Deliver to the laboratory in as short a time as practical to avoid overgrowth by contaminants.

II. SPECIMEN COLLECTION, TRANSPORT, AND STORAGE *(continued)*

2. Refrigerate specimens if transport is delayed more than 1 h (8, 10).
3. For blood, collect in BACTEC 13A bottle or Isolator tube. Do not refrigerate. Hold at room temperature.

C. Storage

1. Specimens (except blood) not processed within 1 h of collection should be refrigerated (2 to 8°C).
2. If gastric lavage specimens are delayed more than 4 h, add approximately 100 mg of sodium carbonate to the container to neutralize the acidity.

D. Specimens for decontamination

1. Contaminated specimens requiring decontamination include sputum, bronchial wash, skin, soft tissue, gastric lavage, and urine.
2. Sterile sites not requiring decontamination include CSF, bone marrow, blood, and biopsy sites.

III. MATERIALS

Include QC information on reagent container and in QC records.

Equipment that applies to all procedures listed includes centrifuges with aerosol-free sealed centrifuge canisters, class I or II biological safety cabinets inspected and certified at least annually, and vortex mixers. All reagents must be labeled with the date of preparation or receipt, contents, concentration, storage requirements, and expiration date.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Monitor the percentage of contaminated specimens. To determine the contamination rate, decontaminate four to six sputum samples, concentrate them by centrifugation, and inoculate onto general bacteriology media (e.g., blood agar) in addition to the mycobacteriology media normally used. Only minimal or no contaminants should grow after 24 to 48 h of incubation at 37°C. The acceptable range is 3 to 5% with media without antimicrobial agents. A contamination rate significantly less than 3% in the non-antimicrobial-agent-containing media suggests overly harsh contamination. Greater than 5% growth suggests inadequate decontamination or incomplete digestion.

V. PROCEDURES

With all procedures listed, use only sterile distilled water for all steps to prevent contamination with environmental mycobacteria (*see* Appendix 7.1.2–1).

Observe standard precautions.

A. NACL-NaOH method

1. Add an equal volume of working NALC-NaOH solution (no more than 24 h old) in a sterile 50-ml conical polypropylene screw-cap centrifuge tube (aerosol free and graduated). If more than 10 ml of specimen is submitted, use a sterile transfer pipette to select 10 ml of the most purulent, bloody, or mucoid portion. Secure the cap tightly.
2. Mix contents of the tube on a vortex mixer for not more than 30 s.
3. Incubate tube for 15 min at room temperature to decontaminate the specimen.
4. Dilute the mixture to the 50-ml mark with either sterile distilled water or sterile 0.067 M phosphate buffer (pH 6.8). This will reduce the continued action of the NaOH and lower the viscosity of the mixture.
5. Recap the tube tightly, and invert several times to mix the contents.
6. Centrifuge the tubes at $\geq$3,000 $\times$ *g* for 15 to 20 min. Use aerosol-free sealed centrifuge cups.
7. Pour off the supernatant into a splash-proof discard container filled with a suitable disinfectant (e.g., Amphyl).

V. PROCEDURES *(continued)*

8. Use a sterile 3-mm-diameter bacteriological loop or the end of a sterile wooden applicator stick to remove a portion of the sediment. Prepare a smear over an area of 1 by 2 cm on a new, clean, unscratched glass slide. If the amount of sediment is very small, delay making the smear after the addition of the 0.2% bovine albumin fraction V. (Bovine albumin fraction V [0.2%] buffers the inoculum and increases the adhesion of the sample.)
9. Suspend the remaining sediment in 1 to 2 ml of sterile 0.85% NaCl or sterile distilled water if media are to be inoculated immediately. Otherwise, suspend the sediment in 1 to 2 ml of sterile 0.2% bovine albumin fraction V. If the amount of sediment is very small, reconstitute the sediment to 1 ml only.
10. Use half of the suspension to make a 1:10 dilution in sterile distilled water (1 part suspension plus 9 parts water) (9).
11. Inoculate each suspension (diluted and undiluted) onto the appropriate media.

B. Sodium hydroxide method

1. Add a volume of 2% NaOH solution equal to the volume of the specimen in a sterile 50-ml conical polypropylene screw-cap centrifuge tube (aerosol free and graduated). If more than 10 ml of specimen is submitted, use a sterile transfer pipette to select 10 ml of the most purulent, bloody, or mucoid portion. Secure the cap tightly.
2. *Note:* Sodium hydroxide should be used at the lowest concentration that will effectively digest and decontaminate the specimen. If excessive contamination occurs with the use of 2% NaOH, increase the concentration to 3 or 4% rather than increasing the time of exposure.
3. Mix the tube on a vortex mixer. Incubate the tube for 15 min at room temperature to decontaminate the specimen, or shake vigorously on a mechanical shaker for 15 min.
4. Dilute the mixture to the 50-ml mark with either sterile distilled water or sterile 0.067 M phosphate buffer (pH 6.8). This will reduce the continued action of the NaOH and lower the viscosity of the mixture.
5. Recap the tube tightly, and invert several times to mix the contents.
6. Centrifuge the tubes at $\geq$3,000 $\times$ *g* for 15 to 20 min. Use aerosol-free sealed centrifuge cups.
7. Pour off the supernatant into a splash-proof discard container filled with a suitable disinfectant (e.g., Amphyl).
8. The sediment may be tested with a drop of phenol red indicator solution. Add 2 N HCl dropwise until the color of the sediment turns from red to *persistent* yellow. The use of lower working concentration of NaOH (e.g., 2%) together with a dilution step should obviate this step.
9. Use a sterile 3-mm-diameter bacteriological loop or the end of a sterile wooden applicator stick to remove a portion of the sediment. Prepare a smear over an area of 1 by 2 cm on a new, clean, unscratched glass slide. If the amount of sediment is very small, delay making the smear after the addition of the 0.2% bovine albumin fraction V.
10. Suspend the remaining sediment in 1 to 2 ml of sterile 0.85% NaCl or sterile distilled water if media are to be inoculated immediately. Otherwise, suspend the sediment in 1 to 2 ml of sterile 0.2% bovine albumin fraction V. If the amount of sediment is very small, reconstitute the sediment to 1 ml only.
11. Inoculate the suspension onto the appropriate media.

C. Z-TSP method

1. Add a volume of Z-TSP reagent equal to the volume of the specimen in a sterile 50-ml conical polypropylene screw-cap centrifuge (aerosol free and graduated). If more than 10 ml of specimen is submitted, use a sterile trans-

V. PROCEDURES *(continued)*

fer pipette to select 10 ml of the most purulent, bloody, or mucoid portion. Secure the cap tightly.

2. Shake vigorously on a mechanical shaker for 30 min.
3. Incubate the tube for 20 to 30 min at room temperature *without* additional shaking.
4. Centrifuge the tubes at ≥3,000 × *g* for 15 to 20 min. Use aerosol-free sealed centrifuge cups.
5. Pour off the supernatant into a splash-proof discard container filled with a suitable disinfectant (e.g., Amphyl).
6. Suspend the sediment in 20 ml of neutralizing buffer, pH 6.6 (2). Agitate thoroughly on a vortex mixer for 30 s.
7. Centrifuge the suspension at ≥3,000 × *g* for 15 to 20 min.
8. Pour off most of the supernatant. Retain a small amount of supernatant for resuspension of the sediment.

   ◩ **NOTE:** The buffer will neutralize any remaining Z-TSP. This is especially critical if inoculation of an agar-based medium is intended.
9. Use a sterile 3-mm-diameter bacteriological loop or the end of a sterile wooden applicator stick to remove a portion of the sediment. Prepare a smear over an area of 1 by 2 cm on a new, clean, unscratched glass slide.
10. Inoculate the suspension onto the appropriate media.

D. Oxalic acid method

1. Add an equal volume of 5% oxalic acid to the specimen in a sterile 50-ml conical polypropylene screw-cap centrifuge tube. If more than 10 ml of specimen is submitted, use a sterile transfer pipette to select 10 ml of the most purulent, bloody, or mucoid portion. Secure the cap tightly.
2. Mix the tube on a vortex mixer for 30 s.
3. Incubate the tube for 20 to 30 min at room temperature to decontaminate the specimen. Shake the tube occasionally.
4. Dilute the mixture to the 50-ml mark with sterile 0.85% NaCl.
5. Recap the tube tightly, and invert several times to mix the contents.
6. Centrifuge the tubes at ≥3,000 × *g* for 15 to 20 min. Use aerosol-free sealed centrifuge cups.
7. Pour off the supernatant into a splash-proof discard container filled with a suitable disinfectant (e.g., Amphyl).
8. Add a few drops of phenol red indicator to the sediment.
9. Neutralize the sediment with 4% NaOH until a pale pink color is obtained.
10. Use a sterile 3-mm-diameter bacteriological loop or the end of a sterile wooden applicator stick to remove a portion of the sediment. Prepare a smear over an area of 1 by 2 cm on a new, clean, unscratched glass slide.
11. Resuspend the sediment and inoculate the suspension onto the appropriate media.

E. CPC-sodium chloride method

1. Add an equal volume of CPC-NaCl reagent to the specimen in a sterile 50-ml conical polypropylene screw-cap centrifuge tube (aerosol free and graduated). If more than 10 ml of specimen is submitted, use a sterile transfer pipette to select 10 ml of the most purulent, bloody, or mucoid portion. Secure the cap tightly.
2. Shake by hand until the specimen liquefies.
3. Package the tube as required by postal and International Air Transport Association regulations and send to a processing laboratory or place in a suitable holding area pending local processing. *Be sure to allow at least 24 h for digestion-decontamination to be completed!*
4. Dilute the mixture to the 50-ml mark with either sterile distilled water or neutralizing buffer (4).

V. PROCEDURES *(continued)*

5. Recap the tube tightly, and invert several times to mix the contents.
6. Centrifuge the tubes at ≥3,000 × *g* for 15 to 20 min. Use aerosol-free sealed centrifuge cups.
7. Pour off the supernatant into a splash-proof discard container filled with a suitable disinfectant (e.g., Amphyl).
8. Use a sterile 3-mm-diameter bacteriological loop or the end of a sterile wooden applicator stick to remove a portion of the sediment. Prepare a smear over an area of 1 by 2 cm on a new, clean, unscratched glass slide. If the amount of sediment is very small, delay making the smear after the addition of the 0.2% bovine albumin fraction V.
9. Suspend the sediment in 1 to 2 ml of sterile 0.85% NaCl or sterile distilled water if media are to be inoculated immediately. Otherwise, suspend the sediment in 1 to 2 ml of sterile 0.2% bovine albumin fraction V. If the amount of the sediment is very small, reconstitute the sediment to 1 ml.
10. Inoculate the suspension onto the appropriate media.
11. *Note:* Residual CPC may inhibit mycobacteria inoculated onto agar-based media. Phospholipids found in egg-based media neutralize this inhibitory action. It has been reported that growth inhibition on agar-based medium may not occur if residual CPC is inactivated with Bacto neutralizing buffer (Difco) (10).

REFERENCES

1. **Cooper, H. J., and N. Uyei.** 1930. Oxalic acid as a reagent for isolating tubercle bacilli and a study of the growth of acid-fast nonpathogens on different mediums with their reaction to chemical reagents. *J. Lab. Clin. Med.* **15:**348–369.
2. **Della-Latta, P.** 1996. Workflow and optimal protocols for laboratories in industrialized countries. *Clin. Lab. Med.* **16:**677–695.
3. **Garner, J.** 1996. *CDC Guideline for Isolation Precautions in Hospitals, Hospital Infection Control Advisory Committee*. Centers for Disease Control and Prevention, Atlanta, Ga.
4. **Good, R. C., V. Silcox, and J. O. Kilburn.** 1981. Tuberculosis and other mycobacterioses, p. 675–703. *In* A. Balows and W. J. Hausler (ed.), *Diagnostic Procedures for Bacterial, Mycotic and Parasitic Infections*, 6th ed. American Public Health Association, Washington, D.C.
5. **Gullans, C. R., Sr.** 1992. Preparation of specimens for mycobacterial culture, p. 3.3.1–3.3.6. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook*, vol. 1. American Society for Microbiology, Washington, D.C.
6. **Kent, P. T., and G. P. Kubica.** 1985. *Public Health Mycobacteriology. A Guide for the Level III Laboratory.* U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta, Ga.
7. **Kubica, G. P., W. E. Dye, M. L. Cohn, and G. Middlebrook.** 1963. Sputum digestion and decontamination with N-acetyl-L-cysteine-sodium hydroxide for culture of mycobacteria. *Am. Rev. Respir. Dis.* **87:**775–779.
8. **Patterson, R. A., T. L. Thompson, and D. H. Larsen.** 1956. The use of Zephiran in the isolation of *M. tuberculosis. Am. Rev. Tuberc.* **74:**284–288.
9. **Pfyffer, G. E., B. A. Brown-Elliott, and R. J. Wallace, Jr.** 2003. *Mycobacterium*, p. 532–584. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
10. **Smithwich, R. W.** 1976. *Laboratory Manual for Acid-Fast Microscopy*, 2nd ed. Center for Disease Control, Atlanta, Ga.

SUPPLEMENTAL READING

Tenover, F. C., J. T. Crawford, R. E. Huebner, L. J. Geiter, C. R. Horsburgh, Jr., and R. C. Good. 1993. The resurgence of tuberculosis: is your laboratory ready? *J. Clin. Microbiol.* **31:**767–770.

APPENDIX 7.1.2–1

Materials

A. NALC-NaOH method

1. NaOH: sodium citrate stock solution (0.1 M)
   Solution 1

   sodium citrate dihydrate ……29 g
   (or sodium citrate, anhydrous) ……26 g
   distilled water ……to 1,000 ml

   Solution 2: 4% NaOH solution (1 N)

   NaOH pellets ……40 g
   distilled water ……to 1,000 ml

   Combine equal volumes of solutions 1 and 2, and autoclave the mixture at 121°C for 15 min in screw-cap flasks. This solution may be stored at room temperature, but refrigeration is preferred.
   Caution: *Sodium hydroxide is caustic. Always wear appropriate clothing and eye protection.*
2. Working NALC-NaOH solution
   Just before use, combine NALC with the NaOH-sodium citrate solution (Table 7.1.2–A1). The working solution can be used for up to 24 h, after which it must be discarded.
3. 0.067 M (M/15) phosphate buffer (pH 6.8) (6)
   Stock alkaline buffer

   Na_2HPO_4 (anhydrous) …… 9.47 g
   distilled water ……to 1,000 ml

   Stock acid buffer

   KH_2PO_4 …… 9.07 g
   distilled water ……to 1,000 ml

   Add dry buffer powder to separate 1,000-ml volumetric flasks. Add distilled water to the 1,000-ml mark. Each buffer may be transferred to screw-cap containers and sterilized at 121°C for 15 min to be mixed at a later time, or the buffers may be immediately combined and sterilized. Storage under refrigeration is preferred.
   Working phosphate buffer (pH 6.8)
   Combine equal volumes of stock alkaline and acid buffers. Check the pH of the working solution with a pH meter. Titrate with appropriate buffer to the correct pH.
4. 0.2% Bovine albumin fraction V solution (6)
   Stock 2% bovine albumin solution

   NaCl …… 0.85 g
   bovine albumin fraction V …… 2.00 g
   distilled water …… 100 ml

   Prepare a solution of 0.85% NaCl by dissolving the NaCl in distilled water. Slowly add the bovine albumin to the NaCl solution, and mix by swirling or gentle agitation on a vortex mixer. Adjust the pH to 6.8 with 4% NaOH. Sterilize by passing the solution through a membrane filter (0.45-μm pore size). Use aseptic technique to dispense 10- to 20-ml aliquots into sterile screw-cap tubes. Store the stock solution in a refrigerator (2 to 8°C) until needed.

Table 7.1.2–A1 Preparation of NALC-NaOH digestant-decontaminant

Total vol needed (ml)	Amt of:	
	NaOH-sodium citrate (ml)	NALC (g)
50	50	0.25
100	100	0.50
250	250	1.25
500	500	2.50
1,000	1,000	5.00

APPENDIX 7.1.2–1 *(continued)*

Working 0.2% bovine albumin solution

stock 2% bovine albumin solution ..... 1 part
sterile 0.85% NaCl
(or sterile distilled H_2O) ..... 9 parts

Dilute the stock solution to the desired volume. Prepare enough for use on 1 day. Discard the remainder at the end of the day.

5. Sterile 0.85% NaCl or sterile distilled water

B. Sodium hydroxide method

1. 2% NaOH solution (0.5 N) (4, 5)

NaOH pellets .....20 g
distilled water .....to 1,000 ml

2. 4% NaOH solution (1 N)

NaOH pellets .....40 g
distilled water .....to 1,000 ml

Caution: *Sodium hydroxide is caustic.*
Prepare the reagent by rapidly weighing the NaOH in less than 1 liter of distilled water in a beaker. Keep the beaker cool to minimize heat buildup. Transfer to a volumetric flask, and add distilled water to the 1,000-ml mark. Sterilize at 121°C for 15 min. Store at room temperature.

3. 0.067 M (M/15) phosphate buffer (pH 6.8) (6)
Stock alkaline buffer

Na_2HPO_4 (anhydrous) ..... 9.47 g
distilled water .....to 1,000 ml

Stock acid buffer

KH_2PO_4 ..... 9.07 g
distilled water .....to 1,000 ml

Add dry buffer powder to separate 1,000-ml volumetric flasks. Add distilled water to the 1,000-ml mark. Each buffer may be transferred to screw-cap containers and sterilized at 121°C for 15 min to be mixed at a later time, or the buffers may be immediately combined and sterilized. The solution may be stored at room temperature, but refrigeration is preferred.
Working phosphate buffer (pH 6.8)
Combine equal volumes of stock alkaline and acid buffers. Check the pH of the working solution with a pH meter. Titrate with appropriate buffer to the correct pH.

4. 2 N HCl solution (6)

HCl, concentrated (approximately 12 N or 36% [wt/vol]) .....33 ml
distilled water .....to 200 ml

Add approximately 100 ml of distilled water to a volumetric flask. *Slowly* add HCl to the water by running the acid down the inside of the flask. *Never add water to acid!* Wear an acid-resistant apron, gloves, and eye protection when working with concentrated HCl. In the event of a spill, immediately flood any area in contact with acid with copious amounts of water. Seek medical attention if required. Sterilize the acid solution at 121°C for 15 min. The solution may be stored at room temperature.

5. Phenol red indicator (6)

Phenol red .....8 mg
4% NaOH .....20 ml
distilled water .....to 1,000 ml

Dissolve the phenol red powder in 4% NaOH using a magnetic stirrer. Gentle heat may be required. Transfer the solution to a 1,000-ml volumetric flask. Add distilled water to the 1,000-ml mark. The solution may be stored at room temperature.

C. Z-TSP method

1. Z-TSP reagent (3, 7)

$Na_3PO_4 \cdot 12H_2O$ .....1 kg
hot distilled water .....4 liters

APPENDIX 7.1.2–1 *(continued)*

2. Zephiran (17% benzalkonium chloride) ……7.5 ml
Dissolve the trisodium phosphate ($Na_3PO_4 \cdot 12H_2O$) in hot water. Add Zephiran while stirring. Mix well. Store at room temperature.
3. Neutralizing buffer (2)

Bacto neutralizing buffer, dehydrated (Difco) ……5.2 g
distilled water ……1,000 ml

4. Rehydrate by dissolving the powder in distilled water on a magnetic stirrer. Gentle heat may be required. Dispense into screw-cap containers, and sterilize at 121°C for 15 min. Refrigerate.

D. Oxalic acid method

1. 5% Oxalic acid reagent (1, 2)

oxalic acid ($C_2H_2O_4$) ……50 g
distilled water ……to 1,000 ml

Dissolve the oxalic acid in a beaker of distilled water. Transfer the solution to a 1,000-ml volumetric flask. Add distilled water to the 1,000-ml mark. Sterilize at 121°C for 15 min. Store at room temperature.
2. Phenol red indicator

phenol red ……8 mg
4% NaOH ……20 ml
distilled water ……to 1,000 ml

Dissolve the phenol red powder in 4% NaOH on a magnetic stirrer. Gentle heat may be required. Transfer the solution to a 1,000-ml volumetric flask. Add distilled water to the 1,000-ml mark. Store at room temperature.
3. 4% NaOH solution (1 N)

NaOH pellets ……40 g
distilled water ……to 1,000 ml

Caution: *Sodium hydroxide is caustic.*
Prepare the reagent by rapidly weighing the NaOH in less than 1 liter of distilled water in a beaker. Keep the beaker cool to minimize heat buildup. Transfer to a volumetric flask, and add distilled water to the 1,000-ml mark. Sterilize at 121°C for 15 min. Store at room temperature.
4. Sterile 0.85% NaCl

E. CPC-sodium chloride method

1. CPC-NaCl reagent (6, 8)

CPC ……10 g
NaCl ……20 g
distilled water ……1,000 ml

Dissolve 10 g of CPC and 20 g of NaCl in a beaker of distilled water on a magnetic stirrer. Gentle heat may be required. The solution is self-sterilizing and is stable for long periods. Store at room temperature. Protect from light, excess heat, and evaporation. If crystals form, dissolve them with gentle heat.
2. Neutralizing buffer (2)

Bacto neutralizing buffer, dehydrated (Difco) ……5.2 g
distilled water ……1,000 ml

Rehydrate by dissolving the powder in distilled water on a magnetic stirrer. Gentle heat may be required. Dispense into screw-cap containers and sterilize at 121°C for 15 min. Refrigerate.
3. Sterile 0.85% NaCl

APPENDIX 7.1.2–1 *(continued)*

References

1. **Centers for Disease Control.** 1989. A strategic plan for the elimination of tuberculosis in the United States. *Morb. Mortal. Wkly. Rep.* **38**(Suppl. S-3):1–25.
2. **Difco Laboratories.** 1984. *Difco Manual. Dehydrated Culture Media and Reagents for Microbiology*, 10th ed. Difco Laboratories, Detroit, Mich.
3. **Garner, J.** 1996. *CDC Guideline for Isolation Precautions in Hospitals, Hospital Infection Control Advisory Committee*. Centers for Disease Control and Prevention, Atlanta, Ga.
4. **Gullans, C. R., Sr.** 1992. Digestion-decontamination procedures, p. 3.4.1–3.4.14. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook*, vol. 1. American Society for Microbiology, Washington, D.C.
5. **Gullans, C. R., Sr.** 1992. Preparation of specimens for mycobacterial culture, p. 3.3.1–3.3.6. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook*, vol. 1. American Society for Microbiology, Washington, D.C.
6. **Kent, P. T., and G. P. Kubica.** 1985. *Public Health Mycobacteriology. A Guide for the Level III Laboratory*. U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta, Ga.
7. **Pfyffer, G. E., B. A. Brown-Elliott, and R. J. Wallace, Jr.** 2003. *Mycobacterium*, p. 532–584. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
8. **Smithwich, R. W.** 1976. *Laboratory Manual for Acid-Fast Microscopy*, 2nd ed. Center for Disease Control, Atlanta, Ga.

7.1.3 Reporting

State and local authorities vary in regulations governing reporting of *Mycobacterium tuberculosis* detection. CDC guidelines advocate reporting all positive acid-fast bacterial smears and culture results for *M. tuberculosis* complex rapidly to all appropriate authorities as well as the physician in charge of the patient (1, 2, 3).

A. Communicate with the state and/or local tuberculosis control officer regarding laws governing the reporting of positive acid-fast smears and cultures.
B. Telephone diagnostic information as soon as possible to the clinician, the infection control officer, and the public health authorities. Document date, time, and person notified.
C. Issue a preliminary report each time new information is obtained and whenever isolates are referred to another laboratory for identification and susceptibility testing.
D. Verbally communicate the existence of the first positive culture (each patient, each episode) and subsequent results that may suggest dissemination, such as a sputum culture isolate followed by a blood culture isolate of suspected *Mycobacterium avium* complex.
E. Send written reports of preliminary information, final identification, and drug susceptibility results to the clinician, the infection control officer, and the medical records department.
F. Verbally communicate the first evidence of *M. tuberculosis* in culture. Call in results of additional culture-positive specimens if they were obtained during the time the patient is known or presumed to have been receiving treatment. Positive cultures at this stage will alert the public health officer to possible poor compliance with antituberculosis therapy.
G. Send a written report of the final identification and drug susceptibility result. This level of reporting will be unnecessary to laboratories that send isolates to their local public health laboratory for identification and/or antimicrobial susceptibility testing.

Turnaround time expectations in typical mycobacteriology laboratory

Time to results from specimen receipt	Positive reports to physician and public health
1 day	Acid-fast smear, direct amplification test
1–2 wks	Culture report
3 wks	*M. tuberculosis* identification
4 wks	Susceptibility results

REFERENCES

1. **Centers for Disease Control.** 1989. A strategic plan for the elimination of tuberculosis in the United States. *Morb. Mortal. Wkly. Rep.* **38**(Suppl. S-3):1–25.
2. **Della-Latta, P.** 1996. Workflow and optimal protocols for laboratories in industrialized countries. *Clin. Lab. Med.* **16:**677–695.
3. **Tenover, F. C., J. T. Crawford, R. E. Huebner, L. J. Geiter, C. R. Horsburgh, Jr., and R. C. Good.** 1993. The resurgence of tuberculosis: is your laboratory ready? *J. Clin. Microbiol.* **31:**767–770.

7.2 Acid-Fast Stains

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Mycobacteria are difficult to stain due to the presence of large amounts of lipid (mycolic acid) in the cell wall; hence, the use of the traditional Gram stain is of little to no value since the dyes do not usually permeate the mycobacterial cell walls. Instead, the most widely used methods to determine acid-fastness in a clinical specimen are the carbol fuchsin stains (Kinyoun or Ziehl-Neelsen) and the fluorochrome stain (e.g., auramine O or auramine-rhodamine). The fluorochrome stains are recommended for the examination of clinical specimens because of their increased sensitivity and speed, since they may be examined at a lower magnification than the carbol fuchsin stain (5, 7).

The acid-fast stain remains the most rapid method for the detection of mycobacteria. It can determine the presence of mycobacteria in a clinical specimen, monitor the progress of patients on antimycobacterial therapy, clarify a need for appropriate infection control procedures, dictate the appropriate dilution of sediments for drug susceptibility testing, and confirm the presence of acid-fast bacilli (AFB) in culture. Due to its poor sensitivity and specificity (5), the acid-fast smear is an adjunct and not a substitute for culture.

II. SPECIMENS

It is imperative that these cultures be handled in a biological safety cabinet.

A. Clinical specimens suspected of harboring mycobacteria or pure cultures are stained for acid-fastness. The value of staining gastric aspirates and urine is controversial. A smear directly from a clinical specimen (not concentrated) is discouraged because it lacks the sensitivity of a concentrated smear. A negative result from a direct smear must be followed by testing with a concentrated smear.

B. Specimens for the diagnosis of mycobacterial infection that are routinely acid-fast stained are from the respiratory tract (mostly sputa but also bronchial washings and bronchoalveolar lavage specimens) but may include sterile body fluids or tissue.

C. AFB smears are made from the sediments of specimens that have been decontaminated, when appropriate, and concentrated by centrifugation.

D. Preparation of smears for staining

1. Prepare a smear from the centrifuged specimen with either a bacteriological loop (3 mm in diameter), a sterile wooden applicator stick, or 1 drop from a sterile capillary pipette.

2. Allow smears to air dry in a biological safety cabinet (1).

3. Save sediments from each specimen and refrigerate as a backup for stain failure, a doubtful smear result, or contamination of culture. A smear directly from a clinical specimen (not centrifuged) is discouraged because it lacks the sensitivity of a concentrated smear.

4. Smears must be heat fixed before staining by using an electric staining rack or plate or with a Bunsen burner.

III. MATERIALS

Prepare as indicated in *Clinical Microbiology Procedures Handbook,* vol. 1, pages 3.5.3 to 3.5.5 (4), or purchase from ENG Scientific, Inc., Clifton, N.J.

A. Carbol fuchsin-stained smears (Kinyoun or Ziehl-Neelsen)
1. Carbol fuchsin
2. 3% Acid-alcohol
3. Methylene blue or brilliant green counterstain
4. Filter paper strips, Bunsen burner, or, preferably, an electric staining rack

B. Fluorochrome acid-fast stains
1. Auramine O (with or without rhodamine)
2. 0.5% Acid-alcohol
3. Potassium permanganate or acridine orange counterstain

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Positive and negative controls
1. Positive and negative control slides must be included whenever the AFB stain is performed and upon receipt of each new lot of materials, reagents, and media to verify the correct performance of the procedure. *Escherichia coli* is commonly used for the negative control and *Mycobacterium tuberculosis* H37Ra ATCC 25177 is the positive control.
2. Record the results of the control slides before reviewing the smears from clinical specimens. If the control slide is unacceptable, review procedures and reagent preparations.

B. Kinyoun carbol fuchsin stain
1. Positive: *Mycobacterium* species stain red or magenta against a blue or green background depending upon the counterstain used.
2. Negative: *E. coli* stains blue or green depending upon the counterstain used.

C. Fluorochrome stain (auramine O or auramine-rhodamine)
1. Positive: *Mycobacterium* species show a yellow to orange fluorescence (color may vary with the filter system used).
2. Negative: *E. coli* will show no fluorescence.

V. PROCEDURES

Discard slides after examination into a sharps receptacle; wear gloves when handling the slides. To avoid false-negative results, a minimum of 300 microscopic fields should be examined before the slide is reported as negative in industrialized countries and at least 100 fields should be examined in low-income countries (4).

A. Kinyoun carbol fuchsin stain
1. Flood the entire fixed slide with Kinyoun's carbol fuchsin.
2. Allow the smear to stain for 5 min.
3. Rinse the slide with water.
4. Flood the slide with 3% acid-alcohol to decolorize for 2 min and until no more color drains from the slide.
5. Rinse the slide with water; drain excess water from the slide.
6. Flood the slide with counterstain (methylene blue or brilliant green).
7. Counterstain for 2 min.
8. Rinse the slide thoroughly with water; drain excess water from the slide.
9. Air dry. Do not blot.
10. Examine with a 100× oil immersion objective (×1,000 total magnification) using a light microscope.

B. Ziehl-Neelsen (a hot carbol fuchsin stain)
1. Cover the heat-fixed slide with filter paper at 2 by 3 cm to minimize precipitation of crystals due to the heating and to keep the stain on the slide.

V. PROCEDURES *(continued)*

2. Flood the filter paper strip with the carbol fuchsin stain.
3. Heat the slide to steaming on an electric staining rack or with a Bunsen burner.
4. Let stand for 5 min. Add more stain if the smear dries, but do not reheat.
5. Remove filter paper strips with a forceps and discard into a container to be autoclaved later.
6. Wash slides with water.
7. Flood smear with acid-alcohol and allow destaining for 2 min.
8. Wash smear with water and drain.
9. Flood smear with the counterstain (methylene blue or brilliant green) for 1 to 2 min.
10. Rinse with water, drain, and then air dry.
11. Examine with a 100× oil immersion objective (×1,000 total magnification) using a light microscope.

C. Fluorochrome

1. Flood the slide with the fluorochrome stain.
2. Stain for 15 min.
3. Rinse the slide with water; drain the excess water from the slide.
4. Flood with 0.5% acid-alcohol.
5. Decolorize for 2 min.
6. Rinse the slide with water; drain the excess water from the slide.
7. Flood the slide with the counterstain (potassium permanganate or acridine orange).
8. Counterstain for precisely 2 min (a longer period may quench the fluorescence of the AFB when using the permanganate as counterstain).
9. Rinse with water; drain the excess water from the slide.
10. Air dry; do not blot.
11. Examine the smear with a fluorescent microscope with a 25× or 40× objective (×250 or ×450 total magnification).
12. The morphology is confirmed under oil immersion at a ×1,000 or ×450 magnification.
13. Fluorochrome-stained slides may be directly restained with the carbol fuchsin stain after immersion oil is removed with xylene.

VI. RESULTS

A. Fluorochrome stain

1. Positive: yellow to orange fluorescence (depending upon the filter system used) against a black background. Artifacts may fluoresce, so morphology must be carefully scrutinized.
2. Negative: no fluorescence

B. Carbol fuchsin (Kinyoun, Ziehl-Neelsen) stain

1. Positive: red-stained rods against blue or green background, depending on the counterstain used
2. Negative: no red-stained rods observed.

C. Mycobacteria typically appear as slender rods, curved, bent or filamentous, 1 to 10 μm long. Morphology of mycobacteria from liquid medium may assist in presumptive differentiation of *M. tuberculosis* complex from other mycobacteria as follows: *M. tuberculosis* and *Mycobacterium bovis* form typical serpentine cords and clumps, while other mycobacteria (except *Mycobacterium kansasii*) do not form cords.

D. Data are number of AFB per indicated number of microscopic fields. This takes into account the larger number of fields covered with the lower magnification examined under the fluorescent microscope.

VI. RESULTS *(continued)*

Fluorochrome		Carbolfuchsin, ×1,000	Report
×250	×450		
0	0	0	No AFB seen
1–9/10 fields	2–18/50 fields	1–9/100 fields	1+
1–9/field	4–36/10 fields	1–9/10 fields	2+
10–90/field	4–36/field	1–9/field	3+
>90/field	>36/field	>9/field	4+

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Negative: when no AFB are observed, report as "Negative for acid-fast bacilli."
B. Positive: report as "Positive for acid-fast bacilli" and provide the count.
C. Recommended reporting is listed on the chart above (3).

VIII. LIMITATIONS

A. The acid-fast stain is not specific for mycobacteria. Also, nonmycobacterial microorganisms such as *Nocardia, Rhodococcus, Legionella micdadei,* cysts of *Cryptosporidium* species, and *Isospora* species and other microsporidia may exhibit various degrees of acid-fastness (6).
B. The acid-fast stains cannot differentiate *M. tuberculosis* from nontuberculous mycobacteria.
C. The acid-fast smear lacks sensitivity. Compared with culture, sensitivities ranging from 22 to 78% have been reported (2). Although slow-growing mycobacteria like *M. tuberculosis* are consistently acid-fast, the rapidly growing mycobacteria may be variably acid-fast or even negative for acid-fastness especially with the fluorochrome stain. Also, a negative acid-fast smear does not rule out mycobacterial infection, because estimated detection levels for AFB in sputum are only 5,000 to 10,000 bacilli per ml of sputum (5). Culture detects 10 to 100 viable mycobacteria (2).

REFERENCES

1. **Centers for Disease Control and Prevention.** 1999. *Biosafety in Microbiological and Biomedical Laboratories,* 4th ed. Centers for Disease Control and Prevention, Atlanta, Ga.
2. **Daniel, T. M.** 1990. The rapid diagnosis of tuberculosis: a selective review. *J. Lab. Med.* **116:**277–282.
3. **Della-Latta, P., and I. Weitzman.** 1998. Acid-fast stain procedures, p. 176–178. *In* H. D. Isenberg (ed.), *Essential Procedures for Clinical Microbiology.* ASM Press, Washington, D.C.
4. **Ebersole, L. L.** 1992. Acid-fast stain procedures, p. 3.5.1–3.5.11. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook,* vol. 1. American Society for Microbiology, Washington, D.C.
5. **Kent , P. T., and G. P. Kubica.** 1985. *Public Health Mycobacteriology. A Guide for the Level III Laboratory,* p. 57–68. U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta, Ga.
6. **Pfyffer, G. E., B. A. Brown-Elliott, and R. J. Wallace, Jr.** 2003. *Mycobacterium,* p. 532–584. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology,* 8th ed. ASM Press, Washington, D.C.
7. **Salfinger, M., and G. E. Pfyffer.** 1994. The new diagnostic mycobacteriology laboratory. *Eur. J. Clin. Microbiol. Infect. Dis.* **13:**961–979.
8. **Yeager, H. J., Jr., J. Lacy, L. Smith, and C. LeMaistre.** 1967. Quantitative studies of mycobacterial populations in sputum and saliva. *Am. Rev. Respir. Dis.* **95:**998–1004.

7.3 Solid Media for Isolation

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Both liquid and solid media are recommended for optimal recovery of mycobacteria. The advantage of solid media (tubed or in plates) is that they enable detection of mixed cultures and contaminants. Egg-based and agar-based media may be used. The main advantage of an egg-based medium is that it supports the growth of most mycobacteria and permits niacin testing. However, contamination occurs more easily involving the total surface of the medium. The main advantages of agar-based media are less contamination and earlier and easier visibility of colonial morphology. Colonial morphology aids in the identification of mycobacteria. Use of both nonselective and selective media is needed for isolation, the latter containing one or more antimicrobial agents to prevent overgrowth by contaminating bacteria or fungi (2).

II. SPECIMEN

It is imperative that these cultures be handled in a biological safety cabinet.

A. Only pure cultures that are acid-fast bacillus (AFB) positive are identified.

B. Discard contaminated cultures. If the patient's specimen is still available, decontaminate by another method or request another specimen. Specimens repeatedly contaminated with *Pseudomonas* species or other organisms could be decontaminated by the oxalic acid, *N*-acetyl-L-cysteine–NaOH–oxalic acid, or other methods (1, 6).

III. MATERIALS

A. Media

Table 7.3–1 lists the selective and nonselective media commonly available. Nonselective media are recommended for use with specimens from normally sterile sites. Selective media are recommended for use with contaminated specimens.

B. Supplies

1. Bactericidal-mycobactericidal disinfectant
2. Gloves, masks, and gowns
3. Cotton or gauze pads
4. Sterile Pasteur pipettes

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Prepared media

1. Laboratories purchasing commercially prepared media that are quality controlled by the manufacturers in accordance with NCCLS-proposed standards need not perform QC checks for sterility, growth, selectivity, or biochemical response on Lowenstein-Jensen and Middlebrook media (3).
2. Documentation of the manufacturer's QC procedures should be obtained, by placing the lot number of each medium in a log book.
3. All other media, including user-prepared media, must be quality controlled with the appropriate positive and negative control organisms to ensure sat-

Table 7.3–1 Media commonly available for recovery of mycobacteria (2)[a]

Medium	Components	Inhibitory agent
Nonselective		
Lowenstein-Jensen	Coagulated whole eggs, defined salts, glycerol, potato flour	Malachite green, 0.025 g/100 ml
Petragnani	Coagulated whole eggs, egg yolks, whole milk, potato flour, glycerol	Malachite green, 0.052 g/100 ml
American Thoracic Society	Coagulated fresh egg yolks, potato flour, glycerol	Malachite green, 0.02 g/100 ml
Middlebrook 7H10	Defined salts, vitamins, cofactors, oleic acid, albumin, catalase, glycerol, glucose	Malachite green, 0.0025 g/100 ml
Middlebrook 7H11	Defined salts, vitamins, cofactors, albumin, catalase, glycerol, 0.1% casein hydrolysate	Malachite green, 0.025 g/100 ml
CHOC	Hemolyzed blood agar with IsoVitaleX and bovine hemoglobin enrichment	
Selective		
Gruft modification of Lowenstein-Jensen	Coagulated whole eggs, defined salts, glycerol, potato flour, RNA (5 mg/100 ml)	Malachite green, 0.025 g/100 ml Penicillin, 50 U/ml Nalidixic acid, 35 μg/ml
Mycobactosel Lowenstein-Jensen	Coagulated whole eggs, defined salts, glycerol, potato flour	Malachite green, 0.025 g/100 ml Cycloheximide, 400 μg/ml Lincomycin, 2 μg/ml Nalidixic acid, 35 μg/ml
Mycobactosel Middlebrook 7H10	Defined salts, vitamins, cofactors, oleic acid, albumin, catalase, glycerol, glucose	Malachite green, 0.025 g/100 ml Cycloheximide, 360 μg/ml Lincomycin, 2 μg/ml Nalidixic acid, 20 μg/ml
Selective 7H11 (Mitchison's medium)	Defined salts, vitamins, cofactors, oleic acid, albumin, catalase, glycerol, glucose, casein hydrolysate	Carbenicillin, 50 μg/ml Amphotericin B, 200 U/ml Polymyxin B, 200 U/ml Trimethoprim lactate, 20 μg/ml

[a] Reproduced and modified from **H. M. Sommers,** 1984. Mycobacterial diseases. *In* J. B. Henry (ed.), *Clinical Diagnosis and Management by Laboratory Methods,* 17th ed. The W. B. Saunders Co., Philadelphia, Pa., with permission of Elsevier.

IV. QUALITY CONTROL *(continued)*

isfactory performance and examined for signs of deterioration (color, dehydration, presence of contamination, bubbles, etc.).

4. Do not use media beyond the expiration date.
5. User-prepared media may be checked for sterility by incubating 1 to 3% of each batch and must be quality controlled and documented for performance. The microorganisms chosen for QC depend on the media. Generally, ATCC stock cultures of *Mycobacterium tuberculosis, Mycobacterium kansasii, Mycobacterium scrofulaceum, Mycobacterium intracellulare,* and *Mycobacterium fortuitum* are used. *Escherichia coli* can be the test organism to determine the inhibitory effectiveness of selective antimicrobial media.

IV. QUALITY CONTROL *(continued)*

6. All QC results must be documented and reviewed by a designated supervisor. Documentation of corrective action, resolution, and follow-up of the situation is required.

B. Inoculation

1. Make a suspension of the organism in 7H9 broth equal to a 0.5 McFarland standard.
2. For tubed media, use a calibrated loop or pipette to inoculate each tube to be tested with 10 μl of suspension. Incubate at 35 to 37°C in 5 to 10% CO_2 for up to 21 days.

C. Interpretation of results

Expected results of QC testing of mycobacterial media are shown below.

Control type and recommended ATCC strains	Result
Positive controls	
M. tuberculosis ATCC 25177	Growth on all media
M. kansasii ATCC 12478	Growth on all media
M. scrofulaceum ATCC 19981	Growth on all media
M. intracellulare ATCC 13950	Growth on all media
M. fortuitum ATCC 2841	Growth on all media
Negative control	
E. coli ATCC 25922	Partial or total inhibition

V. PROCEDURE

A. Add 3 drops of sediment to Lowenstein-Jensen agar with a sterile transfer pipette, allowing the sediment to spread over the surface of the slant. Store slants with caps loosened and place in a slanted rack.

B. Add 1 drop of sediment to plated agar (Middlebrook 7H10 and/or 7H11) and streak sediment over the surface of the agar. Place each plate uninverted into a CO_2-permeable plastic bag. Invert plates after 24 h to allow sediment to be absorbed into the agar.

C. Incubate all cultures in the dark at 35 to 37°C, with the exception of skin or soft tissue suspected to be infected with *Mycobacterium marinum, Mycobacterium ulcerans,* or any specimen suspected to be infected with *Mycobacterium haemophilum* for which an additional separate set of media is incubated at 30°C.

D. Examine solid media within 3 to 5 days after incubation, twice a week up to week 4, and weekly thereafter until week 8 (4).

E. Before examining agar plates, seal with Parafilm and examine these plates microscopically to facilitate the early observation of microcolonies.

VI. RESULTS AND INTERPRETATION

Morphological features

A. AFB smear-positive colonies growing in pure culture are considered positive.

B. These pure cultures should be identified (5).

C. Colonies on clear agar media in petri dishes should be inverted and examined under a microscope for cording and presence of filaments. Slow-growing, dry, corded colonies are suggestive of *M. tuberculosis* or *M. kansasii* (1).

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report as culture positive only when confirmed by acid-fast smear.
B. Report as "Culture positive for mycobacteria, identification pending."
C. Immediate reporting of AFB-positive results to the physician in charge and the local or state department of health is required.

REFERENCES

1. **Kent, P. T., and G. P. Kubica.** 1985. *Public Health Mycobacteriology. A Guide for the Level III Laboratory*. U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta, Ga.
2. **Lambi, E. A.** 1992. Medium selection and incubation for the isolation of mycobacteria, p. 3.6.1.–3.6.8. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook*, vol. 1. American Society for Microbiology, Washington, D.C.
3. **NCCLS.** 1990. *Quality Assurance for Commercially Prepared Microbiological Culture Media.* NCCLS document M-22A, vol. 16, no. 16. NCCLS, Villanova, Pa.
4. **Pfyffer, G. E., B. A. Brown-Elliott, and R. J. Wallace, Jr.** 2003. *Mycobacterium*, p. 532–584. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
5. **Silcox, V.** 1992. Identification of mycobacteria, p. 3.11.1–3.11.11. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook*, vol. 1. American Society for Microbiology, Washington, D.C.
6. **Whittier, S., R. L. Hopfer, M. R. Knowles, and P. H. Gilligan.** 1993. Improved recovery of mycobacteria from respiratory secretions of patients with cystic fibrosis. *J. Clin. Microbiol.* **31:**861–864.

7.4.1 BACTEC 460TB Radiometric System

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

In the BACTEC 460TB radiometric system (Becton Dickinson Diagnostic Systems, Sparks, Md.), growth medium for culturing mycobacteria is supplemented with a substrate labeled with radioactive carbon (^{14}C). This substrate is utilized by mycobacteria, and during metabolism, carbon dioxide ($^{14}CO_2$) is produced from the substrate. The $^{14}CO_2$ is detected quantitatively by counting the radioactivity with a BACTEC 460 instrument. The rate and amount of $^{14}CO_2$ produced are directly proportional to the rate and amount of growth occurring in the medium. This principle is applied for isolation of mycobacteria from clinical specimens, differentiation of the *Mycobacterium tuberculosis* complex from other mycobacteria, and antimicrobial susceptibility testing.

II. SPECIMENS

Observe standard precautions.

A. Pulmonary and extrapulmonary specimens can be inoculated in BACTEC 12B medium for mycobacterial isolation. Blood, bone marrow, and sometimes CSF are inoculated into BACTEC 13A medium.

B. Blood specimens are collected as follows.

1. If the BACTEC 13A medium is to be inoculated at the patient's bedside, collect 5 ml of blood aseptically with a syringe without any anticoagulants.

2. If the specimen is to be transported, collect blood in a tube with an anticoagulant that is acceptable for bacterial cultures, such as sodium polyanethol sulfonate (e.g., WAMPOLE ISOLATOR tube, Wampole Laboratories, Cranbury, N.J.).

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Media

1. Non-blood specimens

a. BACTEC 12B (Middlebrook 7H12) medium
This is a broth medium with 7H9 broth base, bovine serum albumin, casein hydrolysate, catalase, and ^{14}C-labeled substrate (1 μCi/ml). A 20-ml vial containing 4 ml of broth is sealed with a rubber septum. BACTEC 12B medium may be used as a stand-alone medium, but for a maximum recovery of mycobacteria, an additional solid medium is recommended.

b. Antimicrobial mixture PANTA
PANTA is a mixture of five antimicrobial agents (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) provided in lyophilized form and should be added to 12B medium for suppression of contaminating bacteria and fungi. PANTA is used for primary isolation only.

c. Reconstituting fluid
This fluid is used to reconstitute lyophilized PANTA. It is an aqueous solution of polyoxyethylene stearate (POES) that is known to enhance growth of mycobacteria, especially the *M. tuberculosis* complex.

III. MATERIALS *(continued)*

d. Diluting fluid
This fluid (0.2% Tween 80 and 0.2% fatty acid-free bovine serum albumin in water) is used for diluting cultures for susceptibility testing.

2. Blood specimens
- **a.** BACTEC 13A, Middlebrook 7H13 medium: hypotonic medium, 30 ml in a 50-ml vial sealed with a rubber septum
- **b.** BACTEC 13A enrichment: to be added to 13A medium for support of mycobacterial growth
- **c.** Antimicrobial mixture PANTA: to suppress the growth of contaminating bacteria
- **d.** Reconstituting fluid: to reconstitute lyophilized PANTA

B. Supplies

1. Disposable allergist's or tuberculin syringes with permanently attached needles
2. Bactericidal-mycobactericidal disinfectant (examples: Amphyl, Cidex)
3. Gloves, respirators, and gowns
4. Cotton or gauze pads
5. Alcohol swabs

C. Equipment

1. BACTEC 460 instrument
This semiautomated instrument can test 60 vials at a time at a rate of 82 ± 2 s per vial. The headspace gas from the vial is aspirated by two needles, and the quantity of $^{14}CO_2$ is determined on a scale of 0 to 999 called the growth index (GI). These numbers are displayed on the front panel and printed on a tape along with information about the position of the vial on the instrument.
2. A carbon dioxide tank containing 5 to 10% CO_2 and 90 to 95% air is attached to the instrument. During testing, the instrument introduces fresh 5 to 10% CO_2, which is required for the growth of many mycobacteria.
3. BACTEC TB hood
The BACTEC 460 instrument should be used with a BACTEC TB hood, which is placed on top of the instrument. The hood is equipped with a UV light, a HEPA filter, and a laminar-flow system.
4. Biological safety cabinet
5. Incubator, 37 ± 1°C
6. Vortex mixer

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. BACTEC 12B medium

1. Prepare a uniform suspension of *M. tuberculosis* H37Rv ATCC 27294 with turbidity equivalent to a McFarland no. 1 standard. A standardized suspension frozen at −70°C may also be used.
2. Dilute the suspension 1:500 by using the diluting fluid. Mix well.
3. Inoculate 0.1 ml per vial into four vials of 12B medium.
4. Incubate at 37 ± 1°C, and test daily on a BACTEC 460 instrument for 12 to 14 days.
5. The average peak growth index should be 400 or above and should be achieved in 12 ± 2 days.
6. If results do not meet the specification, check the incubation temperature and adjust to 37 ± 1°C. Check the culture suspension. It should be made from a fresh viable culture that is not more than 3 weeks old. If the suspension is frozen at −70°C, it should be used within 6 months of storage and should contain at least 2×10^6 CFU/ml.

B. Instrument QC

1. Performance test
- **a.** Performance test should be done each day of testing by following the procedure given in the *BACTEC 460 Operation and Maintenance Manual* (supplied by the manufacturer).
- **b.** A growth index of 50 to 60 should be obtained.

IV. QUALITY CONTROL
(continued)

c. If the growth index is outside this range, repeat the test with the same acid vial.

d. If the repeat test fails, do not test patient vials. Refer to the *BACTEC 460 Operation and Maintenance Manual.*

2. Instrument maintenance

a. The maintenance schedule for the BACTEC 460 instrument should be strictly followed. Good maintenance of the needle heater, filter, medium trap, and UV light will help prevent cross-contamination.

b. Change needles daily. Proper maintenance of the instrument needles is especially critical.

V. PROCEDURE

Observe standard precautions.

A. Non-blood specimen processing

1. The *N*-acetyl-L-cysteine–sodium hydroxide procedure for specimen decontamination is the method of choice.

2. The sodium hydroxide and oxalic acid methods can also be used.

3. Do not use the Zephiran-trisodium phosphate, benzalkonium chloride, lauryl sulfate, or cetylpyridinium chloride method with the BACTEC procedure, as residual quantities of these substances in the inoculum inhibit mycobacterial growth.

B. Inoculation of non-blood specimens

1. Prior to inoculation, flush 12B vials with 5 to 10% CO_2 by testing them on a BACTEC 460 instrument. Do not use vials with a growth index of $\geq$20, as this may indicate contaminated medium.

2. Addition of PANTA

Prior to the inoculation of concentrated, decontaminated specimens, add PANTA to the vial to suppress contamination.

a. Add 5 ml of reconstituting fluid to the lyophilized PANTA vial.

b. Mix thoroughly. When PANTA is completely dissolved, add 0.1 ml of the solution to each 12B vial. The same syringe may be used to add PANTA into several vials.

c. The remaining PANTA solution may be stored at 2 to 8°C for 3 days, -5 to -20°C for 3 months, or -70°C for 6 months.

d. It is not necessary to add PANTA to specimens (such as certain extrapulmonary specimens) that are expected to be free of contaminating organisms. In such a case, add only 0.1 ml of diluting fluid to introduce POES in the medium. If in doubt, use two 12B medium vials, one with and one without PANTA, especially with extrapulmonary specimens.

3. Inoculation of specimen

Use a tuberculin syringe with a permanently attached needle to inoculate 0.1 to 0.5 ml of processed specimen into a 12B vial supplemented with PANTA. For tissue or other particulate specimens, use a larger-gauge needle (20 to 22 gauge) with a Luer-Lock. Use a separate syringe for each specimen.

4. Clean the top of the rubber septum with an appropriate mycobactericidal disinfectant and then wipe the septum with a 70% isopropyl alcohol swab.

C. Blood specimen processing

Blood is collected aseptically, and thus, a digestion-decontamination step is not required. Because of the large volume of the medium, up to 5 ml of specimen can be inoculated into the medium, which is hypotonic, thus resulting in lysis of the blood cells (8).

D. Inoculation of blood specimens

1. Inoculate up to 5 ml of blood directly into 13A medium after disinfecting the top of the vial with an alcohol swab.

V. PROCEDURE *(continued)*

2. Disinfect the top of the vial with an alcohol swab, and add 0.5 ml of 13A enrichment to a 13A vial before or after specimen inoculation.
3. Use a separate syringe for each vial if the enrichment is added after the specimen is inoculated.
4. Do not test the vial on a BACTEC 460 instrument before or just after inoculation. The vials contain a CO_2 atmosphere and a vacuum sufficient to accommodate 5 to 7 ml of blood.
5. Add BACTEC antimicrobial supplement PANTA if the specimen is expected to be contaminated with other bacteria. Reconstitute PANTA with 2.5 ml of reconstituting fluid, and add 0.4 ml to a 13A vial.
6. Clean the top of the vial septum with an alcohol swab before inoculation and with an appropriate mycobactericidal disinfectant. Then use an alcohol swab to wipe after inoculation.

E. Incubation

1. Incubate vials at 37 ± 1°C.
2. The temperature of incubation is critical. Check temperature with an accurate thermometer right where the vials are placed.
3. Specimens taken from wounds or ulcers should be incubated in duplicate, one at 37°C and the other at 30°C, because certain mycobacteria such as *Mycobacterium marinum* grow at the lower temperature.
4. It is not necessary to incubate vials in the dark, in a CO_2 incubator, or with shaking.

F. Testing schedule

The testing schedule should be decided according to the workload.

1. Maximum testing (low-volume laboratories): three times per week, with a 2- to 3-day interval for the first 3 weeks, and weekly thereafter for a total of 6 weeks.
2. Minimum testing (high-volume laboratories): twice weekly, with a 3- to 4-day interval for the first 2 weeks, and weekly thereafter for a total of 6 weeks.
3. With more frequent testing, earlier detection of positive cultures is expected.
4. Once the growth index reaches 10 or more, test the vial daily.

VI. RESULTS

A. Non-blood specimens: detection of growth

1. Negative: growth index less than 10.
2. Positive: growth index of 10 or more. Separate these vials and test daily.
3. Examine all positive vials for the presence of contamination, which is indicated by the presence of turbidity or a very rapid increase in growth index.
4. If contaminated, the culture can be salvaged by reprocessing the medium.
   - **a.** Remove the medium from the vial.
   - **b.** Add an equal quantity of 4% sodium hydroxide solution.
   - **c.** Mix well, and let stand for 15 to 20 min.
   - **d.** Add phosphate buffer (pH 6.8) to the 40-ml line indicated on the tube.
   - **e.** Concentrate by centrifugation (3,000 × *g*) for 20 min.
   - **f.** Suspend the sediment in 0.5 ml of pH 6.8 phosphate buffer, and inoculate 0.5 to 1.0 ml into a fresh 12B vial with PANTA.
   - **g.** Incubate, and continue testing on the BACTEC 460 instrument.

B. Non-blood specimens: expected values

1. Growth index of ≥10
   Culture is presumptive positive. Test the vial daily, and rule out contamination.
2. Growth index of 50 to 100
   Perform BACTEC NAP test.

VI. RESULTS *(continued)*

3. Growth index of ~100
Make a smear, stain for acid-fast bacilli, and confirm if it is positive for mycobacteria. BACTEC 12B medium does not stick to the slide well unless additional proteins are added to the smear. Therefore, a 20% solution of rabbit serum (in sterile water with 0.02% thimerosal and 0.5% phenol) as a smear-fixing solution yields the best results.

4. Growth index of ≥500
Perform antimicrobial susceptibility test if desired. The rate of growth in 12B medium depends on the species of mycobacteria isolated, *M. tuberculosis* and *Mycobacterium bovis* being the slowest. Among mycobacteria other than the *M. tuberculosis* complex commonly isolated from specimens, *Mycobacterium kansasii* is the slowest grower. Most other mycobacteria, including those that grow slowly on solid media, grow rapidly in 12B medium.

5. BACTEC 12B medium has been reported to yield more positive cultures from clinical specimens than other media (5, 7, 10). This is particularly significant with smear-negative specimens and specimens from chronic, treated patients (6, 7). Improved detection of *Mycobacterium avium* complex in 12B medium has also been reported (2).

6. Time to detection for positive growth is significantly shorter than with any other medium (1, 2, 6, 7). The detection time could vary because of the following factors.

- **a.** Type of specimen
  The average detection time is longer for certain specimens, such as extrapulmonary specimens, that harbor smaller numbers of bacteria.
- **b.** Degree of smear positivity
  Organisms in a smear showing numerous acid-fast bacilli may be detected as early as 24 h, while organisms in smear-negative, culture-positive specimens may take 5 to 6 weeks to be detected.
- **c.** Therapy status of the patient
  Generally, organisms in specimens from patients receiving antituberculosis treatment take a longer time to grow.
- **d.** Decontamination procedure
  Higher sodium hydroxide concentrations or harsh treatment damages mycobacterial cells, and they take a longer time to grow. Generally, laboratories using lower concentrations of sodium hydroxide or milder decontamination procedures report results within an average of 7 to 9 days.
- **e.** Incubation temperature
  Temperature is critical for the BACTEC system, since the system is based on metabolism of mycobacteria, which is optimum at 37.0 to 37.5°C. Lower temperatures slow down the detection time. Generally, it has been reported that positive cultures are detected between 9 and 12 days (6, 7, 10).

7. If contamination in 12B medium is higher than 5%, check all procedures and reagents thoroughly. If a higher contamination rate exists, take the following steps.

- **a.** Increase the sodium hydroxide concentration to 5%. (Do not increase time of exposure to sodium hydroxide.)
- **b.** Increase the concentration of PANTA by reconstituting PANTA with 3 or 4 ml (instead of 5 ml) of reconstituting fluid. Add 0.1 ml of the reconstituted PANTA. This will result in a higher concentration of PANTA in the 12B medium.
- **c.** Increase the concentration of *N*-acetyl-L-cysteine if specimens are more mucoid and hard to liquefy.

VI. RESULTS *(continued)*

C. Blood vials: detection of growth

1. Background/negative growth index: 10 to 15
2. Positive growth index: 20 or more
3. Prepare a smear at this point, and stain for acid-fast bacteria.
4. If the smear is positive, pull out the vial and incubate separately for further investigation. If the smear is negative, continue incubation and testing.
5. If the growth indices are high and the acid-fast smear is negative, check for contamination by Gram stain, and subculture on a blood agar plate.
6. Mycobacterial growth and other bacterial growth may be present.
7. Add the recommended concentration of BACTEC antimicrobial supplement PANTA if bacterial growth is observed. Continue incubation and testing.

D. Blood specimens: expected values

1. BACTEC 13A is a sensitive medium and can detect blood specimens having a very low concentration of viable acid-fast bacilli (11).
2. The detection of growth is slightly slower in 13A medium than in 12B medium because of the presence of blood. The smear made at a growth index of 20 is usually positive.
3. A positive culture can usually be detected in 12 to 16 days (3, 4, 9).

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Blood and non-blood specimens

1. Report as culture positive only when the growth index is positive and is confirmed by acid-fast smear made from the positive broth.
2. Report as "Culture positive for mycobacteria, identification pending."

B. Blood only

Subculture in 12B medium and/or Lowenstein-Jensen medium for further identification and antimicrobial susceptibility testing.

VIII. LIMITATIONS

A. Colony morphology cannot be observed.
B. Infection with more than one *Mycobacterium* species or the presence of both mycobacteria and bacteria cannot be detected separately.
C. If even a single contaminating bacterium is present, the entire medium will be contaminated.
D. A subculture on a Middlebrook agar plate is needed to isolate a mixed culture.

REFERENCES

1. **Fadda, G., and S. Roe.** 1984. Recovery and susceptibility testing of *Mycobacterium tuberculosis* from extrapulmonary specimens by the BACTEC radiometric method. *J. Clin. Microbiol.* **19:**720–721.
2. **Hoffner, S. E.** 1988. Improved detection of *M. avium* complex with the BACTEC radiometric system. *Diagn. Microbiol. Infect. Dis.* **10:**1–6.
3. **Kiehn, T. E., and R. Cammarata.** 1986. Laboratory diagnosis of mycobacterial infections in patients with acquired immunodeficiency syndrome. *J. Clin. Microbiol.* **24:**708–711.
4. **Kiehn, T. E., and R. Cammarata.** 1988. Comparative recoveries of *Mycobacterium avium-M. intracellulare* from isolator lysis-centrifugation and BACTEC 13A blood culture systems. *J. Clin. Microbiol.* **26:**760–761.
5. **Kirihara, J. M., S. L. Hillier, and M. B. Coyle.** 1985. Improved detection times for *Mycobacterium avium* complex and *Mycobacterium tuberculosis* with the BACTEC radiometric system. *J. Clin. Microbiol.* **22:**841–845.
6. **Morgan, M. A., C. D. Horstmeier, D. R. DeYoung, and G. D. Roberts.** 1983. Comparison of a radiometric method (BACTEC) and conventional culture media for recovery of mycobacteria from smear-negative specimens. *J. Clin. Microbiol.* **18:**384–388.

REFERENCES *(continued)*

7. **Roberts, G. D., N. L. Goodman, L. Heifets, H. W. Larh, T. H. Lindner, J. K. McClatchy, M. R. McGinnis, S. H. Siddiqi, and P. Wright.** 1983. Evaluation of BACTEC radiometric method for recovery of mycobacteria and drug susceptibility testing of *Mycobacterium tuberculosis* from acid-fast smear-positive specimens. *J. Clin. Microbiol.* **18:**689–696.
8. **Siddiqi, S. H., and C. Hwangbo.** 1987. A new medium (Middlebrook 7H13) for recovery of mycobacteria from blood specimens, abstr. U-78. *Program Abstr. Annu. Meet. Am. Soc. Microbiol. 1987.* American Society for Microbiology, Washington, D.C.
9. **Strand, C. L., C. Epstein, S. Verzosa, E. Effatt, P. Hormozi, and S. H. Siddiqi.** 1989. Evaluation of a new blood culture medium for mycobacteria. *Am. J. Clin. Pathol.* **91:**316–318.
10. **Takahashi, H., and V. Foster.** 1983. Detection and recovery of mycobacteria by a radiometric procedure. *J. Clin. Microbiol.* **17:**380–381.
11. **Witebsky, F. G., J. F. Keiser, P. S. Conville, R. Bryan, C. H. Park, R. Walker, and S. H. Siddiqi.** 1988. Comparison of BACTEC 13A medium and DuPont Isolator for detection of mycobacteria. *J. Clin. Microbiol.* **26:**1501–1505.

7.4.2 BACTEC MGIT 960 Automated System

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The mycobacteria growth indicator tube (MGIT tube) is designed for the rapid detection of mycobacteria in all types of clinical specimens except blood and urine. The BACTEC MGIT 960 automated system, by Becton Dickinson Diagnostic Systems (Sparks, Md.) includes a liquid culture medium (modified Middlebrook 7H9 broth), a growth supplement, and an antimicrobial agent mixture. The antimicrobial agent mixture suppresses the growth of contaminating bacteria.

A fluorescent compound is embedded in silicone on the bottom of each MGIT tube, which is sensitive to the presence of oxygen dissolved in the broth. Initially, the large amount of dissolved oxygen quenches the emissions of fluorescence from the compound and little fluorescence can be detected. Later, actively respiring microorganisms consume the oxygen and the depletion of oxygen allows the compound to fluoresce.

II. SPECIMENS

The MGIT tubes are used for the isolation of mycobacteria from pulmonary and extrapulmonary specimens. Use of urine and blood specimens is not validated. Specimens that are expected to contain other bacteria, such as sputum, must be digested and decontaminated. Aseptically collected specimens, which are not expected to contain contaminating bacteria, can be inoculated without decontamination.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Media and instrumentation

1. BBL MGIT
   This medium contains 7 ml of modified Middlebrook 7H9 broth base with casein peptone.
2. MGIT 960 supplement kit
   The kit contains the following items.
   a. Growth supplement containing bovine albumin with glucose, catalase, oleic acid, and polyoxyethylene stearate
   b. BBL MGIT PANTA
      A lyophilized mixture of antimicrobials (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin)
3. BACTEC MGIT 960 system instrument

B. Supplies

1. Automated pipettor for addition of growth supplement into MGIT tube
2. Bactericidal-mycobactericidal disinfectant (examples: Amphyl, Cidex)
3. Safety equipment

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Each new shipment or lot number of MGIT tubes may be tested using the following strains and dilutions. The test culture should not be more than 15 days old. Prepare a suspension from growth on solid medium, which is well dispersed, free of large clumps, and with a turbidity comparable with a McFarland 0.5 standard. When 0.5 ml of diluted suspension is inoculated into an MGIT tube supplemented with enrichment, it should be detected as instrument positive within the time frames shown below.

Species	Dilution of 0.5 McFarland in saline	Days to instrument positivity
Mycobacterium tuberculosis ATCC 27294	1:500	6–10
Mycobacterium kansasii ATCC 12478	1:50,000	6–11
Mycobacterium fortuitum ATCC 6841	1:5,000	1–3

All the inoculations should be done under a biological safety cabinet with all the standard safety precautions.

B. The following is performed daily on the BACTEC MGIT 960 instrument.

1. Check temperature readout of each drawer (37 ± 2°C).

2. Check all three external indicator lamps on all three drawers by pressing "test drawer indicators" soft key.

3. For each drawer, test red and green light-emitting diode (LED) lights by pressing appropriate soft keys.

4. Check printer paper supply and print out "Quality Control Report."

5. Clean and replace filters monthly.

V. PROCEDURE

Observe standard precautions.

It is imperative that these cultures be handled in a biological safety cabinet.

A. Specimen preparation

1. For those specimens requiring digestion and decontamination, use established conventional procedures for digestion and decontamination that are compatible with non-egg-based media. The *N*-acetyl-L-cysteine–sodium hydroxide method is the method of choice. Concentrate by established centrifugation procedure. If using the Petroff's NaOH method, a very high pH for the inoculum may result in a temporary fluorescence.

2. Do not use the Zephiran-trisodium phosphate, lauryl sulfate, benzylkonium chloride, or cetylpyridinium chloride method with the MGIT medium.

3. For specimens that do not require decontamination, inoculate 0.5 ml after the process of concentration by centrifugation.

B. Specimen inoculation

1. Reconstitute a lyophilized vial of BBL MGIT PANTA antimicrobial agent mixture with 15 ml of BACTEC MGIT growth supplement.

2. Label the MGIT tube with the specimen number.

3. Aseptically add 0.8 ml of growth supplement-antimicrobial agent mixture into the 7-ml MGIT tube just prior to specimen inoculation.

4. Add 0.5 ml of specimen suspension (digested, decontaminated, and concentrated if required); tightly recap and mix well.

5. Enter tubes into the instrument by scanning the bar codes.

6. Insert tubes into the instrument in the slots assigned by the BACTEC MGIT instrument, indicated by a green light.

V. PROCEDURE *(continued)*

C. Instrument-positive cultures

1. The positive indicator lamp on the front of the drawer illuminates and the audible alert sounds until acknowledged.
2. Press the silence alarm key and open appropriate drawer.
3. Remove positive tubes whose stations illuminate with flashing green and red indicators.
4. Scan the positive tube's bar code to extinguish LED light for that tube's station.
5. Prepare an acid-fast stain.
6. An acid-fast bacillus (AFB) smear-positive BBL MGIT tube can be subcultured to both selective and nonselective mycobacterial media for isolation, identification, and susceptibility testing.
7. Contamination may also cause instrument-positive results. Contaminated cultures may be reprocessed by decontamination/concentration and inoculated into a fresh MGIT tube.
8. Smear-negative "instrument-positive" tubes with no contamination may be returned to the instrument within 5 h of their removal.

D. Out-of-protocol negatives

1. Press the "remove negative tubes" soft key.
2. Remove tubes from flashing green stations.
3. The flashing red light on the front panel indicates station error.

VI. RESULTS AND INTERPRETATION

A. Positive culture: positive tubes are detected by the instrument and confirmed by an acid-fast smear.

B. Negative culture: negative tubes are not detected by the instrument and are removed as "out-of-protocol" negatives after 42 or 56 days.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. If AFB smear from MGIT tube is positive, report as "AFB present; identification to follow."

B. If AFB negative and contaminated, report as contaminated.

C. All out-of-protocol negative tubes should be observed visually before being reported as negative.

VIII. LIMITATIONS

A. Recovery and time to detection of positive culture for mycobacteria are dependent on the number of organisms present in the specimen, specimen quality, specimen collection method, method of specimen processing, and patient factors such as history of antituberculosis treatment.

B. Colony morphology and pigmentation can be determined only on solid media. Mycobacteria may vary in acid-fastness depending on species, age of culture, and other variables.

C. BBL MGIT tubes which are instrument positive may contain other nonmycobacterial species. Nonmycobacterial species may overgrow mycobacteria present in the medium.

D. BBL MGIT tubes which are instrument positive may contain one or more species of mycobacteria. Faster-growing mycobacteria may be detected prior to slower-growing mycobacteria; therefore, it is important to subculture positive MGIT tubes onto solid media to ensure proper identification of all mycobacteria present in the sample.

VIII. LIMITATIONS *(continued)*

E. Due to the richness of the BBL MGIT broth and to the nonselective nature of the MGIT indicator, it is important to strictly follow the recommended digestion-decontamination procedure to reduce the possibility of contamination. Adherence to procedural instruction, which includes use of the recommended inoculum volume (0.5 ml), is critical for optimal recovery of mycobacteria.

F. The use of BBL MGIT PANTA antimicrobial agent mixture, although necessary for all nonsterile specimens, may have inhibitory effects on some mycobacteria.

SUPPLEMENTAL READING

Bloom, B. R., and C. J. L. Murray. 1992. Tuberculosis: commentary on a re-emergent killer. *Science* **257:**1055–1064.

Cohn, M. L., R. F. Waggoner, and J. K. McClatchy. 1968. The 7H11 medium for the cultivation of mycobacteria. *Am. Rev. Respir. Dis.* **98:**295–296.

Horsburgh, C. R., Jr. 1991. *Mycobacterium avium* complex infection in the acquired immunodeficiency syndrome. *N. Engl. J. Med.* **324:**1332–1338.

Isenberg, H. D. (ed.). 1992. *Clinical Microbiology Procedures Handbook*, vol. 1. American Society for Microbiology, Washington, D.C.

Kent, P. T., and G. P. Kubica. 1985. *Public Health Mycobacteriology. A Guide for the Level III Laboratory*. U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta, Ga.

Occupational Safety and Health Administration. 1991. Occupational exposure to bloodborne pathogens: final rule. 29CFR part 1910.1030. *Fed. Regist.* **56:**64175–64182.

Youmans, G. P. 1979. *Tuberculosis*, p. 25–35. The W. B. Saunders Company, Philadelphia, Pa.

7.4.3 VersaTREK (ESP Culture System II)

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The VersaTREK (ESP Culture System II) (Trek Diagnostic Systems, Inc., Westlake, Ohio) for the isolation of mycobacteria from clinical specimens consists of a modular arrangement of incubator and detection instruments and medium-containing culture bottles. The VersaTREK instrument consists of a series of drawers, each containing locations for the placement of culture bottles. The Myco bottles contain 7H9 broth and cellulose sponges utilized as a growth platform. The Myco bottles are supplemented with Myco growth supplement (GS) (Middlebrook oleic acid-albumin-dextrose-catalase [OADC] enrichment) and an antimicrobial agent solution prior to inoculation with the specimen. Each location contains a sensor to which the inoculated culture bottle is attached via a plastic connector. The VersaTREK (ESP) instrument detects mycobacterial growth by automatically monitoring (every 24 min) the rate of consumption of oxygen within the headspace of the culture bottle. This information is used to generate a curve (graph) for each bottle. Internal algorithms analyze these changes and signal a positive culture when appropriate. Positive culture bottles are smeared and stained to confirm the presence of acid-fast bacilli (AFB) (2). Aliquots from the bottles containing AFB may be tested directly with nucleic acid probes to rapidly identify the majority of mycobacteria growing in the culture system.

II. SPECIMENS

All specimen types, including blood and bone marrow specimens, can be utilized in the VersaTREK (ESP) system. Blood specimens must be collected in either an ISOLATOR tube or a tube containing EDTA or sodium polyanethol sulfate and inoculated into the Myco bottle after processing.

Decontaminate specimens obtained from body sites normally contaminated with bacterial microbiota prior to inoculation into the Myco culture bottles. Viscous specimens like sputa must also be digested prior to inoculation. Utilize standard laboratory protocol for the digestion and decontamination of clinical specimens for mycobacterial culture.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents (*see* Appendix 7.4.3–1)

B. Equipment

1. VersaTREK (ESP) instrument
2. VersaTREK (ESP) Myco bottles
3. VersaTREK (ESP) Myco GS
4. VersaTREK (ESP) Myco AS or PVNA
5. VersaTREK (ESP) bottle connectors
6. 1-ml tuberculin syringe with fixed needles
7. Alcohol pads (70% isopropyl alcohol)
8. 12- by 75-mm sterile capped tubes
9. Lowenstein-Jensen (LJ) slants
10. LJ-Gruft slants
11. Middlebrook 7H11 agar plates
12. Middlebrook 7H10 (0.4%) hemoglobin plates

C. Storage

The Myco bottles should be stored at 15 to 30°C protected from light. VersaTREK Myco GS, AS, and PVNA must be stored at 2 to 8°C in the dark.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Temperature QC

1. Verify that the temperature in each unit is as displayed on the controller. Each readout should be verified by the use of an NBS-certified thermometer.
2. Print out the temperature report on a daily basis for review. Each module must be at 35 ± 1°C.

   ◩ The temperature printout will contain daily temperature readings for 31 days. Each daily sheet may be discarded the following day. The printout should be saved on the last day of each month and placed in a QC book.

B. Sensor QC

1. QC of each sensor is performed whenever a new bottle is placed in that location.
2. If an error message should be obtained for any location, remove the bottle, let it come to room temperature, fit the bottle with a new connector (perform under a biological safety hood), and place it back into the instrument.
3. If the sensor fails a second time, disable the location (refer to VersaTREK [ESP] manual), place the bottle in a new location, and notify a supervisor.
4. Additional sensor QC need not be performed if each location is used at least once every 90 days.
5. QC must be performed on any sensor that has been replaced (*see* system manual).

C. ESP Myco Bottle QC

1. Add 1 ml of ESP Myco GS and 0.5 ml of rehydrated ESP Myco AS or ESP Myco PVNA to an ESP Myco culture bottle.
2. QC organisms may be used from frozen aliquots or solid medium (Table 7.4.3–1).
3. Prepare a suspension in sterile 0.85% saline equivalent to a McFarland standard no. 1.
4. Allow clumps to settle for 30 min.
5. Dilute the organism to 10^4 CFU/ml in sterile saline.
6. Inoculate the bottle with 1 ml of diluted organism.

V. PROCEDURE

Observe standard precautions.

It is imperative that these cultures be handled in a biological safety cabinet.

A. Preparation of Myco bottles

1. Disinfect the septum of each VersaTREK (ESP) Myco bottle with 70% isopropyl alcohol.
2. Aseptically reconstitute Myco AS or PVNA by injecting 25 ml of sterile distilled or deionized water through the disinfected septum using a needle and syringe.
3. Disinfect the bottle stopper with 70% isopropyl alcohol.
4. Aseptically add 1 ml of VersaTREK (ESP) Myco GS by injecting through the septum with a needle and syringe.
5. Aseptically add 0.5 ml of VersaTREK (ESP) Myco AS or PVNA solution by injecting through septum with needle and syringe.

   ◩ **NOTE:** If the bottles are not to be inoculated within 2 h after the addition of supplements, they may be stored refrigerated for up to 8 h.

Table 7.4.3–1 Expected QC results

Organism	ATCC no.	CFU/ml	Days to positivity
Mycobacterium tuberculosis	27294	10^4	6–10
Mycobacterium avium	25291	10^4	4–8
Mycobacterium intracellulare	13950	10^4	4–8

V. PROCEDURE *(continued)*

B. Specimen processing and inoculation

1. All specimens should be processed as per standard protocol.
2. Place approximately 1.2 ml of each specimen into a labeled sterile 12- by 75-mm capped tube.
3. Disinfect the septum of a Myco bottle which has been previously labeled with the specimen number.
4. Inoculate each Myco bottle with up to 1.0 ml of processed sample.
5. Inoculate solid media according to the following schedule.
   - **a.** Respiratory, urine, gastric, and stool specimens are inoculated into an LJ-Gruft slant or other mycobacterial solid agar or egg-based medium.
   - **b.** Blood, bone marrow, CSF, and other normally sterile body fluids are inoculated onto an LJ plain slant or other mycobacterial solid agar or egg-based medium.
   - **c.** Tissues, biopsy specimens, and specimens from skin lesions are inoculated onto an LJ plain slant and a 7H10 hemoglobin plate. As an alternative, the specimen can be inoculated onto a 7H10 or 7H11 agar plate to which an (hemin-containing) X factor strip (BBL) is added.
   - **d.** Incubate all solid media at 35°C except the 7H10 hemoglobin or X factor-containing plate, which is incubated at 30°C.

C. Placement of the ESP-Myco bottles into the ESP Culture System II

1. Disinfect the top of each inoculated Myco bottle with standard disinfectant solution (70% isopropyl alcohol). Allow to air dry and place a connector onto each bottle.
2. Transport the bottle with connector to the instrument area in the carrier.
3. Place the bottles into the next available slots in the instrument, taking care to fill all locations on a rotating basis.
4. Accession the bottles in the ESP computer as follows.
   - **a.** From the Main Menu select Specimen Entry; press Enter. The instrument will search for unidentified bottles. The statement "Unidentified Bottles Exist" will appear on the screen.
   - **b.** Hit any key; the specimens' entry screen will appear.
   - **c.** Press Enter. The first unidentified location will appear.
   - **d.** Enter the accession number; press Enter to move the cursor to the next box. Enter the patient name (last name, comma, space, first name) and chart number, hitting the enter key each time to advance the cursor. Press page down after entering the chart number to go to the next patient. The length of incubation will default to 42 days.
   - **e.** After the last specimen is accessioned, press the end key to accept all entered patient information.

D. Workup of positive specimens

1. When the ESP signals a positive culture, check the graph to look for a declining slope indicating the consumption of oxygen. A rising graph indicates the production of carbon dioxide, which implies a contaminated culture.
2. Remove the bottle from the ESP instrument and transport to the AFB laboratory with the connector in place.

   ◩ **NOTE:** Connectors may not be removed from the bottle unless under a biological safety hood.
3. Disinfect the septum with disinfectant (70% isopropyl alcohol).
4. Vortex the bottle. Using a sterile 1-ml syringe with a fixed needle, remove a small aliquot and place 1 or 2 drops on a clean glass slide. Perform an acid-fast stain.
5. If AFB smear negative and without signs of contamination, place the bottle into the instrument in its original location after fitting the bottle with a new connector.

V. PROCEDURE *(continued)*

6. If AFB smear positive, using a sterile 1-ml syringe with a fixed needle, remove approximately 0.15 ml of medium and inoculate onto a 7H11 agar plate.
7. Add 1 ml of medium to a sterile centrifuge tube. Centrifuge at 3,500 × *g* for 15 min to obtain a pellet for AccuProbe mycobacterial identification.
8. Resuspend pellet with 7H9 broth to a McFarland no. 1 equivalent and assay using the AccuProbe kit, following manufacturer's instructions for the broth culture method. It is recommended that if the relative light unit (RLU) value falls within the equivocal range (10,000 to 29,999 RLUs) the specimen be retested. Specimens containing blood may also be tested directly from a positive bottle following three washings with phosphate-buffered saline (1).
9. For susceptibility testing, remove 0.1 to 0.5 ml from the Myco bottle and inoculate it directly onto the appropriate susceptibility testing medium.
10. Examine all corresponding medium slants for growth of AFB. If negative, place the tubes in a separate rack and examine weekly for growth.

E. Reprocessing of contaminated specimens

In the case of smear-positive specimens (with the exception of stool specimens) that become contaminated with bacteria, the Myco bottle must be reprocessed. Cultures should be reprocessed as follows.

1. Vortex the ESP bottle and withdraw all of the liquid using a sterile 5-ml syringe.
2. Place the aspirated culture medium into a 50-ml sterile centrifuged tube.
3. Add an equal volume of sterile 4% NaOH; vortex.
4. Allow to stand for 15 min.
5. Neutralize with an equal volume of 0.067 M phosphate buffer, pH 6.8 (e.g., M/15 phosphate buffer).
6. Centrifuge at 3,500 × *g*.
7. Pour off all but 1 ml of the supernatant.
8. Vortex and inoculate into a freshly supplemented Myco bottle.
9. Place the Myco bottle in a new location, allowing for a full 42-day incubation period. If the bottle should become contaminated a second time, discard the bottle. If the corresponding medium slant is also contaminated, finalize the culture as "contaminated." If the slant is not contaminated, continue to incubate the slant as per standard protocol.

F. Removal of culture bottles from the ESP Culture System II

1. Positive cultures

   Bottles that have been physically removed from the instrument must be removed from the computer system as well. This may be accomplished as follows.

   a. From the Main Menu, select Remove Specimen, and press Enter.
   b. Select Bottles Removed, and hit Enter.
   c. Any location from which a bottle has been removed will appear on a worksheet.
   d. Either select all by pressing the function key F4 or press F3 to mark one and arrow down for each record you wish to remove.
   e. Press End. Records will be removed from the system one at a time.
2. Contaminated specimens

   Those specimens determined to be terminally contaminated are removed from the system as described above.

V. PROCEDURE *(continued)*

3. Negative cultures
All cultures are incubated for 42 days before it is determined that they are negative. Bottles that have completed their incubation time may be removed from the system as follows.
- **a.** From the Main Menu select Remove Specimen; press Enter.
- **b.** Select Completed Removal; press Enter. All bottles that have completed their designated incubation time will appear on the worksheet.
- **c.** Press the function key F4 to mark all of the bottles for removal.
- **d.** Press End. The Light-emitting diode light on each drawer from which bottles are to be removed will light a steady red light. Each individual location will flutter to easily identify those bottles that must be removed. Remove the bottle by pushing down on the bottle below the connector. Once removed, the indicator light will cease fluttering. Dispose of the bottle-connector combination as per laboratory protocol. The confirmation screen will be blank if all of the bottles have been removed.
- **e.** Return to Main Menu.

VI. RESULTS AND INTERPRETATION

A. Negative cultures
Cultures negative after 6 weeks in the VersaTREK (ESP) with a negative-to-date solid medium culture are to be assigned a preliminary report: "Broth culture negative 6 weeks, solid media pending." If the solid medium remains negative after 8 weeks, the culture result is finalized: "No growth in 8 weeks."

B. Positive VersaTREK (ESP), positive solid medium
If identification was made by probing the Myco bottle directly, examine the solid medium. If the growth on the solid medium agrees with the identification made from the Myco bottle, the report may be finalized. Always examine for mixed cultures.

C. Positive VersaTREK (ESP), negative solid medium
If probe positive, report the results but leave in preliminary report status. The report is kept preliminary until the subculture plate grows and the morphology is consistent with the probe identification. All subculture plates must be carefully examined for mixed mycobacterial cultures.

D. Negative VersaTREK (ESP), positive solid medium
Report the culture as per standard protocol.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Preliminary report
- **1.** Positive: when the contents of the bottle are confirmed as acid-fast smear positive, report as "AFB present, identification to follow."
- **2. Negative:** when a bottle is negative after 6 weeks, report as "Broth culture AFB negative after 6 weeks, solid medium pending."

B. Final report
- **1.** Positive: when the mycobacteria are identified to species level, report as "Positive for *Mycobacterium.*"
- **2.** Negative: when a bottle is negative after 8 weeks, report as "No growth in 8 weeks."

VIII. LIMITATIONS

A. One specimen may not be sufficient to diagnose a mycobacterial infection. In general three first-morning sputum specimens should be obtained.

B. Patients on antitubercular therapy may have smear-positive but culture-negative specimens.

C. Patients suspected of having infections due to *Mycobacterium haemophilum* will require their specimens to be plated onto a hemoglobin- or heme-containing medium (e.g., CHOC).

D. Patients with infections due to *Mycobacterium marinum* or *Mycobacterium ulcerans* may require that solid media be incubated at 30°C for isolation.

E. Due to different growth rates, mixed mycobacterial infections may not be obvious in liquid cultures. Both the primary slant and agar plate subculture must be examined to detect mixed cultures.

REFERENCES

1. **LaBombardi, V. J., L. Carter, and S. Massarella.** 1997. Use of nucleic acid probes to identify mycobacteria directly from Difco ESP-Myco bottles. *J. Clin. Microbiol.* **35:**1002–1004.

2. **Trek Diagnostic Systems, Inc.** 2000. ESP Culture System II Product insert. Trek Diagnostic Systems, Westlake, Ohio.

SUPPLEMENTAL READING

Kent, P. T., and G. P. Kubica. 1985. *Public Health Mycobacteriology. A Guide for the Level III Laboratory.* U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta, Ga.

Nord, J. A., and V. J. LaBombardi. 1998. The effect of a continuously monitored culture system on the isolation rates of *Mycobacterium avium* complex (MAC), abstr. U82. *Abstr. Gen. Meet. Am. Soc. Microbiol.* American Society for Microbiology, Washington, D.C.

Shay, W. E., and V. J. LaBombardi. 1999. Diagnosis of disseminated *Mycobacterium scrofulaceum* in an AIDS patient using a continuously monitored culture system. *Int. J. STD AIDS* **10:**413–416.

APPENDIX 7.4.3–1

Reagents

A. VersaTREK (ESP) Myco (12.5 ml of broth culture medium and compressed sponge)
Formula per liter:

Middlebrook 7H9 broth	5.66 g
Casitone	1.2 g
glycerol	2.4 g

B. VersaTREK (ESP) Myco GS (50 ml of Middlebrook OADC enrichment)
Formula per liter:

bovine serum albumin	75 g
glucose	30 g
oleic acid	0.9 ml
catalase	72,000 U
sodium chloride	12.75 g

C. VersaTREK (ESP) Myco AS and PVNA contain lyophilized mixtures of antimicrobial agents that are rehydrated to 25 ml.
Formula per milliliter:
VersaTREK (ESP) Myco AS

polymyxin B	1,500 U
azlocillin	75 µg
fosfomycin	540 µg
nalidixic acid	420 µg
amphotericin B	150 µg
stabilizer	1 %
filler	7.7 %

VersaTREK (ESP) Myco PVNA

polymyxin B	1,500 U
nalidixic acid	600 µg
amphotericin B	150 µg
vancomycin	90 µg
solubilizing agent	5 %

7.4.4 MB/BacT Mycobacterial Detection

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The MB/BacT mycobacterial detection system (bioMérieux, Hazelwood, Mo.), in conjunction with BacT/ALERT MP process bottles and BacT/ALERT MB blood culture bottles, is designed to recover mycobacterial species commonly isolated from patient specimens. The BacT/ALERT MP process bottles are designed for use with MB/BacT antimicrobial agent supplement and MB/BacT reconstitution fluid. The MB/BacT antimicrobial agent supplement is intended to reduce breakthrough contamination due to bacteria that may survive the decontamination process. The MB/BacT reconstitution fluid contains components that are necessary to ensure optimal growth of mycobacteria. The MB/BacT microbial detection system utilizes a colorimetric sensor, which detects the production of CO_2 dissolved in the culture medium as microorganisms grow. If microorganisms are present in the sample, CO_2 is produced as the organisms metabolize the substrate in the culture medium. As growth of the microorganisms produces CO_2, the gas-permeable, liquid emulsion sensor on the bottom of each culture bottle changes from a blue-green to yellow color. Positive bottles are determined by software algorithms and are indicated as yellow sensors and high reflectance units. Negative bottles result from darker sensors and low reflectance units. Bottles are read every 10 min and processed through the software algorithms to measure changes in reflected light.

II. SPECIMENS

Observe standard precautions.

A. Pulmonary and extrapulmonary specimens

Follow recommended specimen collection, transport, and processing procedures. Standard safety precautions must be followed (1).

B. Blood specimens

1. Proper skin disinfection is essential to reduce the incidence of contamination.
2. BacT/ALERT MB blood culture bottle septa must be disinfected with 70% isopropanol or iodine before being inoculated.
3. Inoculate 3 to 5 ml of whole blood into the blood culture bottles. Blood specimens may be drawn using the bioMérieux blood collection adapter system or collected into a sterile Vacutainer tube containing sodium polyanethol sulfonate (SPS) or heparin.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. BacT/ALERT MP process bottle (3)

1. Middlebrook 7H9 broth, pancreatic digest of casein, bovine serum albumin, catalase
2. Atmosphere of CO_2, nitrogen, and oxygen under vacuum
3. 10 ml of medium in bottle
4. Color coded with red labels and seals

B. MB/BacT antimicrobial agent supplement kit

MB/BacT antimicrobial agent supplement (to reduce contamination)

Lyophilized antimicrobial agent supplement containing amphotericin B, azlocillin, nalidixic acid, polymyxin B,

III. MATERIALS *(continued)*

trimethoprim, vancomycin, and bulking agent

1. MB/BacT reconstitution fluid
   oleic acid, glycerol, amaranth, and bovine serum albumin in purified water
2. Preparation
   a. Prior to use, aseptically add 10 ml of MB/BacT reconstitution fluid to each vial of MB/BacT antimicrobial agent supplement required for testing. Swirl to mix the contents.
   b. Once reconstituted, the antimicrobial agent supplement has a shelf life of 7 days when stored at 2 to 8°C.

C. BacT/ALERT MB (blood) culture bottle

1. Middlebrook 7H9 broth, pancreatic digest of casein, SPS in purified water
2. Atmosphere of CO_2 in oxygen under vacuum
3. 29 ml of medium in bottle
4. Color coded with black printing and seals

D. MB/BacT enrichment fluid

1. Bovine serum albumin, sodium chloride, oleic acid, and saponin (lytic agent) in purified water
2. 5.5 ml of colorless fluid in each bottle

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. A certificate of conformance is provided with each case of BacT/ALERT MP (process) media indicating satisfactory growth performance of *Mycobacterium tuberculosis* ATCC 25177 and *Mycobacterium intracellulare* ATCC 13950. A certificate of conformance is provided with each case of BacT/ALERT MB (blood) culture media indicating satisfactory growth performance of *Mycobacterium avium* ATCC 25291 and *M. intracellulare* ATCC 13950 (2). Upon receipt, new lots or shipments of BacT/ALERT MP and MB bottles may be tested for QC as follows.

1. Add 0.5 ml of rehydrated MB/BacT antimicrobial agent supplement to each BacT/ALERT MP (process) bottle required or 1.0 ml of MB/BacT enrichment fluid to each BacT/ALERT MB (blood) culture bottle for testing.
2. Inoculate representative BacT/ALERT MP (process) bottles with 0.5 ml of selected control organisms listed below, diluted to 10^4 CFU/ml in sterile physiological saline or sterile unsupplemented Middlebrook 7H9 broth. Subculture onto Middlebrook 7H11 plates to determine viability and count. Load bottles into BacT/ALERT 3D.
3. Inoculate representative BacT/ALERT MB (blood) culture bottles with 5.0 ml of selected control organisms listed below, diluted to 10^3 CFU/ml in sterile sheep blood, defibrinated. Subculture onto Middlebrook 7H11 plates to determine viability and count. Load bottles into BacT/ALERT 3D.

Organism	ATCC no.	Days to positivity
M. tuberculosis (MP)	27294	11–13
M. tuberculosis (MP)	25177	11–13
M. intracellulare (MP and MB)	13950	7–14
Mycobacterium kansasii (MP)	12478	11–15
Mycobacterium fortuitum (MP)	6841	2–5
M. avium (MB)	25291	7–14

B. Instrument calibration and preventive maintenance

1. Record temperature.
2. Cell calibration is performed with a set of four calibration standards. The system will alert the user when cells are due to be calibrated. Refer to the operator's manual for calibration instructions as needed.

V. PROCEDURE

Observe standard precautions.

A. BacT/ALERT MP inoculation: specimens other than blood

1. BacT/ALERT MP (process) bottles must be at room temperature before inoculation.
2. Disinfect the top of each bottle septum with an alcohol pad.
3. Using a syringe and needle, aseptically add 0.5 ml of reconstituted antimicrobial agent supplement to each process bottle. For sterile fluids, inoculate the bottles with 0.5 ml of reconstitution fluid only. A pink tinge will indicate that the supplement or reconstitution fluid was added.
4. Using a 3-ml syringe and needle, transfer 0.5 ml of each processed or sterile patient sample into the appropriately labeled MP bottle.
5. Swab bottle septa with an appropriate mycobactericidal agent.
6. Load inoculated BacT/ALERT MP (process) bottles into an MB cabinet.

B. BacT/ALERT MB inoculation: blood specimens

1. Remove the plastic flip-top from the culture bottle and disinfect the septum with 70% isopropyl alcohol. Allow to dry.
2. Enrichment fluid is provided separately and must be added to the blood culture bottle for growth of mycobacteria. This can be done within 24 h before or after inoculation. Using a syringe and needle, aseptically add 1.0 ml of enrichment fluid to each blood bottle.
3. Collect the blood (refer to item II.B above) specimen according to specimen collection procedures.
4. After inoculation of the bottle with 3 to 5 ml of blood, clean the septum with 70% isopropyl alcohol. Allow to air dry.
5. DO NOT VENT THE BOTTLE.
6. Immediately transport the inoculated bottle to the laboratory.
7. Invert and load inoculated BacT/ALERT MB (blood) culture bottles into an MB/BacT MB cabinet.

C. Loading bottles

1. Press the ⟨Load Bottles⟩ button on the monitor screen.
2. Scan the bottle bar code. The cell address appears on the screen. A flashing green light will direct you to the correct cell. Load bottle into the assigned cell. The light will go out and the screen will clear if the bottle is in the correct cell.
3. Touch ⟨CANCEL⟩ to exit the function.
4. Remember to press ⟨Log off⟩ when finished.

D. Unloading negative bottles

1. Touch the ⟨Unload MB Negatives⟩ button on the monitor screen.
2. An automatic unload report will print (optional).
3. Cell lights will illuminate, directing you to the negative bottle.
4. Unload and scan the bottles' bar code labels one at a time. Scanning is optional.
5. Touch the ⟨Test Day Status Report⟩ button on the monitor screen to obtain a status report on final, negative cultures (optional).

It is imperative that these cultures be handled in a biological safety cabinet.

E. Unload and work up positive bottles

1. Touch the ⟨Unload MB Positives⟩ button on the monitor screen.
2. An automatic unload positives report will print (optional).
3. Cell lights will illuminate, directing you to the positive bottle(s).
4. Unload and scan the bottle's bar code label. Scanning is optional.
5. Work up all positive cultures as per laboratory procedures.
6. All culture workup should take place under the biological safety hood.

V. PROCEDURE *(continued)*

7. When bacterial contamination is detected, reprocess by removing entire contents of bottle and decontaminate contents by the *N*-acetyl-L-cysteine–sodium hydroxide or other procedure. Rely on solid medium results, or obtain a new specimen and process. When bacteria are detected in a BacT/ALERT MB blood bottle, consider bacterial septicemia. If the physician still requests a blood culture for mycobacteria, a new blood specimen must be obtained.

VI. RESULTS AND INTERPRETATION

A. Positive: positive bottles are detected by the instrument determined by software algorithms and are indicated as yellow sensors and high reflectance units.

B. Negative: negative bottles are detected by the instrument from darker sensors and low reflectance units.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Positive: when the contents of the bottle are confirmed as acid-fast smear positive, report as "Acid-fast bacilli present; identification to follow."

B. Negative: when a bottle is negative after 42 or 56 days, report as "No acid-fast bacilli isolated after 42/56 days."

REFERENCES

1. **Centers for Disease Control and Prevention and National Institutes of Health.** 1993. *Biosafety in Microbiological and Biomedical Laboratories,* 3rd ed. U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta, Ga., and National Institutes of Health, Bethesda, Md.
2. **NCCLS.** 1996. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 2nd ed., vol. 16, no. 16. Approved standard M22-A2. NCCLS, Wayne, Pa.
3. **Organon Teknika.** 2000. BacT/ALERT MB, MP culture bottles, package inserts. Organon Teknika, Malvern, Pa.

SUPPLEMENTAL READING

Cernoch, P. L., R. K. Enns, M. A. Saubolle, and R. J. Wallace, Jr. 1994. *Cumitech 16A-, Laboratory Diagnosis of the Mycobacterioses.* Coordinating ed., A. C. Weissfeld. American Society for Microbiology, Washington, D.C.

Organon Teknika. 2000. *MB/BacT System Training Manual,* p. 21. Organon Teknika, Malvern, Pa.

7.4.5 Wampole ISOLATOR Tube

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

To detect mycobacteremia in patients, blood is aseptically collected in a 10-ml Wampole ISOLATOR tube (Wampole Laboratories, Cranbury, N.J.). The tube contains saponin, which lyses all blood cells and releases intracellular mycobacteria. Tubes are centrifuged, thus concentrating organisms in the sediment; the supernatant is discarded, and the sediment (approximately 1.5 ml) is inoculated onto the appropriate media (1).

Blood cultures for mycobacteria are most often performed for patients with human immunodeficiency virus infection or for those who are immunosuppressed and have a fever of unknown origin. The use of blood cultures for the diagnosis of tuberculosis has increased (2).

II. SPECIMEN

Observe standard precautions.

Blood (10 ml) is collected in ISOLATOR 10 microbial tubes under aseptic conditions from adults. Blood (1.5 ml) is collected in ISOLATOR 1.5 microbial tubes under aseptic conditions from pediatric patients.

III. MATERIALS

A. Collection tool and media

1. Wampole ISOLATOR tube (store at room temperature)
   - **a.** Saponin—host cell lysis
   - **b.** Polypropylene glycol—foam retardant for saponin
   - **c.** Sodium polyanethol sulfate—anticoagulant
   - **d.** EDTA—anticoagulant
   - **e.** Fluorinert—liquid plastic immiscible with water concentrates bacteria
2. Media for recovery of mycobacteria, e.g., Lowenstein-Jensen; Middlebrook 7H10, 7H11 or 7H11 selective; or BACTEC 13A

B. Supplies

1. ISOLATOR tube
2. ISOSTAT pipettes and caps (two types of pipettes are used: one to remove supernatant and one to aspirate sediment)
3. 70% Isopropanol preparations
4. Providone-iodine
5. 20-gauge needle with 3-ml syringe

C. Equipment

1. Fixed-angle rotor centrifuge (3,000 $\times$ *g*), ambient temperature
2. Centrifuge adapters for 10-ml tubes
3. 5 to 10% CO_2 incubator, 35 to 37°C
4. Vortex mixer
5. ISOSTAT press and rack

ANALYTICAL CONSIDERATIONS

IV. PROCEDURE

Observe standard precautions.

A. ISOLATOR 10

1. Invert the tube several times to mix and lyse cells.
2. Centrifuge the ISOLATOR tube for 30 min at 3,000 × *g* in fixed-angle centrifuge.
3. Remove each ISOLATOR tube carefully from its centrifuge adapter, and place the tubes in the ISOSTAT rack.
4. Disinfect the top stopper with providone-iodine followed by 70% isopropanol. Do not allow the disinfectant to pool in the topper cavity. Allow to dry for 1 min.
5. Place the rack on the base of the ISOSTAT press.
6. Remove an ISOSTAT cap from the sterile package. Place cap over the top stopper of each ISOLATOR tube.
7. Position a tube with its cap under the press head. Gently pull the handle of the press down as far as possible. The spike will penetrate the stopper, and the cap will be firmly seated on the top of the tube.
8. Remove a supernatant pipette from the package. Squeeze the bulb of the ISOSTAT pipette to collapse it and provide a vacuum for withdrawal of the supernatant. Do this before inserting the stem into the tube.
9. Insert the pipette as far as possible into the tube; the base of the bulb should rest on the cap. Remove the pipette and discard.
10. Vortex ISOLATOR for 10 s.
11. Remove a concentrate pipette from its package. Squeeze the bulb to collapse it and create a vacuum for withdrawal of the concentrate. Do this before inserting the stem into the tube.
12. Gradually release pressure on the bulb, and allow the concentrate to be drawn into the pipette.
13. Inoculate onto appropriate media.

B. ISOLATOR 1.5

1. Vortex tube for at least 10 s.
2. Insert 20-gauge needle with 3-ml syringe.
3. Withdraw blood and inoculate onto appropriate media.
4. Discard needle and syringes appropriately.

V. RESULTS AND INTERPRETATION

A. Positive: due to the high sensitivity of the ISOSTAT System, only one or two colonies on streak area is enough to indicate a positive culture (3).

B. Negative: no colonies on streak area.

C. Colonies appearing only outside the inoculated area should be considered contaminants.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. If acid-fast bacillus (AFB) colonies are present, identify and report as "Positive for *Mycobacterium* (indicate the species)."

B. If AFB colonies are absent, report as "No acid-fast bacilli isolated after 56 days."

VII. LIMITATIONS

A. The ISOSTAT system permits recovery and detection of microorganisms at concentrations of less than 1 CFU/ml of blood.
B. Do not refrigerate or incubate the specimen.
C. ISOLATOR tubes may be held for up to 8 h if necessary.
D. Pneumatic transport systems are not recommended.

REFERENCES

1. **Baron, E. J., L. R. Peterson, and S. M. Finegold (ed.).** 1990. *Bailey and Scott's Diagnostic Microbiology,* 9th ed., p. 206–207. Mosby, St. Louis, Mo.
2. **Pfyffer, G. E., B. A. Brown-Eliott, and R. J. Wallace, Jr.** 2003. *Mycobacterium,* p. 532–584. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology,* 8th ed. ASM Press, Washington, D.C.
3. **Wampole Laboratories.** 1991. *ISOSTAT Microbial System Resource Guide.* Wampole Laboratories, Cranbury, N.J.

7.5 Septi-Chek AFB Biphasic Medium

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The BD BBL Septi-Chek AFB mycobacterial culture system (Becton Dickinson Microbiology Systems, Cockeysville, Md.) is a biphasic medium with a self-contained CO_2 environment. It consists of Middlebrook 7H9 broth and a slide of Middlebrook 7H11, modified egg, and chocolate media. The system offers sensitivity comparable to those of conventional agar and broth methods (1, 4). The inclusion of CHOC in this system is advantageous because it detects contaminants and when incubated at 30°C, *Mycobacterium haemophilum* can be detected.

II. SPECIMENS

The Septi-Chek system is used for the detection and isolation of mycobacteria from sputum, bronchial washings or aspirates, body fluids (CSF and pleural, ascites, or synovial fluid), urine, stool, biopsy tissues, or wounds and skin (3) when mycobacteriosis is suspected. For blood specimens, Septi-Chek has been successfully used with ISOLATOR-procured blood concentrated by centrifugation and the addition of 0.75-ml volumes to two sets of Septi-Chek AFB broth (2).

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Media

1. Septi-Chek AFB mycobacterial culture bottle
   - **a.** Store at 15 to 30°C. Protect from light.
   - **b.** Do not use if turbid. Broth should appear pale green to colorless.
   - **c.** Broth to which 1 ml of supplement has been added is stable at 2 to 8°C for 14 days.
   - **d.** Do not freeze.
   - **e.** Do not inoculate after expiration date shown on bottle label.
2. Septi-Chek AFB mycobacterial culture supplement
   - **a.** Store at 2 to 8°C. Protect from light.
   - **b.** Supplement reconstituted with sterile distilled water can be stored at 2 to 8°C or at −20°C for up to 14 days prior to use.
   - **c.** Do not use to supplement culture bottle after the expiration date shown on the bottle label.
3. Septi-Chek AFB slide
   - **a.** Slide 1: nonselective Middlebrook 7H11 agar, modified, suitable for growth of most mycobacteria
   - **b.** Slide 2: egg-based medium, suitable for growth of most mycobacteria
   - **c.** Slide 3: CHOC

   Store at 2 to 8°C. Do not freeze.
   Do not use dehydrated or contaminated Septi-Chek AFB slides.
   Do not attach to bottle after expiration date shown on package.

B. Supplies

1. Bactericidal-mycobactericidal disinfectant (e.g., Amphyl, Cidex)
2. Gloves, masks, and gowns
3. Cotton or gauze pads
4. Sterile Pasteur pipettes

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Upon receipt of a new shipment or lot number, one of the following organisms is recommended for incubation of Septi-Chek AFB mycobacterial culture bottle or Septi-Chek AFB slide at 10^4 CFU/bottle: *Mycobacterium tuberculosis* ATCC 25177, *Mycobacterium kansasii* ATCC 12478, *Mycobacterium scrofulaceum* ATCC 19981, *Mycobacterium intracellulare* ATCC 13950, and *Mycobacterium fortuitum* ATCC 6841. One of the following organisms is recommended for inoculation at 10^5 CFU/bottle: *Escherichia coli* ATCC 25922, *Streptococcus pneumoniae* ATCC 6305, and *Staphylococcus aureus* ATCC 25923.

B. Add 1 ml of reconstituted supplement to each bottle.

C. Add 0.5 ml of the 10^{-4} dilution of each control organism to its culture bottle.

D. Remove the cap from the culture medium bottle and immediately attach the slide. Inspect for tightness of fit. Slowly rotate the system 360° around the vertical axis twice.

E. Incubate in a vertical position at 37°C.

F. Observe the contents daily for the first week. Continue incubation and check for growth at 21 and 28 days.

V. PROCEDURE

Observe standard precautions.

A. All manipulations should be carried out aseptically under a vertical laminar-flow hood using standard safety precautions for handling mycobacteria.

B. Reconstitute the supplement with 9 ml of sterile distilled water. Add 1 ml of supplement to each bottle of culture broth. (Remove screw cap or inject through stopper.)

C. Add 0.5 to 1.0 ml of treated specimen to culture bottle. Invert to mix.

D. Hold the slide upright while unscrewing the white bottom cap of the slide container. Do not touch the inner thread.

E. Remove the cap from the culture medium bottle and immediately attach the slide. Inspect for tightness of fit. Label slide so as not to obscure any bacterial growth.

F. Slowly rotate the system 360° around the vertical axis twice.

G. Incubate in a vertical position at 37°C (except for skin specimens, which require one system at 37°C and another at 30°C). A CO_2 incubator is not required but may be used.

It is imperative that these cultures be handled in a biological safety cabinet.

H. Observe the contents of the bottle daily during the first week to detect rapidly growing mycobacteria or potential contaminants. After 1 week, examine negative cultures weekly for up to 8 weeks. Observed changes may include turbidity, flakes or granules in broth, and colonies of various forms on the slide.

I. Invert and rotate daily during the first week and then weekly for 8 weeks or until growth is observed. Inversion may wash off colonies and is not recommended once any colonies are seen.

J. Autoclave Septi-Chek bottles and slides prior to discarding.

VI. RESULTS AND INTERPRETATION

A. Observe weekly for visible growth on slides and turbidity (or grains or flakes) in broth.

B. Perform acid-fast stain on suspicious colonies and/or broth.

C. If contaminated, culture can be salvaged by processing the broth medium as per standard operating procedure and reincubating another Septi-Chek system.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report as culture positive only when confirmed by acid-fast smear.
B. Report as "Culture positive for mycobacteria; identification pending."

VIII. LIMITATIONS

Mycobacteria are often fastidious, and a single negative culture should not be used to rule out their presence.

The Roche Septi-Chek AFB system is intended for use in the detection, isolation, and subculture of mycobacteria. Conventional identification methods or probe technologies are required for species identification.

REFERENCES

1. **D'Amato, R. F., H. D. Isenberg, L. Hochstein, A. J. Mastellone, and P. Alperstein.** 1991. Evaluation of the Roche Septi-Chek AFB system for recovery of mycobacteria. *J. Clin. Microbiol.* **29:**2906–2908.
2. **Isenberg, H. D., R. F. D'Amato, L. Heifets, P. R. Murray, M. Scardamaglia, M. C. Jacobs, P. Alperstein, and A. Niles.** 1991. Collaborative feasibility study of biphasic system (Roche Septi-Chek AFB) for the rapid detection and isolation of mycobacteria. *J. Clin. Microbiol.* **29:**1719–1722.
3. **Kent, P. T., and G. P. Kubica.** 1985. *Public Health Mycobacteriology. A Guide for the Level III Laboratory*, p. 21–30. U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta, Ga.
4. **Kiehn, T. E., F. F. Edwards, P. Brannon, A. Y. Tsang, M. Maio, J. W. M. Gold, E. Whimbey, B. Wong, J. K. McClatchy, and D. Armstrong.** 1985. Infections caused by *Mycobacterium avium* complex in immunocompromised patients: diagnosis by blood culture and fecal examination, antimicrobial susceptibility tests, and morphological and seroagglutination characteristics. *J. Clin. Microbiol.* **21:**168–173.

7.6.1 Conventional Biochemicals

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Whenever possible, mycobacteria should be identified to the species level. A level II laboratory must routinely process and culture at least 20 colonial specimens per week in order to ensure proficiency in identifying *Mycobacterium tuberculosis.* However, this low workload necessitates referring nontuberculosis mycobacteria to a level III laboratory for identification (3). In addition to colonial morphology (5) and acid-fastness, the identification of mycobacteria is largely based on rapid DNA probes (AccuProbe) and conventional methods. The conventional biochemicals used to identify mycobacteria are discussed in this procedure.

A. Arylsulfatase test

Arylsulfatase is an enzyme that hydrolyzes the bond between the sulfate and the aromatic rings of tripotassium phenolphthalein. Free phenolphthalein can be recognized by the red color formed when an alkali is added. The 3-day test is used to identify and distinguish among rapid growers. The 14-day test aids in the identification of slower-growing species and some rapid growers.

B. Catalase tests

The enzyme catalase splits hydrogen peroxide into water and oxygen. The oxygen appears as bubbles. Two measurements of catalase activity are (i) the heat stability of catalase at 68°C and (ii) the amount of catalase produced (semiquantitative test). All mycobacteria except some isoniazid-resistant strains of *M. tuberculosis* and *Mycobacterium bovis* display catalase activity. However, mycobacteria vary in the amount of catalase produced and in the heat stability of the enzyme.

C. Growth rate and pigment production

Mycobacteria may be separated into two main groups based on growth rate, preferred growth temperature, and pigment production with or without light exposure (1, 2). The growth rates, preferred growth temperatures, and pigment production of mycobacteria can be determined by observing growth on freshly made subcultures on Lowenstein-Jensen agar slants. This medium is preferred because the color contrast makes it easy to observe the presence and pigment of mycobacterial colonies growing on it. Middlebrook 7H10 can be used for mycobacteria that will not grow on Lowenstein-Jensen agar.

D. Growth on MAC without crystal violet

Growth on MAC without crystal violet (2) is used to separate isolates of the rapidly growing mycobacteria. This test is often used in combination with the 3-day arylsulfatase test.

E. Iron uptake

Mycobacterium fortuitum and a few other rapid and slow growers can convert ferric ammonium citrate to iron oxide. The iron oxide is viewed as a rust color in the colonies when they are grown in the presence of ferric ammonium citrate.

F. Sodium chloride (NaCl) tolerance

Few mycobacteria are able to grow in culture media containing 5% sodium chloride, the exceptions including *Mycobacterium triviale* and most of the rapid growers except *Mycobacterium chelonae* and *Mycobacterium mucogenicum.*

G. Niacin accumulation

All mycobacteria produce nicotinic acid during growth. *M. tuberculosis* and some isolates of *Mycobacterium simiae* and *M. chelonae* do not metabolize the nicotinic acid further and therefore accumulate more. The niacin test should not be used alone to distinguish *M. tuberculosis* from *M. bovis* because approximately 4% of *M. bovis* strains are niacin positive (6). The niacin is excreted into the agar, from which it can be extracted and detected (2).

H. Nitrate reduction

Mycobacteria differ in their ability to reduce nitrate. This is denoted by the development of a color when nitrite reacts with the appropriate reagents.

I. Pyrazinamidase

Certain mycobacteria possess the enzyme pyrazinamidase, which deaminates pyrazinamide (PZA) to pyrazinoic acid and ammonia, which can be detected by the addition of ferrous ammonium sulfate. In addition, the mechanism of PZA resistance of *M. tuberculosis* appears to be the inability of the organism to produce pyrazinoic acid, which is assumed to be the active component of PZA. Therefore, this test has been used to determine PZA resistance of *M. tuberculosis.* Once the organism has become pyrazinamidase negative, it is assumed to be PZA resistant as well.

J. Inhibition of thiophene-2-carboxylic acid hydrazide (TCH; T2H)

M. tuberculosis and most of the other slowly growing mycobacteria are resistant to TCH at levels of 1 to 5 μg/ml. This test differentiates niacin-positive *M. bovis* from *M. tuberculosis* since *M. bovis* is susceptible to the low concentrations. The exception exists with strains of *M. bovis* resistant to isoniazid (INH) which may also be resistant to TCH.

K. Tellurite reduction

Most mycobacteria have the ability to reduce tellurite to metallic tellurium (2, 5), visible in liquid cultures of mycobacteria as a fine black precipitate. However, the speed with which most organisms are able to accomplish this reduction varies. *Mycobacterium avium* complex, *Mycobacterium celatum*, and most rapid growers are able to reduce tellurite within 3 days in an actively growing broth culture. This test is most useful, in conjunction with other tests, for identifying *M. avium* complex.

L. Tween 80 hydrolysis

The ability to hydrolyze Tween 80 is a valuable test for the separation of potentially pathogenic slow-growing scotochromogens and nonphotochromogens. In this test, Tween 80 acts as a lipid, binding the neutral red indicator and causing the solution to be yellow. If the mycobacterial lipase hydrolyzes the Tween 80, the neutral red indicator is no longer bound, and it reverts back to its normal red color at pH 7.

M. Urease

The urease test is used to differentiate mycobacteria on the basis of their abilities to hydrolyze urea (1, 2). Use only urea broth made specifically for use with mycobacteria.

II. SPECIMEN

A pure culture of acid-fast bacilli growing on solid medium

III. MATERIALS

Include QC information on reagent container and in QC records.

To perform biochemical tests, biological safety cabinets, incubators, water baths, vortex mixers, and sterile pipettes and screw-cap tubes are required as per individual protocols.

A. Arylsulfatase test

1. Media
   - **a.** Arylsulfatase stock substrate
   - **b.** Arylsulfatase broth: 3-day test
   - **c.** Arylsulfatase broth: 2-week test
2. Reagents
   - **a.** 2 N sodium carbonate (Na_2CO_3)
   - **b.** Arylsulfatase color standards
   - **c.** Prepare the standards per Table 7.6.1–1.

B. Catalase tests

1. Media
   - **a.** Dubos Tween broth or Middlebrook 7H9 broth
   - **b.** Lowenstein-Jensen deeps prepared in screw-cap test tubes (25 by 150 mm)
2. Reagents
   - **a.** 30% H_2O_2
   - **b.** 10% Tween 80
   - **c.** 10% Tween 80–30% H_2O_2 reagent
   - **d.** 10% Tween 80–30% H_2O_2
   - **e.** M/15 phosphate buffer (0.067 M)

C. Growth rate and pigment production

1. Media
   - **a.** Dubos Tween broth or Middlebrook 7H9 broth
   - **b.** Lowenstein-Jensen slants
2. Supplies
   - **a.** Sterile applicator sticks
   - **b.** Sterile Pasteur pipettes
   - **c.** Pieces of heavy brown wrapping paper or aluminum foil approximately 4 by 5 in.
   - **d.** The paper must be large enough to cover the entire slant of medium.
3. Equipment
   - **a.** Slant racks
   - **b.** Lamp with a 60-W light bulb

D. Growth on MAC without crystal violet

Medium: MAC plates without crystal violet

Table 7.6.1–1 Preparation of arylsulfatase standards (5)

Tube no.	Amt of:		Interpretation
	Na_2HPO_4	Phenol red	
1	4 ml	10 drops of stock solution	5+
2	4 ml	1 ml from tube 1	4+
3	3 ml	2 ml from tube 2	3+
4	3 ml	2 ml from tube 3	2+
5	3 ml	2 ml from tube 4	1+
6	3 ml	2 ml from tube 5	±

III. MATERIALS *(continued)*

E. Iron uptake
- **1.** Medium: Lowenstein-Jensen slants
- **2.** Reagent: 20% ferric ammonium citrate

F. Sodium chloride tolerance
- **1.** Media
  - **a.** Lowenstein-Jensen slants without NaCl
  - **b.** Lowenstein-Jensen slants with 5% NaCl
  - **c.** Dubos Tween or Middlebrook 7H9 broth
- **2.** Supplies
  - **a.** Sterile applicator sticks
  - **b.** Sterile 1-ml pipettes
  - **c.** McFarland no. 1 standard

G. Niacin accumulation
- **1.** Reagents
  - **a.** 4% Aniline
  - **b.** 10% Aqueous cyanogen bromide
  - **c.** 10% Sodium hydroxide (NaOH)
  - **d.** Niacin filter strips (impregnated with cyanogen chloride) (Becton Dickinson, Sparks, Md.)
- **2.** Supplies
  - **a.** Sterile screw-cap test tubes (20 by 125 mm)
  - **b.** Sterile Pasteur pipettes
  - **c.** Sterile 5-ml pipettes

H. Nitrate reduction
- **1.** Reagents
  - **a.** Nitrate substrate broth
  - **b.** 1:1 Solution of concentrated HCl and sterile H_2O (nitrate reagent 1)
    Wear goggles/face shields.
  - **c.** 0.2% Sulfanilamide (nitrate reagent 2)
  - **d.** 0.1% β-*N*-(1-naphthyl)ethylenediamine dihydrochloride (nitrate reagent 3)
  - **e.** Crystalline reagents (Lampe's method) (4)
  - **f.** Nitrate reduction color standard
- **2.** Supplies
  - **a.** Sterile applicator sticks
  - **b.** Sterile Pasteur pipettes
  - **c.** Sterile 5-ml pipettes

I. Pyrazinamidase
- **1.** Medium: pyrazinamidase substrate medium
- **2.** Reagent: 1% ferrous ammonium sulfate

J. Inhibition by TCH
- **1.** Media
  - **a.** Conventional method
    - (1) Dubos Tween or Middlebrook 7H9 broth
    - (2) TCH susceptibility medium
  - **b.** BACTEC method
    - (1) BACTEC 12B medium
    - (2) TCH stock medium
- **2.** Supplies
  - **a.** Conventional method
    - (1) 35 to 37°C, 8 to 10% CO_2 incubator
    - (2) Pasteur pipettes
  - **b.** BACTEC method
    - (1) BACTEC 460 instrument
    - (2) 1-ml tuberculin syringes with attached needles

K. Tellurite reduction
- **1.** Medium: Middlebrook 7H9 broth with glycerol
- **2.** Reagent: 0.2% potassium tellurite

L. Tween 80 hydrolysis
- **1.** Media
  - **a.** Tween hydrolysis reagent (Difco)
  - **b.** Sterile water
- **2.** Supplies
  - **a.** Sterile screw-cap tubes
  - **b.** Sterile pipettes

M. Urease: Wayne method
Medium: urea broth

ANALYTICAL CONSIDERATIONS

IV. PROCEDURE, RESULTS, AND QUALITY CONTROL

(See Tables 7.6.1–2 and 7.6.1–3 and Fig. 7.6.1–1.)

It is imperative that these cultures be handled in a biological safety cabinet.

A. Arylsulfatase test (2, 5, 6)
- **1.** Procedure
  - **a.** Inoculate labeled tubes of 3- and 14-day test media with 0.1 ml of a 7-day broth culture, 0.1 ml of a slightly turbid suspension, or a loopful of growth from an actively growing culture.
  - **b.** Incubate at 35 to 37°C in a non-CO_2 incubator.
  - **c.** After 3 days of incubation, add 6 drops of 2 N sodium carbonate to the 3-day test tube.
  - **d.** After 14 days, add 6 drops of 2 N sodium carbonate to the 14-day test tube.

Table 7.6.1–2 Distinctive properties of cultivable mycobacteria encountered in clinical specimens[a]

Descriptive term	Organism	Optimal temp (°C)	Usual colony morphology[b]	Pigmentation[c]	Niacin	Growth on T2H (10 μg/ml)	Nitrate reduction	Semiquantitative catalase (mm of bubbles)	68°C catalase	Tween hydrolysis	Tellurite reduction	Tolerance to 5% NaCl	Iron uptake	Arylsulfatase, 3 day	MAC with crystal violet	Urease[d]	Pyrazinamidase, 4 day	Nucleic acid probes available
Slow growers																		
M. tuberculosis complex	*M. tuberculosis*	37	R	N (100)	+ (95)	+	+ (97)	<45 (89)	− (1)	± (68)	−/+ (36)	− (0)	−	− (0)	−	±64	+	+[e]
	M. africanum	37	R	N	−	V	−	<45	−	−	−	−	−	−	−	+	−	+[e]
	M. bovis	37	Rt	N (100)	− (4)	−	− (9)	<45 (69)	−(2)	−(21)		− (0)	−	− (0)	−	± (50)	−	+[e]
	M. bovis BCG	37	R	N	−	−	−	<45	−	+/−		−	−	−		+	−	+[e]
Nonchromogens	*M. avium complex*	37	St/R	N (87)	− (0)	+	− (4)	<45 (98)	± (60)	− (2)	+ (81)	− (0)	−	− (1)	−/+	−(2)	+	+
	M. xenopi	42	S	S (21)	− (0)	+	− (7)	>45 (85)	± (31)	− (12)	± (65)	− (0)	−	± (36)	−	−(0)	V	−
	M. haemophilum[f]	30	R	N		+	−	<45	−	−	−	−	−	−	−	−	+	−
	M. malmoense	37	S	N (88)	− (0)	+	− (1)	<45 (99)	−/+	+ (99)	+ (74)	− (0)	−	− (0)		− (9)	+	−
	M. shimoidei	37	R	N	−	+	−	<45	−	+		−	−	−	−	−	+	−
	M. genavense	37	St	N	−	+	−	>45	+	+			−	−		+	+	−
	M. celatum	37	S/St	N (100)	−	+	− (0)	<45 (100)	+ (100)	− (0)	+ (100)	− (0)	− (0)	+ (100)	− (0)	− (0)	+ (100)	−
	M. ulcerans	30	R	N	−	+	−	<45	+	−		−		−		V	−	−
	M. terrae complex	37	SR	N (93)	− (1)	+	± (67)	>45 (93)	+ (92)	+ (99)	−/+ (46)	− (2)	−	− (2)	V	− (13)	V	−
	M. triviale	37	R	N (100)	− (0)	+	+ (89)	>45 (100)	+ (100)	+ (100)	−(25)	+(100)	−	±(56)	−	−/+ (33)	V	−
	M. gastri	30	S/SR/R	N (100)	− (0)	+	− (0)	<45 (100)	− (11)	+ (100)	± (50)	− (0)	−	− (0)		−/+ (44)	−	−
Chromogens	*M. kansasii*	37	SR/S	P (96)	− (4)	+	+ (99)	>45 (93)	+ (91)	+ (99)	−/+ (31)	− (0)	−	− (0)	−	−/+ (49)	−	+
	M. marinum	30	S/SR	P (100)	−/+ (21)	+	− (0)	<45	−(30)	+(97)	−/+ (39)	− (0)	−	−/+ (41)[g]	−	+ (83)	+	−
	M. simlae	37	S	P (90)	± (63)	+	− (28)	>45 (93)	+ (95)	− (9)	+ (82)	− (0)	−	− (0)		± (69)	+	−
	M. asiaticum	37	S	P (86)	− (0)	+	− (5)	>45 (95)	+ (95)	+ (95)	− (20)	− (0)	−	− (0)		− (10)	−	−
	M. xenopi	42	S	N/S	−	+	−	<45	+/−	−		−	−	+		−		−
	M. gordonae	37	S	S (99)	− (0)	+	− (1)	>45 (90)	+ (96)	+ (100)	− (29)	− (0)	−	V	−	V (31)	−/+	+
	M. scrofulaceum	37	S	S (97)	− (0)	+	− (5)	>45 (84)	+ (94)	− (2)	± (64)	− (0)	−	V	−	V (31)	±	−
	M. szulgai	37	S or R	S/P (93)	− (0)	+	+ (100)	>45 (98)	+ (93)	−/+ (49)	± (53)	− (0)	−	V	−	+ (72)	+	−
	M. flavescens	37	S	S(100)[h]	−(0)	+	+(92)	>45 (94)	+ (100)	+ (100)	−/+ (44)	± (62)	−	− (0)	−	+ (72)	+	−
Rapid growers																		
Nonchromogens	*M. fortuitum* group[i]	28	R/S	N (100)	−	+	+ (100)	>45 (93)	+ (90)	−/+ (43)	+ (92)	+ (85)	+	+ (97)	+	+ (70)	+	−
	M. chelonae	28	S/R	N (100)	−/+	+	− (1)	>45 (92)	± (53)	−/+ (39)	+ (89)	V	−	+ (95)	+	+ (89)	+	−
	M. abscessus	28	S/R	N	−		−	>45		V		±	−	+	+	+		−
	M. mucogenicum	28	S	N	−		V	>45		+		−		+	+	+		−
	M. smegmatis	28	S/R	S (50)	−	+	+ (95)	<45 (82)	−	+	+	+	+ (100)	− (0)	+ (100)			−
Chromogens	*M. phlei*	28	R	S	−	+	+	>45	+	+	+	+	+	−	−			−
	M. vaccae	28	S	S	−	+	+	>45	+	+	+	V	+	−	−			−

[a] Modified from reference 6. Plus and minus signs indicate the presence and absence, respectively, of the feature; blank spaces indicate either that the information is not currently available or that the property is unimportant. V, variable; ±, usually present; −/+, usually absent. The percentage of CDC-tested strains positive in each test is given in parentheses, and the test result is based on these percentages.

[b] R, rough; S, smooth; SR, intermediate in roughness; t, thin or transparent; f, filamentous extensions.

[c] P, photochromogenic; S, scotochromogenic; N, nonchromogenic (*M. szulgai* is scotochromogenic at 37°C and photochromogenic at 24°C).

[d] Urease test performed by the method of Steadham (*J. Clin. Microbiol.* **10:**134–137, 1979).

[e] Probe identifies *M. tuberculosis* complex.

[f] Requires hemin as growth factor.

[g] Arylsulfatase reaction at 14 days is positive.

[h] Young cultures may be nonchromogenic or possess only pale pigment that may intensify with age.

[i] Includes *M. fortuitum*, *M. peregrinum*, *M. fortuitum* third biovariant complex.

Table 7.6.1–3 Features useful for making a presumptive identification of mycobacteria (2)

Organism	Growth rate (days)	Pigment production in:		Colonial morphology on:	
		Light	Dark	Middlebrook 7H10 agar	Egg-based media (e.g., Lowenstein-Jensen)
M. avium complex	10–21	Buff to yellow	Buff to yellow	Thin, transparent, glistening or matte, smooth, entire, rounded; some rough and wrinkled	Thin, transparent, smooth; sometimes domed; some rough and wrinkled; raised edges
M. bovis	25–90	Colorless to buff	Colorless to buff	Small, thin, often nonpigmented, raised, rough, later wrinkled and dry; some inhibited on this medium	Rough, dry, friable; heaped-up center with thin, trailing transparent edge; sometimes flat and spreading
M. chelonae	3–7	Buff	Buff	Rounded, smooth, matte, periphery entire or scalloped, no branching filaments; some rough and wrinkled	Rounded, smooth, matte, periphery entire or scalloped, no branching filaments; some rough and wrinkled
M. fortuitum	3–7	Buff	Buff	Smooth, domed; sometimes rough branching filaments on periphery are obvious	Smooth and domed; sometimes rough branching filaments on periphery are obvious
M. gastri	10–21	Colorless to buff	Colorless to buff	Round, smooth, convex, glistening; often resemble *M. avium* complex	Round, smooth, convex, glistening; often resemble *M. avium* complex
M. gordonae	10–25	Yellow to orange	Yellow to orange	Round, smooth, convex, yellow to orange, glistening	Round, smooth, convex, yellow to orange, glistening
M. haemophilum	14–28	Buff to gray	Buff to gray	Grayish white, smooth to rough; require hemin for growth	Grayish white, smooth to rough; require hemin for growth
M. kansasii	10–21	Yellow	Buff	Raised, smooth; some rough and wrinkled; carotene crystals numerous after exposure to light	Raised and smooth; some rough and wrinkled; carotene crystals numerous after exposure to light
M. marinum	5–14	Yellow	Buff	Round, smooth; some may be wrinkled	Round, smooth; some may be wrinkled
M. scrofulaceum	10–14	Yellow	Yellow	Smooth, moist, yellow, round	Smooth, moist, yellow, round
M. simiae	7–14	Yellow	Buff	Smooth, domed, slightly pigmented	Smooth, domed, slightly pigmented
M. smegmatis	3–7	Buff to coral	Buff to coral	Raised, rough, wrinkled, edges scalloped	Raised, rough, wrinkled, edges scalloped
M. szulgai	14–28	Yellow to orange	Buff at 25°C; yellow at 37°C	Smooth to rough; periphery somewhat irregular	Smooth to rough, periphery somewhat irregular
M. terrae	10–21	Buff	Buff	Round, smooth to rough, glistening, sometimes colorless	Round, smooth to rough, glistening, sometimes colorless
M. tuberculosis	12–28	Buff	Buff	Flat, rough, spreading; irregular periphery	Rough, dry, friable; heaped-up center with thin, trailing transparent edge; sometimes flat and spreading
M. xenopi	28–42	Yellow	Yellow	Small, domed, yellow, smooth or rough; at 45°C, resemble miniature bird's nest	Small, domed, yellow, smooth or rough; at 45°C, resemble miniature bird's nest

IV. PROCEDURE, RESULTS, AND QUALITY CONTROL *(continued)*

2. Results
- **a.** Positive: immediate color change to pink or red after addition of the carbonate solution
- **b.** Negative: no color change

3. QC
- **a.** 3-day test
  - (1) Positive control: *M. fortuitum* ATCC 6841 (3 to 5+) or *Mycobacterium xenopi* if available (3 to 5+)
  - (2) Negative control: *M. intracellulare* ATCC 13950
  - (3) Uninoculated medium and reagent only: no color

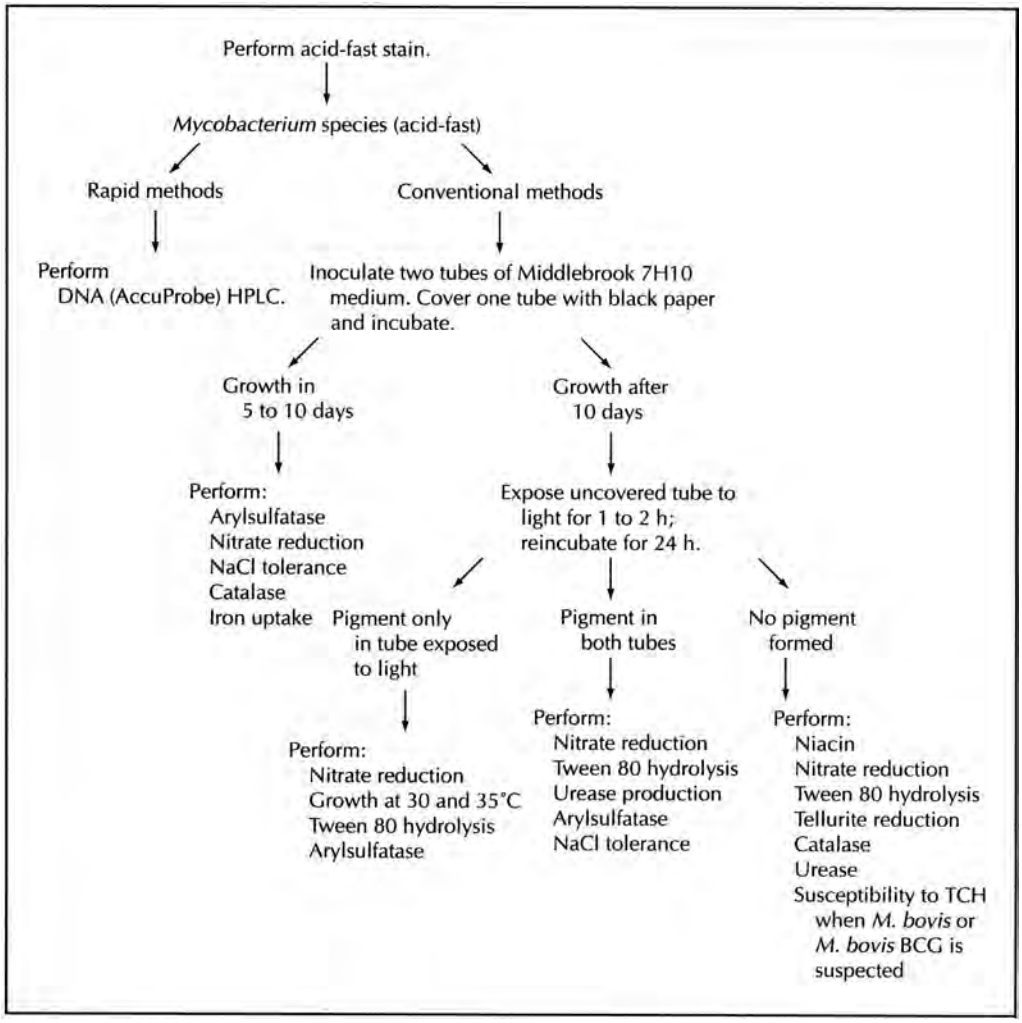
Perform acid-fast stain.
Mycobacterium species (acid-fast)
Rapid methods
Conventional methods
Perform DNA (AccuProbe) HPLC.
Inoculate two tubes of Middlebrook 7H10 medium. Cover one tube with black paper and incubate.
Growth in 5 to 10 days
Growth after 10 days
Perform: Arylsulfatase Nitrate reduction NaCl tolerance Catalase Iron uptake
Expose uncovered tube to light for 1 to 2 h; reincubate for 24 h.
Pigment only in tube exposed to light
Pigment in both tubes
No pigment formed
Perform: Nitrate reduction Growth at 30 and 35°C Tween 80 hydrolysis Arylsulfatase
Perform: Nitrate reduction Tween 80 hydrolysis Urease production Arylsulfatase NaCl tolerance
Perform: Niacin Nitrate reduction Tween 80 hydrolysis Tellurite reduction Catalase Urease Susceptibility to TCH when *M. bovis* or *M. bovis* BCG is suspected

Figure 7.6.1–1 Procedure for identification of acid-fast bacilli from culture (2). HPLC, high-performance liquid chromatography.

IV. PROCEDURE, RESULTS, AND QUALITY CONTROL *(continued)*

b. 2-week test
(1) Positive control: *M. triviale* ATCC 23292 (4 to 5+)
(2) Negative control: *Mycobacterium terrae* ATCC 15755 (0 to 2+)
(3) Uninoculated medium and reagent only: no color

B. Catalase (2, 5, 6)

1. Semiquantitative test

a. Procedure
(1) Inoculate surfaces of Lowenstein-Jensen deep (butt) tubes with 0.1 ml of 7-day-old liquid culture or a loopful of growth from an actively growing slant.
(2) Incubate at 37°C in a 8 to 10% CO_2 incubator for 2 weeks (caps loose).
(3) Add 1 ml of catalase reagent. (Prepare same as with 68°C test, described below.) Observe for 5 min.
(4) Measure the height of the column of bubbles (millimeters) above the surface of the medium.

IV. PROCEDURE, RESULTS, AND QUALITY CONTROL *(continued)*

b. Results
(1) Weak positive: <45 mm
(2) Strong positive: ≥45 mm
(3) Negative: no bubbles

c. QC
(1) Controls
(a) High catalase: *M. fortuitum* ATCC 6841
(b) Low catalase: *M. tuberculosis* ATCC 25177
(c) 0 mm of foam: uninoculated medium and reagent only
(2) Failure of the control cultures to give expected results is usually due to the age or purity of the control cultures. Use only actively growing cultures, and check the purity of control cultures frequently. Old cultures cause false low-catalase results. Cultures contaminated with bacteria other than mycobacteria can cause false high-catalase results.
(3) Use only freshly prepared reagent. Old reagents can cause false-negative results.
(4) False-negative results can also be caused by inadequate growth in the Lowenstein-Jensen deeps due to insufficient inoculation and/or insufficient incubation of the broth or deeps. Inadequate growth can also be due to incubation of the deep in tubes with tightened caps.
(5) For best results, use Lowenstein-Jensen deeps prepared in tubes that are 25 by 150 mm. These tubes allow adequate surface area for growth and adequate height for the column of foam.
(6) If the results of the control cultures are not as expected, repeat the tests.

2. 68°C test

a. Procedure
(1) Make a heavy suspension of the organism in 0.5 ml of 0.067 M phosphate buffer (pH 7) in a screw-cap tube.
(2) Incubate in a 68°C water bath or heating block for 20 min.
(3) Cool to room temperature.
(4) Add 0.5 ml of the catalase reagent (50–50 solution of 10% Tween 80 and 30% H_2O_2). Recap tubes loosely.
(5) Read after 5 min. Hold negative tubes for 20 min before discarding.

b. Results
(1) Positive: formation of bubbles. Rarely, bubbles may be seen rising from bottom in such a small quantity that foam does not form at the surface. Record this as a positive reaction.
◩ **NOTE:** Do not shake tubes, or they may read as false positive.
(2) Negative: no bubbles.

c. QC
(1) Positive control: *Mycobacterium gordonae* ATCC 14470
(2) Negative control: *M. tuberculosis* ATCC 25177
(3) Reagents only will not bubble at all.

C. Growth rate and pigment production (2, 5, 6)

1. Procedure

a. Inoculate one tube of Dubos Tween or Middlebrook 7H9 broth for each culture to be tested with a spadeful of growth.
b. Mix the inoculated broth cultures on a vortex mixer for 10 s.
c. Using a Pasteur pipette, transfer 6 drops from the broth to each of three Lowenstein-Jensen slants.
d. Immediately wrap two of the slants in brown paper or aluminum foil.
e. Loosen the caps of the tubes.
f. Incubate one wrapped slant at room temperature (20 to 25°C).

IV. PROCEDURE, RESULTS, AND QUALITY CONTROL *(continued)*

g. Incubate the other wrapped slant and the unwrapped slant at 37°C (8 to 10% CO_2).

h. Examine the unwrapped slants weekly for 3 weeks for growth and pigment production. Do not remove the covers until the third week.

i. Record the color of the growth on the slant. Mycobacteria are usually described as buff to cream (nonpigmented) or as yellow to orange (pigmented).

j. When the subculture is 3 weeks old, remove the paper or foil.

k. Record the growth on and colors of the covered slants.

l. Light test

For any cultures having orange or yellow pigment on the uncovered slant and no pigment on the covered slant(s), expose the unpigmented slant to light.

(1) Expose the slant to a 60-W bulb at a distance of 10 to 12 in. for 1 h. Incubate the slant at its original incubation temperature.

(2) Examine the exposed slant for pigment production after 24 h of incubation.

2. Results

a. Nonpigmented (buff or cream) covered and uncovered: nonphotochromogenic

b. Pigmented (yellow to orange) covered and uncovered: scotochromogenic

c. Pigmented (yellow to orange) uncovered and nonpigmented covered: photochromogenic

3. QC

a. *M. terrae* ATCC 15755: slow grower, ± growth at room temperature (22 to 25°C), no pigment in light or dark

b. *M. fortuitum* ATCC 6841: rapid grower, good growth at room temperature, no pigment in light or dark

c. *M. gordonae* ATCC 14470: slow grower, slight growth at room temperature, orange pigment in light and dark

d. *Mycobacterium kansasii* ATCC 12478: slow grower; very slight growth at room temperature; orange pigment in light, no pigment in dark, pigment after light test.

D. Growth on MAC without crystal violet (2, 5, 6)

1. Procedure

a. Inoculate rapid growers to be tested onto MAC by placing a 3-mm loopful of 7-day liquid culture, and streak for isolation.

b. Incubate without CO_2 (28°C).

2. Results

M. fortuitum and *M. chelonae* complexes show growth in <7 days.

3. QC

a. Positive control: *M. fortuitum* ATCC 12478

b. Negative controls: *Mycobacterium phlei* ATCC 11758; uninoculated medium

E. Iron uptake (2, 5, 6)

1. Procedure

a. Inoculate Lowenstein-Jensen slant with barely turbid liquid suspension of the organism.

b. Incubate until there is visible growth.

c. Add 1 drop of ferric ammonium citrate solution for each milliliter of Lowenstein-Jensen medium (usually about 8 drops).

d. Reincubate for up to 21 days and read weekly.

IV. PROCEDURE, RESULTS, AND QUALITY CONTROL *(continued)*

2. Results
- **a.** Positive: rusty brown color in the colonies and tan discoloration of the media
- **b.** Negative: no change in color

3. QC
- **a.** Positive control: *M. fortuitum* ATCC 6841
- **b.** Negative controls: *M. chelonae* ATCC 35751; uninoculated medium and reagent only

F. Sodium chloride tolerance (2, 5, 6)

1. Procedure
- **a.** Prepare a barely turbid suspension of growth from Lowenstein-Jensen slant.
- **b.** Inoculate a Lowenstein-Jensen slant with 5% NaCl and a control slant without NaCl with 0.1 ml of inoculum (4 drops).
- **c.** Incubate at 35 to 37°C and 8 to 10% CO_2.
- **d.** Read once a week for growth.
- **e.** Culture may be discarded after 4 weeks of incubation.

2. Results
- **a.** Positive: growth on NaCl
- **b.** Negative: no growth on NaCl and growth on control slant
- **c.** Invalid test: no growth on NaCl control slant

3. QC
- **a.** Positive control: *M. fortuitum* ATCC 6841
- **b.** Negative control: *M. gordonae* ATCC 14470

G. Niacin (2, 5, 6)

1. Procedure
- **a.** Add 1 to 1.5 ml of sterile distilled water or saline to a 4-week-old Lowenstein-Jensen culture slant.
- **b.** Cut and/or stab medium surface several times with a sterile needle or loop.
- **c.** Slant the tube so that the liquid covers the surface of the medium.
- **d.** Allow 30 min to 2 h for niacin extraction.
- **e.** Remove 0.6 ml of extract to a sterile test tube.
- **f.** With forceps, insert a niacin strip into the extract at the bottom of the tube, with the arrows on the strip pointing downward.
- **g.** Stopper the tube tightly.
- **h.** Leave at room temperature for 15 to 20 min, with occasional gentle agitation.
- **i.** Observe the color of the liquid at the bottom of the tube against a white background.

2. Results
- **a.** Positive: yellow color in extract fluid
- **b.** Negative: colorless fluid

3. QC
- **a.** Positive control: *M. tuberculosis* ATCC 25177
- **b.** Negative controls: *M. intracellulare* ATCC 13950; uninoculated medium and reagents only
- **c.** If the controls do not give the expected results, repeat the tests and controls with freshly prepared aniline and cyanogen bromide. Make sure that the aniline is colorless.

  ◩ If the aniline has turned brown, the color will interfere with the interpretation of the test.

IV. PROCEDURE, RESULTS, AND QUALITY CONTROL *(continued)*

H. Nitrate (2, 5, 6)

1. Procedure

a. Inoculate nitrate broth with a loopful of growth from an actively growing culture.

b. Shake to mix, and incubate at 35 to 37°C for 2 h.

c. Add 1 drop of reagent 1.

d. Add 2 drops of reagent 2.

e. Add 2 drops of reagent 3.

2. Results

a. Positive: pink to deep red color

b. Negative: no color change; confirm by adding small amount of zinc dust. If pink to red color develops, then result is a true negative.

3. QC

a. Positive control (more than 3+): *M. tuberculosis* ATCC 25177

b. Negative control (0 or ±): *M. intracellulare* ATCC 13950

c. Negative control (no color): uninoculated medium and reagents only

I. Pyrazinamidase (2, 5, 6)

1. Procedure

a. Include an uninoculated control tube. Place a heavy inoculum from a fresh culture slant on the surface of each of two tubes of medium. Use enough inoculum so that it may be readily seen with the naked eye.

b. Incubate at 35 to 37°C in a non-CO_2 atmosphere.

c. After 4 days, remove one tube from the incubator, and add 1.0 ml of freshly prepared 1% ferrous ammonium sulfate to the tube.

d. Place the tubes at room temperature for 30 min, and then refrigerate at 2 to 8°C to prevent growth of contaminants.

e. After 4 h, examine tubes for a pink band in the agar. To read, hold the tubes against a white background by using incident room light.

f. After 7 days of incubation, remove the second tube from the incubator and proceed as described above.

◩ **NOTE:** If the 4-day test is positive, it is not necessary to hold the second tube for the 7-day test.

2. Results

a. Positive: pink band formed.

b. Negative: no band formed.

3. QC

a. Positive control: *M. intracellulare* ATCC 13950

b. Negative controls: *M. kansasii* ATCC 12478; uninoculated medium and reagent only

J. Inhibition of TCH (T2H) (2, 5, 6)

1. Procedure

a. Prepare a 7- to 10-day-old liquid culture (or prepare a barely turbid suspension of organisms from a drug-free medium).

b. Dilute with sterile saline or distilled water to 1:1,000.

c. With a sterile capillary pipette, inoculate a control and TCH medium with 3 drops of the dilution.

d. Incubate for 3 weeks in a 35 to 37°C, 5 to 10% CO_2 atmosphere.

e. Read in the same manner as drug susceptibility test plates, comparing growth on TCH with that on control medium.

2. Results

Record the organism as susceptible if growth on TCH medium is <1% of the growth on the control.

IV. PROCEDURE, RESULTS, AND QUALITY CONTROL *(continued)*

3. QC
- **a.** Conventional method
  - (1) Susceptible to 10 µg of TCH per ml: *M. bovis* ATCC 35734
  - (2) Resistant to 10 µg of TCH per ml: *M. tuberculosis* ATCC 25177
- **b.** BACTEC method
  - (1) Susceptible to TCH: *M. bovis* ATCC 35734
  - (2) Resistant to TCH: *M. tuberculosis* ATCC 25177

K. Tellurite reduction (2, 5, 6)

1. Procedure
- **a.** Heavily inoculate Middlebrook 7H9 liquid medium.
- **b.** Incubate at 37°C in 5 to 10% CO_2 for 7 days. The broth should contain sediment on the bottom of the tube.
- **c.** Add 2 drops of sterile potassium tellurite solution to each test culture and controls. Shake tubes to mix.
- **d.** Reincubate at 37°C in 5 to 10% CO_2 for 3 days, but do not shake.
- **e.** On the third day, examine sedimented cells in each culture tube, taking care not to shake.

2. Results
- **a.** Positive: black precipitate of metallic tellurium
- **b.** Negative: no black precipitate. A light brown or gray precipitate should be considered negative.

3. QC
- **a.** Positive control: *M. intracellulare* ATCC 13950
- **b.** Negative controls: *M. tuberculosis* ATCC 25177; uninoculated medium and reagent only

L. Tween 80 hydrolysis (2, 5, 6)

1. Procedure
- **a.** Add 2 drops of the Tween 80 hydrolysis substrate concentrate to 1.0 ml of sterile deionized water.
- **b.** Take a loopful of an actively growing culture from a slant and inoculate the above Tween 80 mixture.
- **c.** Incubate aerobically at 35 to 37°C in a non-CO_2 incubator and examine at 1, 5, and 10 days.
- **d.** Observe for the appearance of a pink to red color (positive test) and record. Do not shake tubes while reading.
- **e.** Discard when positive or on the 11th day.

2. Results
- **a.** Positive: a pink or red color in 10 days or less
- **b.** Negative: an amber color remaining after 10 days of incubation

3. QC
- **a.** Positive control: *M. intracellulare* ATCC 13950
- **b.** Negative controls: *M. tuberculosis* ATCC 25177; uninoculated medium and reagent only

M. Urease (2, 5, 6)

1. Procedure
- **a.** Transfer a young, actively growing culture of the mycobacteria to be tested to the urea broth.
- **b.** Inoculate to a visible turbidity.
- **c.** Incubate at 35 to 37°C without CO_2 for up to 7 days.

2. Results
- **a.** Positive: dark pink to red color
- **b.** Negative: no color change

IV. PROCEDURE, RESULTS, AND QUALITY CONTROL *(continued)*

3. QC
- **a.** Positive control: *M. kansasii* ATCC 12478
- **b.** Negative controls: *M. intracellulare* ATCC 13950; uninoculated medium only

POSTANALYTICAL CONSIDERATIONS

V. REPORTING RESULTS

A. While biochemical tests are in progress, send a preliminary report as "acid-fast bacilli present; identification to follow."

B. When identification is known, then report as "Positive for *Mycobacterium* _____."

REFERENCES

1. **California State Department of Public Health, Department of Mycobacteriology. Procedures.** California State Department of Public Health, Berkeley.
2. **Della-Latta, P., and I. Weitzman.** 1998. Identification procedures from culture, p. 187–197. *In* H. D. Isenberg (ed.), *Essential Procedures for Clinical Microbiology*. ASM Press, Washington, D.C.
3. **Kent, P. T., and G. P. Kubica.** 1985. *Public Health Mycobacteriology. A Guide for the Level III Laboratory*. U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta, Ga.
4. **Kubica, G., and L. Wayne.** 1984. *The Mycobacteria, a Sourcebook.* Marcel Dekker, Inc., New York, N.Y.
5. **Lutz, B.** 1992. Identification tests for mycobacteria, p. 3.12.1–3.12.28. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook*, vol. 1. American Society for Microbiology, Washington, D.C.
6. **Pfyffer, G. E., B. A. Brown-Elliott, and R. J. Wallace, Jr.** 2003. *Mycobacterium*, p. 532–584. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.
7. **Roberts, G. D., E. W. Koneman, and Y. K. Kim.** 1991. *Mycobacterium*, p. 304–339. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), *Manual of Clinical Microbiology*, 5th ed. American Society for Microbiology, Washington, D.C.

SUPPLEMENTAL READING

Della-Latta, P. 1996. Workflow and optimal protocols for laboratories in industrialized countries. *Clin. Lab. Med.* **16:**677–695.

Orange County Public Health Department Laboratory, Mycobacteriology/Mycology Section. *Standard Operating Procedures for the Mycobacteriology Laboratory*. Orange County Public Health Department Laboratory, Santa Ana, Calif.

Silcox, V. A. 1992. Identification of mycobacteria, p. 3.11.1–3.11.11. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook*, vol. 1. American Society for Microbiology, Washington, D.C.

7.6.2 Gen-Probe AccuProbe Mycobacterial Culture Identification Test

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Nucleic acid hybridization assays are based on the ability of complementary nucleic acid strands to come together to form stable double-stranded complexes under appropriate conditions (1, 2, 3). The AccuProbe system uses a chemiluminescently labeled, single-stranded DNA probe which is complementary to the rRNAs of the target organisms. The rRNA is released from the organism through action of a lysing reagent and sonication. The DNA probe then combines with the target organism's rRNA to form a stable DNA:RNA hybrid. The labeled DNA:RNA hybrids are measured in the Gen-Probe Leader luminometer (2).

II. SPECIMENS

The Gen-Probe assays are designed to identify members of the *Mycobacterium tuberculosis* complex, *Mycobacterium avium* complex, *Mycobacterium gordonae,* and *Mycobacterium kansasii* growing on standard Lowenstein-Jensen or Middlebrook media or broth (3). Morphology on solid or liquid media can be used to determine which probe will be tested first (4).

III. MATERIALS

A. Materials available from Gen-Probe

1. Each of the following kits contain Probe reagent tubes and lysing reagent tubes:
Gen-Probe *M. tuberculosis* complex culture identification test kit, Gen-Probe *M. avium* complex culture identification test kit, Gen-Probe *M. gordonae* culture identification test kit, and Gen-Probe *M. kansasii* culture identification test kit.

2. Reagent kit
- **a.** Reagent 1 (specimen diluent)
- **b.** Reagent 2 (probe diluent)
- **c.** Reagent 3 (selection reagent)

3. Detection reagents
- **a.** Reagent 1 (0.1% hydrogen peroxide in 0.001 N nitric acid)
- **b.** Reagent 2 (1 N NaOH)

4. Gen-Probe equipment
Gen-Probe Leader luminometer

B. Materials not provided

1. 1-µl plastic, sterile inoculating loops, plastic needles or applicator sticks for selecting colonies, control culture strains

2. Water bath (60 ± 1°C)

3. Heating block (95 ± 5°C)

4. Micropipettes (100 and 300 µl) and Eppendorf pipettes (100 and 300 µl)

5. Vortex mixer

6. Sonicator

7. Positive and negative control cultures

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Positive and negative controls are tested on each day of use. The following organisms are used as controls and are tested in the same manner as patient culture samples.

1. *M. tuberculosis* complex culture identification test
- **a.** Positive control: *M. tuberculosis* ATCC 25177
- **b.** Negative control: *M. avium* ATCC 25291

2. *M. avium* complex culture identification test
- **a.** Positive control: *M. avium* ATCC 25291
- **b.** Negative control: *M. tuberculosis* ATCC 25177

3. *M. gordonae* culture identification test
- **a.** Positive control: *M. gordonae* ATCC 14470
- **b.** Negative control: *M. scrofulaceum* ATCC 19981

4. *M. kansasii* culture identification test
- **a.** Positive control: *M. kansasii* ATCC 12478
- **b.** Negative control: *M. tuberculosis* ATCC 25177

B. A tritium standard is used to test the performance of the optics in the Leader instrument. The ratio of the observed mean to the expected value should be between 0.95 and 1.05.

C. QC results: positive values should be >30,000 relative light units (RLU) and negative values should be <10,000 RLU.

V. PROCEDURE

It is imperative that these cultures be handled in a biological safety cabinet.

A. Sample preparation

1. Pipette 100 µl of reagent 1 and 100 µl of reagent 2 (probe diluent) into all lysing reagent tubes. If broth cultures are to be tested, do not add reagent 1 to the lysing tube.

2. Inoculum
- **a.** Solid medium method: growth can be removed with a 1-µl disposable plastic loop or an applicator stick. Avoid taking any of the solid medium with the cells.
- **b.** Broth culture method: pipette a 100-µl sample from the broth suspension into the lysing reagent tube.

3. Recap the lysing tubes and vortex.

B. Sample lysis

1. Sonicate for 15 min as recommended by the manufacturer.

2. Place the lysing reagent tubes on the heating block for 10 min at 95 ± 5°C.

C. Hybridization

1. Pipette 100 µl of the lysed specimens from the lysing reagent tubes into the corresponding probe reagent tubes.

2. Recap the probe reagent tubes and incubate for 15 min in 59.5 to 60°C water bath or a heating block. The temperature should be observed throughout the process of hybridization to ensure that 59.5 to 60°C is maintained.

D. Selection and detection

1. Remove the probe reagent tubes from the water bath. Pipette 300 µl of reagent 3 (selection reagent) into each tube. Recap the tubes and vortex.

2. Incubate the probe reagent tubes in a 60°C water bath for 10 min for the *M. tuberculosis* complex probe, 8 min for the *M. kansasii* probe, and 5 min for the *M. gordonae* and *M. avium* complex probes.

3. Remove the probe reagent tubes from the water bath and leave them at room temperature for at least 5 min. Read the results in the luminometer within 1 h.

VI. RESULTS AND INTERPRETATION

A. Positive: signals greater than or equal to 30,000 RLU are considered positive.
B. Negative: signals less than 30,000 RLU are considered negative.
C. If the results are between 20,000 and 29,000 RLU, the test should be repeated.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. If the result is positive, then report the following: "Positive for *M. tuberculosis* complex identified by DNA probe technology," "Positive for *M. avium* complex identified by DNA probe technology," "Positive for *M. kansasii* identified by DNA probe technology," or "Positive for *M. gordonae* identified by DNA probe technology."
B. If the result is negative for all probes, then report the following: "Acid-fast bacilli present; identification to follow."

VIII. LIMITATIONS

A. This method has been tested using fresh growth from solid media and from broth cultures. The efficacy of this test has not been demonstrated on direct clinical specimens (e.g., urine, stool, or respiratory specimens).
B. Results from all AccuProbe culture identification tests should be interpreted in conjunction with other laboratory and clinical data available to the clinician.
C. The AccuProbe *M. tuberculosis* complex identification test does not distinguish species within the *M. tuberculosis* complex.

REFERENCES

1. **Ellner, P. D., T. E. Kiehn, R. Cammarata, and M. Hosmer.** 1988. Rapid detection and identification of pathogenic mycobacteria by combining radiometric and nucleic acid probe methods. *J. Clin. Microbiol.* **26:**1349–1352.
2. **Gen-Probe Inc.** 1991. *AccuProbe* Mycobacterium tuberculosis *Complex Culture Identification Test.* Gen-Probe Inc., San Diego, Calif.
3. **Gonzalez, R., and B. A. Hanna.** 1987. Evaluation of Gen-Probe DNA hybridization systems for the identification of *M. tuberculosis* and *M. avium-intracellulare. Diagn. Microbiol. Infect. Dis.* **8:**69–77.
4. **Kaminski, D. A., and D. J. Hardy.** 1995. Selective utilization of DNA probes for identification of *Mycobacterium* species on the basis of cord formation in primary BACTEC 12B cultures. *J. Clin. Microbiol.* **33:**1548–1550.

7.6.3 BACTEC NAP Test

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The BACTEC NAP (Becton Dickinson Diagnostic Systems, Sparks, Md.) test utilizes *p*-nitro-α-acetylamino-β-hydroxypropiophenone (NAP), which selectively inhibits growth of mycobacteria belonging to the *Mycobacterium tuberculosis* complex, while it does not inhibit or partially inhibit growth of mycobacteria other than the *M. tuberculosis* complex. The inhibition in 12B medium results in the reduction of $^{14}CO_2$ production compared to the growth control index readings.

II. SPECIMEN

A. Only actively growing cultures at an early phase of growth in BACTEC 12B medium are recommended for the NAP test.

B. If the culture is old, overgrown, or on solid medium, subculture onto 12B medium, incubate at 37 ± 1°C, and test on a BACTEC 460 instrument daily.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Media and reagents obtained from Becton Dickinson Diagnostic Systems
1. BACTEC 12B medium. The NAP test cannot be carried out with 13A medium because of the presence of blood.
2. BACTEC NAP vial containing a 5-µg NAP disk

B. Supplies
1. Tuberculin syringe with permanently attached needle
2. Control organisms (*M. tuberculosis* ATCC 27294 and *Mycobacterium kansasii* ATCC 35775)
3. Bactericidal-mycobactericidal disinfectant (e.g., Amphyl, bleach)
4. Gloves, respirators, and gowns
5. Cotton or gauze pads
6. Alcohol swabs

C. Equipment
1. BACTEC 460 instrument
2. Instrument attached to 5 to 10% CO_2 and 90 to 95% air
3. BACTEC TB hood
4. Biological safety cabinet
5. Incubator, well calibrated at 37 ± 1°C
6. Vortex mixer

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Include *M. tuberculosis* ATCC 27294 and *M. kansasii* ATCC 35775 as controls. Grow the organisms on solid medium, and store a homogeneous suspension frozen at −70°C in aliquots.

B. Test these cultures along with the test cultures at least once a month and every time a new lot of NAP is used. *M. tuberculosis* should be inhibited by NAP, while *M. kansasii* should have increasing growth indices in the presence of NAP.

C. Do not report test results if the control results are unsatisfactory. Repeat the test.

V. PROCEDURE

Observe standard precautions.

A. Inoculation

1. If the growth index in 12B medium from the primary isolation or subculture vial is ≥10, test daily until it is 50 to 100.

2. Homogenize the culture by pulling the medium up into a syringe (with permanently attached needle) and pushing it back into the vial several times. Then aseptically transfer 1 ml of the medium into the NAP vial. The remaining 3 ml of medium serves as a control.

3. If the daily growth indices are >100, dilute the culture with fresh 12B medium according to the following table. Transfer 1 ml of inoculum into the NAP vial after diluting the culture. The remaining medium acts as a growth control.

Growth index	Vol (ml) transferred into 12B vial
50–100	None
101–200	0.8
201–400	0.6
401–600	0.4
601–800	0.3
801–999	0.2
999 for >1 day	0.1

4. Mix the contents of the vial thoroughly.

5. Clean the tops of inoculated vials with an appropriate disinfectant.

6. Wipe them with an alcohol swab.

B. Incubation and testing

1. Incubate the NAP and the control vial at 37 ± 1°C, unless another optimal incubation temperature is indicated.

2. Test daily on the BACTEC 460 instrument for about 2 to 7 days.

VI. RESULTS AND INTERPRETATION

A. Growth indices in the control vial should increase steadily, 2 to 2.5 times daily for the *M. tuberculosis* complex and higher with other mycobacteria. If there is no satisfactory steady increase in the growth indices, do not interpret the results.

B. The following criteria are applicable to all mycobacteria.

1. *M. tuberculosis* complex (*M. tuberculosis, Mycobacterium bovis, Mycobacterium africanum*): two consecutive significant (≥20%) decreases in growth indices after inoculation.

2. Mycobacteria other than the *M. tuberculosis* complex: increase in growth indices to ≥400 within 4 days or slight decrease or no increase in the first 1 to 3 days, followed by two consecutive daily significant (≥20%) increases.

C. A growth index of ≤10 is a background reading and is not considered in the interpretation.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Reporting is done within 2 to 7 days, with an average of 4 days (2, 3).

B. The *M. tuberculosis* complex should not be reported in fewer than 4 days.

C. Among mycobacteria other than the *M. tuberculosis* complex, certain strains of *M. kansasii, Mycobacterium gastri, Mycobacterium szulgai, Mycobacterium terrae,* and *Mycobacterium triviale* are partially inhibited by NAP (1). In such instances, there is a longer lag phase in the presence of NAP, and results interpreted in the first 2 to 4 days may be misleading. Incubate further, and test for an additional 2 to 3 days before reporting.

D. Mycobacteria with an optimum temperature other than 37°C should not be incubated at 37°C for the test. The NAP test for specimens originating from wounds or skin should be done in duplicate, with incubation at 37 and 30°C. The growth control vial should show satisfactory growth and increase in growth index.

VIII. LIMITATIONS

A. Confirmation of the NAP test and further identification require additional testing by conventional or other methods.

B. Mixed cultures of two mycobacteria or contaminated cultures give erroneous results.

REFERENCES

1. **Laszlo, A., and S. H. Siddiqi.** 1984. Evaluation of a rapid radiometric differentiation test for the *Mycobacterium tuberculosis* complex by selective inhibition with *p*-nitro-α-acetylamino-β-hydroxy-propiophenone. *J. Clin. Microbiol.* **19:**694–695.
2. **Morgan, M. A., K. A. Doerr, H. O. Hempel, N. M. L. Goodman, and G. D. Roberts.** 1985. Evaluation of the *p*-nitro-α-acetylamino-β-hydroxy-propiophenone differential test for identification of *Mycobacterium tuberculosis* complex. *J. Clin. Microbiol.* **21:**634–635.
3. **Siddiqi, S. H., C. C. Hwangbo, V. Silcox, R. C. Good, D. E. Sneider, Jr., and G. Middlebrook.** 1984. Rapid radiometric methods to detect and differentiate *M. tuberculosis/M. bovis* from other mycobacterial species. *Am. Rev. Respir. Dis.* **130:**634–640.

7.7 Susceptibility Tests by Modified Agar Proportion

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Drug susceptibility testing should be performed on all initial *Mycobacterium tuberculosis* complex isolates and in relapse or retreatment cases and when primary drug resistance is suspected. The modified agar proportion method is used to determine the susceptibility of slow-growing mycobacteria (e.g., *M. tuberculosis* complex, *Mycobacterium kansasii*), either directly from decontaminated acid-fast smear-positive sputum or indirectly from a pure culture. The agar medium is contained in quadrant plates; one is the control without antimicrobial agents and the other quadrants contain antimicrobial agents. The primary drugs, i.e., isoniazid (INH), ethambutol, and rifampin, are commonly selected for testing; however, pyrazinamide cannot be tested by this method (3, 4).

Direct susceptibility testing is based on inoculation of drug-containing media with only those processed sputum specimens that are acid-fast smear positive to determine the proportion or percentage of resistant *M. tuberculosis* complex strains. The advantages include the ability to obtain results within 3 weeks from the time of specimen receipt in the laboratory, cost-effectiveness, and the belief that the proportion of resistant bacteria recovered is a better representation of the patient's bacterial population (3).

II. SPECIMENS

Susceptibility testing should be performed on the first isolate of *M. tuberculosis* complex obtained from each patient, when patient cultures fail to convert to negative after 3 months of therapy, or in cases of therapeutic failure (1, 3).

A. Direct testing

Digested, decontaminated specimens that are acid-fast smear positive

B. Indirect testing

Pure culture of *M. tuberculosis* complex from solid medium

III. MATERIALS

Include QC information on reagent container and in QC records.

A panel of four primary drugs used for susceptibility testing of *M. tuberculosis* complex includes INH at two concentrations (critical and higher), rifampin, ethambutol, and pyrazinamide. Often streptomycin is tested based on the patient population served, prevalence of drug resistance, and standard drugs used for treatment in the community (3).

A. Drug-containing disks

1. INH with 1 μg/ml for final concentration of 0.2 μg/ml
2. INH with 5 μg/ml for final concentration of 1 μg/ml
3. Ethambutol with 25 μg/ml for final concentration of 5 μg/ml
4. Streptomycin with 10 μg/ml for final concentration of 2 μg/ml
5. Streptomycin with 50 μg/ml for final concentration of 10 μg/ml
6. Rifampin with 5 μg/ml for final concentration of 1 μg/ml
7. Pyrazinamide—see procedure 7.8.2.

B. Media and reagents

Store reagents at room temperature unless otherwise indicated.

1. Middlebrook 7H10 agar base
2. Middlebrook oleic acid-albumin-dextrose catalase (OADC)
3. Glycerol
4. Antimicrobial stock solutions prepared at 1,000 μg/ml (−70°C)

III. MATERIALS *(continued)*

5. Solvents are shown in parentheses for the drugs listed below.
   a. Ethambutol (sterile distilled water)
   b. INH (sterile distilled water)
   c. Rifampin (methanol or dimethyl sulfoxide)
   d. Streptomycin (sterile distilled water)
6. Blood agar plate (2 to 8°C)

C. Supplies

1. Sterile plastic petri plates (15 by 100 mm) with four compartments (quadrant plates)
2. 1.0 McFarland turbidity standard
3. Glass beads (3-mm diameter)
4. 1-, 5-, and 10-ml sterile serological pipettes
5. Sterile cotton-plugged glass Pasteur pipettes
6. CO_2-permeable plastic polyethylene bags (6 by 8 in.) for plate incubation (15 by 100 mm)
7. 0.22-μm-pore-size filters
8. Sterile polypropylene tubes (17 by 100 mm)

D. Equipment

1. Pipettors
2. Hot plate with magnetic stirring mechanism to create vortex that optimizes mixing
3. 50 to 56°C water bath
4. Vortex mixer
5. Stereoscopic (dissecting) microscope ($\times 30$ to $\times 60$)
6. 36 ± 1°C incubator with 5 to 10% CO_2
7. Biological safety cabinet

E. Plate preparation

1. Drug-impregnated disk method
   The disk elution method is a simplified procedure in which paper disks containing specified amounts of antituberculous drugs are placed in individual quadrants of 100-mm plastic petri plates (5). The submerged-disk method obviates problems of imprecision in weighing and dilution drugs and mislabeling media and is less labor-intensive.
   a. Paper disks containing appropriate concentrations of drugs are available commercially (BBL Sensi-Disc; Becton Dickin Cockeysville, Md).
   b. Place disks aseptically into individual quadrants of sterile petri dishes. Omit disk from one quadrant per plate to be used as the growth control.
   c. Dispense 5 ml of OADC-enriched 7H10 medium into each quadrant, overlaying the disks to keep them centered.
   d. Allow to solidify at room temperature with lids partially removed to allow excess moisture to evaporate.
   e. Close lids and incubate at room temperature overnight to permit drug to diffuse uniformly.
   f. Store for up to 28 days in sealed plastic bags at 4 to 8°C.

Drug	Concn in disk (μg/ml)	Final concn in medium (μg/ml)
INH	1	0.2
	5	1.0
Streptomycin	10	2.0
	50	10.0
Rifampin	5	1.0
	25	5.0
Ethambutol	25	5.0
	50	10.0
Ethionamide	25	5.0
Kanamycin	30	6.0

2. Preparation of drug-containing media
   a. Heat water bath to 50 to 56°C.
   b. Prepare 7H10 agar base.
      (1) Weigh out the amount of Middlebrook 7H10 agar powder specified by the manufacturer, and slowly add it to the water.
      (2) Heat on hot plate (with constant stirring, with magnetic stirrers) until the medium clears (approximately 20 min).
      (3) Add 10 ml of glycerol to the flask, and continue to mix for another 3 to 5 min.
      (4) Remove flask from hot plate.
      (5) Cover flask with either plastic screw caps or sponge plugs and then cover with aluminum foil, and autoclave for 15 min at 121°C on slow exhaust.
      (6) After autoclaving, place flask in 50 to 56°C water bath to cool.
      (7) If desired, agar can be allowed to solidify at room temperature and stored in tightly capped containers protected from light at 2 to 8°C for up to 2 weeks. Prior to plate preparation, melt the agar by placing container in a boiling water bath.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Strains
- **1.** *M. tuberculosis* H37Rv (ATCC 27294) (4)
- **2.** A strain of *M. tuberculosis* resistant to each of the test drugs may also be selected from clinical isolates as a secondary QC strain.
- **3.** Test a QC strain by following the routine procedure each time a new batch of medium or antimicrobial agent is used or at least once a week. Test the QC organism at 10^{-2} and 10^{-4} dilutions.

B. *M. tuberculosis* H37Rv must be susceptible (at <1% growth) to all the primary and secondary antituberculosis drugs (4).

C. Growth controls

Quadrant I (control; no drug) usually yields 200 to 400 colonies on the 10^{-2} dilution and 20 to 40 colonies on the 10^{-4} dilution.

D. Sterility check of medium
- **1.** Incubate 10% of each batch of plates prepared at 36°C (CO_2) for 48 h.
- **2.** Following incubation, examine for bacterial and fungal contamination. Discard batch of plates if more than one plate is contaminated (one contaminated plate is probably due to sporadic contamination).

E. Acceptable results
- **1.** QC strains performance within acceptable limits.
- **2.** Growth and inoculum controls show adequate growth.

V. PROCEDURE

Observe standard precautions.

A. Direct susceptibility testing
- **1.** Perform on acid-fast bacillus (AFB) smear-positive specimens.
- **2.** Stain and examine smears of concentrated clinical specimens.
- **3.** Make dilutions of the sediment in 7H9 broth according to the following table based on the number of AFB per oil immersion field.

No. of AFB	Dilution inocula
Less than 1/field	Undiluted and 10^{-2}
1–10/field	10^{-1} and 10^{-3}
More than 10/field	10^{-2} and 10^{-4}

- **4.** Inoculate 3 drops of each dilution onto each control and drug quadrant.
- **5.** Keep lids of plates slightly open until drops have dried. Thereafter, plates may be inverted. Place in CO_2-permeable plastic bags.
- **6.** Incubate at 35 to 37°C under 5 to 10% CO_2.
- **7.** Read initially after 2 weeks. Report at 3 weeks.

It is imperative that these cultures be handled in a biological safety cabinet.

B. Indirect susceptibility tests from culture
- **1.** Prepare a no. 1 McFarland suspension from culture.
- **2.** Dilute suspension to 10^{-2} and 10^{-4}.
- **3.** Continue as with direct susceptibility procedure.

VI. RESULTS AND INTERPRETATION

A. The results are obtained by counting the number of colonies growing within the plate quadrant, as follows.

No. of colonies	Report
0–50	Actual count
50–100	1+
100–200	2+
200–500	3+
>500 (confluent growth)	4+

B. Susceptibility is defined as growth on the control quadrant of 3+ to 4+ and no growth in the drug-containing quadrant.

C. Resistance is defined as growth on drug-containing quadrants that is greater than 1% of the number of colonies that grow on the drug-free quadrant (2). The formula to calculate the percentage for resistant colonies is as follows: (no. of colonies on the drug quadrant/no. of colonies on the control quadrant) × 100.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. If there is no growth of AFB on the drug-containing media and growth on the control quadrant, then report as "Susceptible."

B. If there is >1% growth of AFB on the drug-containing media and growth on the control quadrant, then report as "Resistant."

C. When an organism is tested against two concentrations of INH, to the lower concentration of which the organism is resistant and to the higher of which it is susceptible, add the following comment to the report: "These test results indicate low-level resistance to INH. Some evidence indicates that patients infected with strains exhibiting this level of INH resistance may benefit from continuing therapy with INH. Consultation with a specialist is advised concerning appropriate dosages and therapeutic regimen" (3).

REFERENCES

1. **Centers for Disease Control and Prevention.** 1993. Initial therapy for tuberculosis in the era of multidrug resistance. Recommendations of the Advisory Council for the Elimination of Tuberculosis. *Morb. Mortal. Wkly. Rep.* **42**(RR-7):1–8.
2. **Kent, P. T., and G. P. Kubica.** 1985. *Public Health Mycobacteriology. A Guide for the Level III Laboratory.* U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta, Ga.
3. **NCCLS.** 2000. *Susceptibility Testing of Mycobacteria, Nocardia, and Other Aerobic Actinomycetes,* 2nd ed., vol. 20, no. 26. Tentative standard M24-T2. NCCLS, Wayne, Pa.
4. **Pfyffer, G. E., B. A. Brown-Elliott, and R. J. Wallace, Jr.** 2003. *Mycobacterium,* p. 532–584. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology,* 8th ed. ASM Press, Washington, D.C.
5. **Wayne, L. G., and I. Krasnow.** 1966 Preparation of tuberculosis susceptibility testing medium by means of impregnated discs. *Am. J. Clin. Pathol.* **45:**769–771.

7.8.1 BACTEC 460TB (Radiometric) System—Indirect Susceptibility Testing for *Mycobacterium tuberculosis*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The BACTEC 460TB radiometric antimicrobial susceptibility test (Becton Dickinson Diagnostic Systems, Sparks, Md.) is a rapid method based on the principle used for primary isolation of mycobacteria. A ^{14}C-labeled substrate is incorporated in BACTEC 12B medium. When mycobacteria grow in the medium, $^{14}CO_2$ is produced during metabolism, which is measured as the growth index (GI). If a test drug to which mycobacteria are susceptible is added to this medium, the growth is inhibited, resulting in diminishing GIs as measured by the BACTEC 460 instrument. Radiometric antimicrobial susceptibility testing of *Mycobacterium tuberculosis* has been well documented and widely used (2, 3, 6, 7). Results show good agreement with the agar proportion method and are available within 4 to 6 days, compared with 3 weeks with the conventional method.

Primary and secondary agents that should be considered for testing against *M. tuberculosis* include the following (4).

A. Primary: ethambutol, isoniazid (INH), rifampin, and pyrazinamide (PZA)
B. Secondary: capreomycin, ethionamide, kanamycin, and streptomycin
C. Newer antimicrobials: amikacin, ofloxacin, rifabutin, and clarithromycin

Generally, only the primary agents are recommended for testing. Consider testing the secondary agents, which is usually done in reference laboratories (5), when an isolate is resistant to one or more primary agents or if the patient has failed therapy. Additionally, testing of secondary agents would be warranted with isolates from patients who may have become infected in an area in which resistant isolates are endemic.

The BACTEC method for susceptibility testing of isolated *M. tuberculosis* cultures is based on the modified proportion method (1). Mycobacteria are inoculated into vials of BACTEC 12B (Middlebrook 7H12) medium with and without antimycobacterial agents and incubated in a 37°C ambient-air incubator.

The number of organisms added to the control medium (without antimicrobial agent) is 100-fold less than that added to medium containing antimicrobial agent. The inoculated vials are incubated and subsequently read daily on the BACTEC 460 instrument, and the results are interpreted by evaluating the rate of increase in the GI of the control compared with the rate of increase in the GI in the antimicrobial agent-containing medium. If the GI increase in the antimicrobial medium vial is less than that in the control, the isolate is considered susceptible; if it is greater than the increase in the control, the isolate is considered resistant.

II. SPECIMEN

Use pure culture grown on solid medium with growth not more than 4 weeks old. If liquid medium is used, note the following: BACTEC 12B medium (GI of >500 or within 1 week after the peak GI is achieved; peak is the highest GI after which the GI starts declining).

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Media and reagents (storage conditions)
1. BACTEC 12B with Middlebrook 7H12 medium (22 to 25°C): 4 ml of liquid medium in a 20-ml glass vial sealed with a rubber septum
2. BACTEC diluting fluid (22 to 25°C)
3. BACTEC SIRE kit, which includes separate vials of lyophilized streptomycin, INH, rifampin, and ethambutol (2 to 8°C)

B. Equipment
1. BACTEC 460 instrument with a BACTEC TB hood

III. MATERIALS *(continued)*

2. Biological safety cabinet
3. CO_2 tank (5 to 10% CO_2 in air)
4. 37 ± 1°C ambient-air incubator
5. Vortex mixer

C. Other supplies (storage conditions)

1. Disposable tuberculin syringes with permanently attached 25- to 26-gauge needles
2. Disposable 5.0-ml syringes with 18-gauge needles
3. 0.5 and 1.0 McFarland turbidity standards
4. Sterile disposable 1- and 10-ml pipettes and pipette bulb
5. Blood agar plate (2 to 8°C)
6. Middlebrook 7H10 or 7H11 agar plates (2 to 8°C)
7. Other antimicrobial powders: capreomycin, ethionamide, kanamycin, etc. (desiccator, 2 to 8°C).

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Strains

1. *M. tuberculosis* H37Rv (ATCC 27294) and any monoresistant strain may also be selected from clinical isolates as a secondary QC strain (store at −70°C).
2. Test one or more QC strains by following the routine procedure used with patient isolates.

B. Expected results

1. *M. tuberculosis* H37Rv must be completely susceptible to all antituberculosis drugs tested, and monoresistant strains must be resistant to a selected drug. (A well-homogenized suspension devoid of large clumps and equal to a McFarland no. 1 standard may be frozen at −70 ± 10°C.)
2. Growth control vial (including that for *M. tuberculosis* H37Rv) must yield a GI of >30 in 3 to 5 days. Purity plates must be free of contamination.
3. Growth and inoculum controls must show adequate growth.

V. PROCEDURE

Observe standard precautions.

A. Preparation of antimicrobial stock solutions

1. Primary antimicrobial agents
   - **a.** Disinfect septum top of each vial with an alcohol swab.
   - **b.** Using a 5-ml syringe, reconstitute lyophilized antimicrobials by adding 5 ml of sterile deionized water to each vial. Mix until completely dissolved.
   - **c.** Generally, only one critical concentration of each antimicrobial agent is tested; however, higher concentrations may also be tested if resistance to the critical concentration is observed.
2. Secondary antimicrobial agents
   These antimicrobials are not available in lyophilized form and must be obtained in pure powder form from the manufacturer or a supplier. Different antimicrobials are dissolved in different solvents. Prepare a stock solution of a test antimicrobial as recommended by the manufacturer or described in literature (5). In a multicenter study, the concentrations listed in Appendixes 7.8.1–2 to 7.8.1–4 have been recommended (5).

B. Preparation of antimicrobial agent-containing medium

1. Using a disposable tuberculin syringe, add 0.1 ml of each well-mixed antimicrobial solution to a BACTEC 12B vial (one vial per concentration).
2. Once the antimicrobial agent has been added, use the BACTEC 12B medium the same day. If storage is necessary, refrigerate (2 to 8°C), and use within 3 days.
3. Include one BACTEC 12B vial without any antimicrobial agent as a growth control.

V. PROCEDURE *(continued)*

C. Inoculum preparation

1. Preparation of inoculum from a positive BACTEC 12B vial
Use the growth in the vial as the inoculum source for the susceptibility tests once the specified GI is attained.
- **a.** If a primary isolation vial is used, once the GI is >10, test the vial daily until the GI reaches >500, or test until the GI reaches 300 and then incubate for one more day.
- **b.** If a subculture is made onto BACTEC 12B medium, test the vial daily until the GI is >500.
- **c.** If the GI is >800, dilute the suspension 1:1 by adding 1 ml of the culture to 1 ml of BACTEC diluting fluid in a sterile tube.

2. Preparation of inoculum from growth in 7H9 broth
Adjust the turbidity of the culture with BACTEC diluting fluid to match that of a McFarland no. 1.0 standard for the daily schedule.

3. Preparation of inoculum from growth on solid medium
- **a.** Use a spatula or hard loop to scrape off numerous representative colonies, being careful not to transfer medium with the colonies.
- **b.** Transfer colonies to a sterile screw-cap round-bottom glass test tube containing glass beads and 3 ml of BACTEC diluting fluid.
- **c.** Emulsify and vortex until the suspension is well dispersed and moderately turbid (more than McFarland no. 1 standard). Allow the suspension to stand for about 30 min.
- **d.** Using a sterile pipette, transfer the supernatant into a sterile tube. Discard the sediment.
- **e.** Adjust the turbidity of the suspension with BACTEC diluting fluid to match that of a McFarland no. 1.0 standard for a daily schedule.

4. For the control vial, dilute the standardized inoculum suspension 1:100 by adding 0.1 ml of the standardized suspension to 9.9 ml of BACTEC diluting fluid. Mix well by inverting the bottle several times.

It is imperative that these cultures be handled in a biological safety cabinet.

D. Inoculation and incubation

1. Establish a 5% CO_2 atmosphere in each vial by initially testing all BACTEC 12B vials to be used on the BACTEC 460 instrument. The 5% CO_2, which is connected to the instrument via tubing, is infused into each bottle when they are probed for ^{14}C production. Do not use any vial with a GI of ≥20 on initial testing.

2. Disinfect the rubber septum on each of the BACTEC 12B vials with an alcohol swab.

3. Mix the inoculum suspension well, and use a tuberculin syringe to add 0.1 ml of the undiluted standardized suspension to each of the 12B vials containing antimicrobial agent.

4. Subculture a few drops of the inoculum suspension to a blood agar plate and a 7H10 or 7H11 agar plate to check for purity.

5. Inoculate 0.1 ml of the well-mixed 1:100-diluted suspension into the control vial.

6. Disinfect the rubber septum of each vial with an appropriate disinfectant, followed by an alcohol swab.

7. Incubate all vials in the dark in a 37 ± 1°C incubator.

E. BACTEC testing schedule

1. Daily schedule
- **a.** Test at approximately the same time (±2 h) each day.
- **b.** Test for a minimum of 4 days (5 days of incubation).
- **c.** Once the GI in the control vial is ≥30, interpret results.
- **d.** If the GI is ≥30 before day 4, the inoculum is probably too heavy. Repeat the test. Never report results earlier than 4 days of incubation.

V. PROCEDURE *(continued)*

e. If the GI does not reach 30 within 14 days of incubation, consider the test uninterpretable.

f. Generally, with a completely susceptible culture, there is a sharp decline in the daily GIs within a few days in the presence of streptomycin, INH, and rifampin. Ethambutol is not as active, and thus there is a slight initial increase in the GI before the decline.

2. Non-weekend schedule

For those laboratories where the weekend coverage is not available, a non-weekend schedule can be followed. For the non-weekend schedule the basic test procedure is similar to that used for the daily schedule, with the following changes.

a. All the tests are batched and are performed on Friday only.

b. For a positive BACTEC 12B vial, the inoculum is the same as in daily testing, such as a GI of >500 or a GI of 300 plus one additional day of incubation. For growth on solid medium, prepare inoculum equivalent to a McFarland no. 0.5 standard. Emulsify and vortex until the suspension is well dispersed and matches that of a McFarland 0.5 standard.

c. Testing on the BACTEC 460 instrument starts on Monday, skipping the weekend.

d. The vials should be tested on Monday, Tuesday, and Wednesday (minimum of 5 days). If the GI is not 30 or more on Wednesday, continue testing until it reaches 30 but not for more than a total of 14 days.

e. Disregard GIs of the first Monday for interpretation of results; this means that if the GI is 30 or more on Tuesday, the vials still have to be tested on Wednesday to interpret results.

VI. RESULTS AND INTERPRETATION

A. Providing QC is acceptable, interpret results as follows. At the time when the growth control shows a GI of 30 or more, calculate the difference in the GIs (ΔGIs) from the previous day for the control and the drug-containing vials. Negative ΔGIs indicate a decrease in growth and ΔGI output, while positive ΔGIs indicate an increase in growth and GI output.

1. Interpret results as follows.

a. Susceptible: ΔGI of control > ΔGI of drug vial

b. Resistant: ΔGI of control < ΔGI of drug vial

c. Borderline or partially resistant: ΔGI of control ~ ΔGI of drug vial

2. If the GI in the drug-containing vial exceeds 500 and remains >500 on the next day, interpret the test result as resistant regardless of the ΔGIs. (First make certain that the test was not overinoculated, as indicated by a GI of >30 in control vial in less than 3 days, or that it is not contaminated.)

3. If borderline results are obtained, test the vial for two to three additional days. This additional testing will often establish a trend, and the ΔGI either increases or decreases compared with that of the control. If no trend is observed, the isolate may truly be borderline or partially resistant.

4. Because there is a 100-fold-higher inoculum in the drug vials than in the control vial, the GI readings are usually higher in the drug vials than in the control vial for the first day or two.

5. The ΔGIs of the control and drug vials depend on the degree of susceptibility of the test organism and the potency and inhibitory effect of the antimicrobial agent.

6. If the strain is susceptible, the GI levels off or decreases on subsequent days. If the strain is resistant, the GI continues to increase until it is equal to or greater than the ΔGI of the control. Therefore, resistant strains can be detected and reported earlier than susceptible strains.

VI. RESULTS AND INTERPRETATION *(continued)*

B. Susceptibility: growth is inhibited in media with antituberculosis drugs with susceptible mycobacteria. This results in a decrease in the daily $^{14}CO_2$ production or no increase that can be noted by GI readings compared to those of the growth control.

C. Resistance: if there is no or very little inhibition of growth in the drug-containing medium, a culture is resistant to the test drug, and the GI output will thus increase daily.

D. If 1% of the bacterial population in a test culture is resistant to an antimicrobial agent, 99% of the bacteria will be inhibited and only 1% will grow in the presence of the antimicrobial agent. Since the inoculum in the control is 1/100 of that in the antimicrobial medium, the rates of growth (GI increases) in the control and the drug-containing medium will be equal, resulting in approximately equal GI outputs.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

Report drug concentration tested and interpretation according to laboratory policy and format. The following is an example.

M. tuberculosis		
Drug	Amt (μg/ml)	Interpretation
Ethambutol	2.5	S
INH	0.1	R
Rifampin	2.0	S
Streptomycin	2.0	S

VIII. LIMITATIONS

A. Quantitative results that describe percentage of resistance cannot be reported.

B. Susceptibility results are valid only if the culture is pure.

C. Results cannot be reported if there is poor growth in the control vial.

REFERENCES

1. **Canetti, G., W. Fox, A. Khomenko, N. Mahlor, N. K. T. Menon, D. A. Mitchinson, N. Rist, and J. A. Smelev.** 1969. Advances in techniques of testing mycobacterial drug susceptibility, and the use of sensitivity tests in tuberculosis programs. *Bull. W. H. O.* **41:**21–43.
2. **Hawkins, J. E.** 1986. Non-weekend schedule for BACTEC susceptibility testing of *Mycobacterium tuberculosis. J. Clin. Microbiol.* **23:**934–937.
3. **Libonati, J. P., C. C. Stager, J. R. Davis, and S. H. Siddiqi.** 1988. Direct drug susceptibility testing of *M. tuberculosis* by the radiometric method. *Diagn. Microbiol. Infect. Dis.* **101:**41–48.
4. **NCCLS.** 2000. *Susceptibility Testing of Mycobacteria, Nocardia, and Other Aerobic Actinomycetes*, 2nd ed., vol. 20, no. 26. Tentative standard M24-T2. NCCLS, Wayne, Pa.
5. **Pfyffer, G. E., D. A. Bonato, A. Ebrahimzadeh, W. Gross, J. Hotaling, J. Kornblum, A. Laszlo, G. Roberts, M. Salfinger, F. Wittmer, and S. Siddiqi.** 1999. Multicenter laboratory validation of susceptibility testing of *Mycobacterium tuberculosis* against classical second-line and newer antimicrobial drugs by using the radiometric BACTEC 460 technique and the proportion method with solid media. *J. Clin. Microbiol.* **37:**3179–3186.
6. **Robert, G. D., N. L. Goodman, L. Heifets, H. W. Larsh, T. H. Lindner, J. R. McClatchy, M. R. McGinnis, S. H. Siddiqi, and P. Wright.** 1983. Evaluation of BACTEC radiometric method for recovery of mycobacteria and drug susceptibility testing of *Mycobacterium tuberculosis* from acid-fast smear-positive specimens. *J. Clin. Microbiol.* **18:**689–696.
7. **Siddiqi, S. H., J. P. Libonati, M. E. Carter, N. M. Hooper, J. F. Baker, C. C. Hwangbo, and L. E. Warfel.** 1988. Enhancement of mycobacterial growth in Middlebrook 7H12 medium by polyoxyethylene stearate. *Curr. Microbiol.* **17:**105–110.

APPENDIX 7.8.1–1

BACTEC Mycobacterial Susceptibility Test QC

QC strain: _____

Setup date: _____ Tech: _____ Result date: _____ Tech: _____

Inoculum source (date inoculated): _____

Primary specimen: _____

BACTEC bottle: _____ GI: _____

7H9 broth: _____

Slant: _____ Medium: _____

Antimicrobial (µg/ml)	Day/GI															Results	Expected results
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Δ GI		
Control																	
Streptomycin, 6.0																	
Isoniazid, 0.1																	
Rifampin, 2.0																	
Ethambutol, 7.5																	

Lot # 12 B medium: _____

Lot # drug (date reconstituted/frozen): _____

Streptomycin: _____ Rifampin: _____

Isoniazid: _____ Ethambutol: _____

Comments:

APPENDIX 7.8.1–2

Equivalent drug concentrations in BACTEC 12B and solid media: primary drugs

Antimicrobial agent	Concn (µg/ml)			
	BACTEC 12B[a]	Solid medium		
		LJ[b]	7H10[c]	7H11[c]
INH	0.1[d]	0.2	0.2	0.2
	0.4	1.0	1.0	1.0
Rifampin	2.0[d]	40.0	1.0	1.0
Ethambutol	2.5	2.0	5.0	7.5
	7.5[d]	6.0	10.0	NA[e]
PZA	100	—[f]	25–50	50

[a] BACTEC 12B (Middlebrook 7H12) liquid medium. Antimicrobial agents are added to the medium prior to testing.

[b] LJ, Lowenstein-Jensen egg medium. Antimicrobial agents are added before inspissation.

[c] Middlebrook agar medium. Antimicrobial agents are added before plates are poured.

[d] Recommended concentration.

[e] NA, not available.

[f] —, no recommendations due to inconsistent results.

APPENDIX 7.8.1–3

Equivalent drug concentrations in BACTEC 12B and solid media: secondary drugs

Antimicrobial agent	Concn (μg/ml)			
	BACTEC 12B[a]	Solid medium		
		LJ[b]	7H10[c]	7H11[c]
p-Aminosalicylic acid	4	0.5	2	8
Ethionamide	1.25	20	5	10
Kanamycin	5	20	5	6
Capreomycin	1.25	20	10	10
Streptomycin	2.0	4.0	2.0	2.0
	6.0[d]	—[e]	10.0	10.0
Rifabutin	0.5	—[e]	1.0	1.0
Ofloxacin	2.0	—[e]	1.0	1.0

[a] BACTEC 12B (Middlebrook 7H12) liquid medium. Antimicrobial agents are added to the medium prior to testing.

[b] LJ, Lowenstein-Jensen egg medium. Antimicrobial agents are added before inspissation.

[c] Middlebrook agar medium. Antimicrobial agents are added before plates are poured.

[d] Recommended concentration.

[e] —, no recommendations due to inconsistent results.

APPENDIX 7.8.1–4

Equivalent drug concentrations in BACTEC 460 and solid media: secondary drugs

Antimicrobial	Concn (μg/ml)	
	BACTEC 460	Middlebrook 7H10
Capreomycin	1.25	10.0
Cycloserine	Not recommended	Not recommended
Ethionamide	1.25	5.0
Kanamycin	5.0	5.0
Amikacin	1.0	4.0
Clofazimine	0.5	1.0
Ofloxacin	2.0	2.0
Rifabutin	0.5	1.0

7.8.2 BACTEC 460TB System—Indirect Susceptibility Tests with Pyrazinamide for *Mycobacterium tuberculosis*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Pyrazinamide (PZA) is an antimicrobial agent active only against *Mycobacterium tuberculosis* and only at a low pH. Therefore, susceptibility testing of *M. tuberculosis* by the conventional method is performed at a pH of 5.5, at which many cultures fail to grow. Based on the information that the MIC of PZA increases with an increase in the pH of the medium (3), a special modification of the BACTEC susceptibility test procedure has been developed for susceptibility testing of PZA. With the BACTEC PZA method, the test is performed at a pH of 6.0, at which mycobacteria grow better than at pH 5.5. More reportable results can thus be achieved. The BACTEC method correlated well with PZA test results obtained by using alternative methods (3, 4). PZA testing is considered problematic, and there is no uniform standardized method for testing this agent on solid media.

II. SPECIMEN

Same as in procedure 7.8.1

III. MATERIALS

Include QC information on reagent container and in QC records.

Same as in procedure 7.8.1. In addition, the following media and reagents are required (storage conditions).

A. BACTEC PZA susceptibility test medium, which is modified BACTEC 12B at pH 6.0 (22 to 25°C)

B. BACTEC lyophilized PZA obtained from the manufacturer (2 to 8°C)

C. BACTEC reconstituting fluid (2 to 8°C)

BACTEC reconstituting fluid is an aqueous solution of polyoxyethylene stearate (POES) that enhances the growth of mycobacteria (5).

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Use a well-dispersed and standardized suspension of the QC strain *M. tuberculosis* H37Rv with each PZA susceptibility test setup. The growth control of the QC culture must yield a growth index (GI) of ≥30 in 3 to 8 days and must be completely susceptible to PZA.

V. PROCEDURE

A. Preparation of PZA solution

It is recommended that 100 μg/ml of PZA medium should be tested in the BACTEC system. This amount is equivalent to 25 to 50 μg/ml in the conventional method.

1. Reconstitute lyophilized PZA by adding 5 ml of BACTEC reconstituting fluid to the vial. Mix well until dissolved. Once reconstituted, aliquots may be stored at 2 to 8°C for 3 days, −5 to −20°C for up to 3 months, and −70°C for up to 6 months.

V. PROCEDURE *(continued)*

2. If PZA powder is used, prepare stock solution in BACTEC reconstituting fluid by dissolving 40 mg of PZA powder in 10 ml of BACTEC reconstituting fluid (final concentration, 4,000 μg/ml). Dissolve completely, filter sterilize, and store as described above for other stock solutions.

B. Preparation of PZA-containing medium

1. Using a disposable tuberculin syringe, add 0.1 ml of the PZA solution to a BACTEC PZA test medium vial (final concentration, 100 μg/ml).

2. Add 0.1 ml of BACTEC reconstituting fluid to the control BACTEC PZA test medium vial.

Observe standard precautions.

C. Inoculum preparation

1. Use an actively growing culture in BACTEC 12B medium supplemented with 0.1 ml of BACTEC reconstituting fluid. Prepare by subculturing growth from the following.

a. BACTEC 12B medium with a GI of 300 to 999
Add 0.1 ml to a vial of fresh 12B medium supplemented with 0.1 ml of reconstituting fluid.

b. 7H9 broth when turbidity is less than or equal to that of a McFarland no. 1 standard
Add 0.1 ml to a vial of fresh 12B medium supplemented with 0.1 ml of reconstituting fluid.

c. Solid medium
Prepare a suspension with turbidity comparable to that of a McFarland no. 1 standard, and add 0.1 ml of this suspension to a vial of fresh 12B medium supplemented with 0.1 ml of reconstituting fluid.

2. Incubate in a 37 ± 1°C incubator in the dark.

3. Test the subculture daily on the BACTEC 460 instrument.

a. Once the GI is 300 to 500, use this culture as the inoculum source.

b. If the GI is >500, dilute as follows by adding the specified volume of regular BACTEC 12B medium to the vial containing the actively growing organisms.
(1) GI of 300 to 499, no dilution
(2) GI of 500 to 599, add 1.0 ml of 12B medium
(3) GI of 600 to 699, add 1.5 ml of 12B medium
(4) GI of 700 to 799, add 2.0 ml of 12B medium
(5) GI of 800 to 899, add 2.5 ml of 12B medium
(6) GI of 900 to 999, add 3.5 ml of 12B medium

c. Do not use cultures more than 1 day after peak GI or a GI of >999 has been reached in BACTEC 12B vials supplemented with BACTEC reconstituting fluid.

It is imperative that these cultures be handled in a biological safety cabinet.

D. Inoculation and incubation

1. Prepare BACTEC bottles as described in procedure 7.8.1.

2. Mix the inoculum suspension well, and use a tuberculin syringe to add 0.1 ml of the standardized suspension to the PZA-containing BACTEC 12B vial and the control vial (do not dilute inoculum for the control vial).

3. Subculture a few drops of the inoculum suspension onto a blood agar plate and a 7H10 or 7H11 agar plate to check for purity.

4. Incubate in the dark in a 37 ± 1°C incubator.

E. Daily BACTEC testing schedule

1. Examine plates for purity.

2. Test daily on the BACTEC 460.

3. Test at approximately the same time (±2 h) each day.

4. Test for a minimum of 4 days (5 days minimum of incubation).

5. Once the GI in the control vial is ≥200, interpret results.

6. If the GI does not reach 200 within 14 days of incubation, consider the test uninterpretable.

V. PROCEDURE *(continued)*

F. Non-weekend schedule (1)

For those laboratories where weekend coverage is not available, a non-weekend testing schedule has been reported to work well (2). The non-weekend schedule is similar to the one used for SIRE antimicrobials. In this multicenter study, there was an overall 94% agreement between results of daily and non-weekend results, with no significant difference in time to complete the test.

VI. RESULTS AND INTERPRETATION

A. Calculate the percentage of growth in the PZA vial compared with that in the control vial [(GI in PZA vial/GI in control vial) × 100].

B. Interpret results as follows

1. Susceptible: GI in PZA vial <10% of GI in control vial

2. Resistant: GI in PZA vial >10% of GI in control vial

3. Borderline or partially resistant: GI in PZA vial within 5 to 15% of GI in control vial

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Susceptible: report the concentration of PZA tested and report according to laboratory policy and format, such as "*Mycobacterium tuberculosis*, BACTEC PZA susceptibility test, 100 μg/ml, susceptible."

B. Resistance: based on correlation with other methods, resistance is calculated at the 10% level.

C. Generally, results are available in 4 to 7 days, with good correlation with conventional test results (2, 4). Results indicating resistance may be interpreted in less than 4 days, but results indicating susceptibility must never be reported in less than 4 days.

VIII. LIMITATIONS

A. Quantitative results that describe percentage of resistance cannot be reported.

B. Susceptibility results are valid only if the culture is pure. Mixed or contaminated cultures may give erroneous results.

C. Results cannot be reported if there is poor growth in the control vial. Some *M. tuberculosis* strains grow poorly at pH 6.0.

D. Strains of *M. tuberculosis* susceptible to POES at pH 6.0, though rare, have been encountered. In this situation, perform the PZA susceptibility test without POES (reconstitute PZA with sterile deionized water) (2).

REFERENCES

1. **Gross, W. M., J. Ridderhof, I. George, H. Lipman, G. H. Mazurek, B. Metchock, B. Robinson-Dunn, L. Samons, A. Solutsky, G. Washington, and B. Madison.** 2001. A multicenter evaluation of non-weekend reading schedule for radiometric pyrazinamide susceptibility testing of *M. tuberculosis*, abstr. C-247. *Abstr. 101st Gen. Meet. Am. Soc. Microbiol.* American Society for Microbiology, Washington, D.C.
2. **Miller, M. A., L. Thibert, F. Desjardins, S. Siddiqi, and A. Dascal.** 1996. Growth inhibition of *Mycobacterium tuberculosis* by polyoxyethylene stearate present in the BACTEC pyrazinamide susceptibility test. *J. Clin. Microbiol.* **34:**84–86.
3. **Salfinger, M., and L. Heifets.** 1988. Determination of pyrazinamide MICs for *Mycobacterium tuberculosis* at different pHs by the radiometric method. *Antimicrob. Agents Chemother.* **32:**1002–1004.
4. **Salfinger, M., L. B. Reller, B. Demchuck, and Z. T. Johnson.** 1989. Rapid radiometric method for pyrazinamide susceptibility testing of *M. tuberculosis. Res. Microbiol.* **140:**301–309.
5. **Siddiqi, S. H., J. P. Libonati, M. E. Carter, N. M. Hooper, J. F. Baker, C. C. Hwangbo, and L. E. Warfel.** 1988. Enhancement of mycobacterial growth in Middlebrook 7H12 medium by polyoxyethylene stearate. *Curr. Microbiol.* **17:**105–110.

7.8.3 BACTEC 460TB System—Indirect Susceptibility Tests for Slow-Growing Mycobacteria other than *Mycobacterium tuberculosis*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The drugs currently recommended for therapy of *Mycobacterium avium* complex still include those recommended for *Mycobacterium tuberculosis.* However, these are nearly always inactive in vitro when tested by the conventional method. While isoniazid (INH) is usually inactive, susceptibility to streptomycin, rifampin, and ethambutol (especially at higher concentrations) is variable when tested by BACTEC 460TB, with most organisms being susceptible to these drugs (2). The antimicrobial agents and the concentrations tested (particularly for *M. avium* complex) should be determined following consultation with a clinician with expertise in treating infections caused by these organisms.

With some modifications, the BACTEC procedure can be used for testing nontuberculous mycobacteria (NTM). Results for most antimicrobial agents are reproducible but do not correlate well with those obtained by the conventional method. Good correlation between BACTEC and conventional methods has been reported only for *Mycobacterium kansasii* and *Mycobacterium marinum* when testing with INH, rifampin, and ethambutol (1). In general, NTM show greater susceptibility in BACTEC 12B medium than on solid medium (2, 4, 5). Among NTM, only *M. avium* susceptibility testing with the BACTEC method has been thoroughly studied in a multicenter study (2).

The clinical relevance of susceptibility testing for NTM is undetermined. Because of the lack of standardization for testing NTM with BACTEC, this procedure should be performed only in specialized laboratories that have significant experience in testing these organisms and thorough understanding of the test limitations.

II. SPECIMENS

Growth of slow-growing mycobacteria on solid medium, such as Middlebrook agar or egg-based media, or liquid medium, such as Middlebrook 7H9 broth or BACTEC 12B medium (growth index [GI] of $\geq$500)

III. MATERIALS

Same as those required for susceptibility testing of *M. tuberculosis*

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include a culture of *M. avium* that is well characterized and has been tested several times for antimicrobial susceptibility.

V. PROCEDURE

A. In the case of *M. kansasii* follow the procedure for the susceptibility testing of *M. tuberculosis* (1). The procedure for susceptibility testing of *M. avium* complex is very different from that used for *M. tuberculosis* and is strictly experimental. In a multicenter study, BACTEC 460 susceptibility testing of *M. avium* with amikacin, ciprofloxacin, clofazimine, cycloserine, ethambutol, ethionamide, rifampin, and streptomycin yielded reproducible results (6), and the procedure established in this publication should be followed. Since *M. avium* grows

V. PROCEDURE *(continued)*

Observe standard precautions.

relatively rapidly in BACTEC 12B medium, standardization of the inoculum for susceptibility testing is very critical.

B. For *M. avium* three concentrations of a test drug are used since there is variation from strain to strain for susceptibility to any antimicrobial agent (6).

C. Inoculum preparation

1. Liquid medium

Use an actively growing seed culture in BACTEC 12B medium. Prepare a seed vial by subculturing growth from the following.

a. BACTEC 12B medium with a GI of 500 to 999. Add 0.1 ml to a vial of fresh 12B medium.

b. 7H9 broth when turbidity reaches that of a McFarland no. 1 standard. Add 0.1 ml to a vial of fresh 12B medium.

2. Solid medium

a. Prepare a suspension with a turbidity comparable to that of a McFarland no. 1 standard, and add 0.1 ml to a vial of fresh 12B medium.

b. For *M. avium* complex, select as many translucent colonies as possible from 7H10 and 7H11 agar.

3. Incubate in a 37 ± 1°C incubator in the dark.

4. Test BACTEC 12B subculture daily on the BACTEC 460 instrument. Once the GI reaches 999 (usually 24 to 48 h), use as the inoculum source within 24 h.

5. Dilute BACTEC 12B suspension with BACTEC diluting fluid as follows.

a. *M. kansasii* (1:20 dilution): 0.5 ml of suspension in 9.5 ml of diluting fluid

b. *M. avium* complex (1:100 dilution): 0.1 ml of suspension in 9.9 ml of diluting fluid

c. For *M. kansasii* further dilute the inoculum suspension for the control vial 1:100 by adding 0.1 ml of the standardized suspension to 9.9 ml of BACTEC diluting fluid. In the case of *M. avium*, inoculate drug-containing vial and the control vial with 1:100-diluted culture. Use another growth control by diluting the inoculum further 1:100 and inoculating into a 12B vial, control 2 (1:10,000).

It is imperative that these cultures be handled in a biological safety cabinet.

D. Inoculation and incubation

1. Gas BACTEC 12B vials on BACTEC 460 instrument.

2. Prepare a standardized test culture suspension as described above. Mix the inoculum suspension well, and use a tuberculin syringe to add 0.1 ml of the standardized suspension to each of the 12B vials containing antimicrobial agent. For *M. avium* inoculate into the growth control as well (control 1).

3. Subculture a few drops of the inoculum suspension onto a blood agar plate and a 7H10 or 7H11 agar plate to check for purity.

4. Inoculate 0.1 ml of the well-mixed 1:100-diluted suspension into the control vial (*M. avium*, control 2).

5. Disinfect the rubber septum on the top of each vial with a cotton swab or gauze pad soaked with an appropriate disinfectant, and then disinfect it with an alcohol swab.

6. Incubate all vials in a 37 ± 1°C incubator in the dark.

E. Daily BACTEC testing schedule

1. Examine purity plates.

2. Test vials daily at approximately the same time (±2 h) each day.

3. Test for a minimum of 4 days (5 days of incubation).

4. Once the GI in the control for the *M. kansasii* vial is ≥30, interpret results.

5. If the GI of the control does not reach 30 within 10 days of incubation or reaches 30 in less than 3 days, consider the test uninterpretable.

V. PROCEDURE *(continued)*

6. For *M. avium* the MIC is interpreted when the GI of the 1:100 control (control 2) is 20 or more for three consecutive days and the GI of the undiluted control (control 1) reaches 999. These requirements have to be met between days 4 and 8 of incubation (the day of inoculation is considered day 1).

VI. RESULTS AND INTERPRETATION

A. Interpret results of *M. kansasii* as described for *M. tuberculosis.* For *M. avium* the MIC is the lowest concentration of a test drug which inhibits 99% of the bacterial population. In the presence of the lowest concentration of a drug, the daily GI increases (ΔGI) are less than those in the 1:100-diluted control (control 2) and the final GI in the drug-containing vial is not greater than 50 at the time of interpretation.

B. Translucent colonies of *M. avium* complex are more resistant than the opaque variants. Therefore, when they are present, use translucent colonies for susceptibility testing (5).

C. The synergistic effect of several drugs against *M. avium* complex has been tested using the BACTEC system (3).

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

For isolates other than *M. kansasii*, include this note in the report: "To date, no studies have proven that in vitro results correlate with clinical efficacy."

VIII. LIMITATIONS

A. Clinical correlation of in vitro susceptibility test results for NTM has not been determined.

B. It is not known which of the two susceptibility test procedures, BACTEC or the conventional method, yield results for NTM that correlate best with the clinical results. However, it has been suggested that testing of *M. avium* complex be performed by a MIC method (2, 6).

C. There are no standard recommendations for QC of the BACTEC procedure when testing NTM.

D. Because of variation in susceptibilities of different strains within the same species, it is difficult to develop a test procedure using a single concentration of an antimicrobial agent. Thus, more than one concentration of antimicrobial agent should be tested.

REFERENCES

1. **Hawkins, J. E., and W. M. Gross.** 1984. Radiometric drug susceptibility testing of *M. kansasii* and *M. marinum*, abstr. 2300. *Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother.* American Society for Microbiology, Washington, D.C.
2. **Heifets, L.** 1988. MIC as a quantitative measurement of the susceptibility of *Mycobacterium avium* strains to seven antituberculosis drugs. *Antimicrob. Agents Chemother.* **32:**1131–1136.
3. **Heifets, L. B., M. D. Iseman, and P. J. Lindholm-Levy.** 1988. Combinations of rifampin or rifabutin plus ethambutol against *M. avium* complex. *Am. Rev. Respir. Dis.* **137:**711–715.
4. **Lee, C., and L. B. Heifets.** 1987. Determination of minimal inhibitory concentrations of antituberculosis drugs by radiometric and conventional methods. *Am. Rev. Respir. Dis.* **136:**349–352.
5. **Rastogi, N., C. Frehel, A. Ryter, H. Ohayon, M. Lesourd, and H. L. David.** 1981. Multiple drug resistance in *Mycobacterium avium:* is the wall architecture responsible for exclusion of antimicrobial agents? *Antimicrob. Agents Chemother.* **20:**666–667.
6. **Siddiqi, S. H., L. B. Heifets, M. H. Cynamon, N. M. Hooper, A. Laszlo, J. P. Libonati, P. J. Lindholm-Levy, and N. Pearson.** 1993. Rapid microdilution method for determination of MICs for *Mycobacterium avium* isolates. *J. Clin. Microbiol.* **31:**2332–2338.

7.8.4 BACTEC 460TB System—Direct Susceptibility Test for *Mycobacterium tuberculosis*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Direct susceptibility testing of smear-positive specimens of *Mycobacterium tuberculosis* by using the BACTEC method is not widely used and has not been cleared by the Food and Drug Administration. It has been reported to yield rapid and reliable results (1). Direct susceptibility testing may be performed on all acid-fast smear-positive specimens irrespective of the degree of smear positivity. Results are available in 4 to 21 days, depending on the number of viable organisms in the original specimen (1). Direct susceptibility test results may take longer (5 to 21 days; average, 10 to 12 days) than indirect test results. Direct tests are usually set up on the same day that primary isolation media are inoculated, and identification results are often available concurrently with direct susceptibility results. Indirect testing must be performed to confirm all results obtained by direct testing.

II. SPECIMENS

Use concentrated, digested and decontaminated, smear-positive specimens.

III. MATERIALS

Include QC information on reagent container and in QC records.

All the materials and supplies are the same as for the indirect susceptibility testing, except that BACTEC 460 PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) antimicrobial mixture is needed to suppress contamination.

ANALYTICAL CONSIDERATIONS

IV. PROCEDURE

Observe standard precautions.

The test procedures are the same as for indirect susceptibility testing, except for the following.

A. Test only streptomycin, isoniazid, rifampin, and ethambutol.
B. Add 0.1 ml of BACTEC PANTA antimicrobial supplement to control and drug-containing BACTEC 12B vials prior to inoculation.
C. Inoculate 0.1 ml of the well-mixed acid-fast bacillus (AFB) smear-positive sediment directly into the drug-containing BACTEC 12B vials.
D. Prepare a 1:10 dilution of the initial inoculum by using BACTEC diluting fluid (0.1 ml of inoculum plus 0.9 ml of BACTEC diluting fluid), mix well, and inoculate 0.1 ml into the control vial. The inoculum is diluted 1:10 rather than 1:100 because usually the specimen contains a low number of AFB.
E. Incubate in a 37 ± 1°C incubator, and test BACTEC vials every 2 to 3 days for a maximum of 3 weeks. For specimens with numerous acid-fast bacteria on smear, test daily.

IV. PROCEDURE *(continued)*

F. Once the growth index (GI) in the control is ≥10, test vials daily.
G. Interpret results when the GI of the control is ≥20.
H. Occasionally, the organism in the control vial may fail to grow adequately even though resistance is observable in drug vials. Do not report these results.

V. RESULTS AND INTERPRETATION

Usually, results are unequivocally susceptible or resistant because of the smaller number of mycobacteria in the inoculum and the longer incubation time. A GI in the drug vial that is close to the GI in the control vial (within 20%) suggests a 10% resistant population, as the control was diluted 1:10. Report as partially resistant.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report "susceptible" or "resistant" based on the GI interpretation and add the following statement: "Results obtained by direct specimen testing."
B. If borderline results are obtained, report the following: "Based on direct testing, isolate appears partially resistant to (respective drug); confirmatory testing to follow."
C. Do not report results until isolate is confirmed as *M. tuberculosis.*

VII. LIMITATIONS

A. The direct testing method does not include a mechanism for standardizing the number of organisms in the test inoculum.
B. Although initial readings are made every 2 to 3 days, vials must be tested daily once a GI of ≥10 is obtained. Consequently, a daily BACTEC testing schedule must be followed.
C. Only results for *M. tuberculosis* are reportable.
D. Susceptibility tests are set up before mycobacterial identification is established, and sometimes isolates other than *M. tuberculosis* are tested unnecessarily. Confine testing to specimens highly suspicious for *M. tuberculosis.*
E. Direct susceptibility testing has not been extensively validated.

REFERENCE

1. **Libonati, J. P., C. C. Stager, J. R. Davis, and S. H. Siddiqi.** 1988. Direct drug susceptibility testing of *M. tuberculosis* by the radiometric method. *Diagn. Microbiol. Infect. Dis.* **101:**41–48.

7.8.5

BACTEC MGIT 960 SIRE—Nonradiometric Susceptibility Testing for *Mycobacterium tuberculosis*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The BACTEC MGIT 960 SIRE kit is a nonradiometric antimicrobial susceptibility system for testing *Mycobacterium tuberculosis* from isolated culture. It provides susceptibility results for streptomycin (STR), isoniazid (INH), rifampin (RIF) and ethambutol (EMB) in approximately the same time frame as the BACTEC 460TB system. The BBL MGIT 7-ml mycobacteria growth indicator tube (MGIT tube) contains a modified Middlebrook 7H9 broth, which supports the growth and detection of mycobacteria. The MGIT tube contains a fluorescent compound embedded in silicone on the bottom of a 16- by 100-mm round-bottom tube. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. The initial concentration of dissolved oxygen quenches the emission of fluorescence from the compound, and little fluorescence can be detected. Later, actively growing and respiring microorganisms consume the oxygen, which allows the compound to fluoresce.

The BACTEC MGIT 960 SIRE kit is a 4- to 13-day qualitative test. The test is based on growth of the *M. tuberculosis* isolate in a drug-containing tube compared to a drug-free tube (growth control). The MGIT 960 instrument continuously monitors tubes for increased fluorescence. Analysis of fluorescence in the drug-containing tube compared to the fluorescence of the growth control tube is used by the instrument to determine susceptibility results. The BACTEC MGIT 960 instrument automatically interprets these results and reports a susceptible or resistant result.

II. SPECIMENS

Susceptibility tests are performed from pure cultures of *M. tuberculosis.*

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Materials provided

1. BACTEC MGIT 960 SIRE kit containing one vial of each lyophilized drug and eight vials of SIRE supplement (40 tests per drug per kit)
2. BACTEC MGIT 960 STR 4.0 kit containing one vial of lyophilized drug and two vials of SIRE supplement (20 tests per kit)
3. BACTEC MGIT 960 INH 0.4 kit containing one vial of lyophilized drug and two vials of SIRE supplement (20 tests per kit)

B. Materials required and not provided

1. BBL MGIT 7-ml tubes
2. Ancillary culture media, reagents
3. Laboratory equipment as required for this procedure

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Strains

1. Test QC strains, *M. tuberculosis* ATCC 27294, upon receipt of a new shipment or lot number of MGIT 960 SIRE kit vials
2. Maintain QC stock strains at −70°C.
3. The QC AST set should be prepared according to the "Inoculation Procedure for Susceptibility Test" instructions for the drug kits being tested.

IV. QUALITY CONTROL *(continued)*

4. Observation of the proper results within 4 to 13 days, as shown below, indicates that the BACTEC MGIT 960 SIRE kits are ready for use in testing patient isolates. If the proper results are not observed, repeat the test.

Strain	Growth control	MGIT STR	MGIT INH	MGIT RIF	MGIT EMB	MGIT STR 4.0	MGIT INH 0.4
M. tuberculosis ATCC 27294	Positive	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible

B. Troubleshooting

1. The same QC organism should be run once each week when susceptibility testing is performed.

2. If the batch QC fails, do not report patient results for the drug(s) that failed for that testing period. Repeat the QC for the drug(s) and patient isolates affected by the initial QC failure. If the repeat QC does not perform as expected, do not report patient results. Send specimen to reference laboratory for evaluation.

V. PROCEDURE

It is imperative that these cultures be handled in a biological safety cabinet.

A. Specimen preparation

1. Preparation of isolate from solid media

a. Add 4 ml of Middlebrook 7H9 broth or MGIT broth to a sterile tube with cap containing glass beads.

b. Scrape with a sterile loop several colonies and suspend in 7H9 broth.

c. Vortex the suspension for 2 to 3 min to break up the large clumps. The suspension should exceed a 1.0 McFarland standard turbidity.

d. Allow suspension to sit for 20 min.

e. Transfer the supernatant to another sterile tube with cap, and let it sit for an additional 15 min.

f. Transfer the supernatant to another tube. Adjust suspension to a 0.5 McFarland standard.

g. Dilute 1 ml of the adjusted suspension in 4 ml of sterile saline (1:5 dilution).

2. Preparation from a positive BACTEC MGIT 7-ml tube

a. A positive 7-ml MGIT tube should be used the day after it becomes positive on the BACTEC MGIT 960 instrument (day 1), up to and including the fifth day (day 5) after instrument positivity.

b. A tube that has been positive for more than 5 days should be subcultured to a fresh 7-ml MGIT tube containing BACTEC MGIT 960 growth supplement and tested on the BACTEC MGIT 960 instrument until positive and used from 1 to 5 days following positivity.

c. If the tube is a day 1 or 2 positive, mix well and proceed.

d. If the tube is a day 3, 4, or 5 positive, mix well and dilute 1 ml of positive broth in 4 ml of sterile saline and proceed with diluted suspension.

B. Inoculation procedure for BACTEC MGIT 960 SIRE kit susceptibility test

1. Label five 7-ml MGIT tubes for each test isolate. Label one as GC (growth control), one as STR, one as INH, one as RIF, and one as EMB. Place the tubes in the correct sequence in the appropriate-size AST set carrier.

2. Aseptically add 0.8 ml of MGIT SIRE supplement to each tube.

3. Asceptically pipette 100 µl of 83-µg/ml MGIT STR solution to the appropriately labeled MGIT tube.

4. Aseptically pipette 100 µl of 8.3-µg/ml MGIT INH solution to the appropriately labeled MGIT tube.

V. PROCEDURE *(continued)*

5. Aseptically pipette 100 µl of 83-µg/ml MGIT RIF solution to the appropriately labeled MGIT tube.
6. Aseptically pipette 100 µl of 415-µg/ml MGIT EMB solution to the appropriately labeled MGIT tube.
7. No antimicrobial agents should be added to the MGIT growth control tube.

Drug	Drug concn (µg/ml) after reconstitution[a]	Vol (µl) added to MGIT tubes	Final concn (µg/ml) in MGIT tubes
MGIT STR	83	100	1.0
MGIT INH	8.3	100	0.1
MGIT RIF	83	100	1.0
MGIT EMB	415	100	5.0

[a] Reconstitute using 4 ml of sterile distilled and deionized water.

C. Growth control tube preparation and inoculation

1. Aseptically pipette 0.1 ml of the organism suspension into 10 ml of sterile saline to prepare the 1:100 growth control suspension.
2. Mix the growth control suspension thoroughly. Inoculate 0.5 ml of the 1:100 growth control suspension into the MGIT tube labeled "GC."

D. Inoculation of drug-containing tubes

1. Aseptically pipette 0.5 ml of the organism suspension into each of the four remaining drug tubes (STR, INH, RIF, and EMB).
2. Tightly recap the tubes. Mix tubes thoroughly by gentle inversion three or four times.
3. Enter the AST set into the MGIT 960 using the AST set entry feature (refer to the *BACTEC MGIT 960 User's Manual,* "AST Instructions").
4. Streak 0.1 ml of the organism suspension onto a TSA with 5% sheep blood plate. Enclose in a plastic bag. Incubate at 35 to 37°C.
5. Check the blood agar plate at 48 h for bacterial contamination. If the blood agar plate shows no growth, then allow AST testing to proceed. If the blood agar plate shows growth, discard the AST set (refer to the *BACTEC MGIT 960 User's Manual,* "AST Instructions") and repeat testing with pure culture.

E. Inoculation procedure for BACTEC MGIT STR 4.0 and INH 0.4 kits

It is recommended that if resistance occurs at the critical concentration of a test drug, a susceptibility test be performed with a higher concentration of the drug, especially for INH.

1. Label enough 7-ml MGIT tubes for the test isolate to have a MGIT growth control and a MGIT drug tube for each antimicrobial agent tested. Place the tubes in the correct sequence in the appropriate-size AST set carrier (*see BACTEC MGIT 960 User's Manual,* "AST Instructions").
2. Aseptically add 0.8 ml of BACTEC MGIT SIRE supplement to each tube.
   ◩ **NOTE:** It is important to use the supplement supplied with the kit.
3. Aseptically pipette, using a micropipette, 100 µl of the drug solution to the appropriately labeled MGIT tube. It is important to add the correct drug to the corresponding tube. No antimicrobial agents should be added to the MGIT growth control tube.

Drug	Concn (µg/ml) of drug after reconstitution[a]	Vol (µl) added to MGIT tubes	Final concn (µg/ml) in MGIT tubes
MGIT STR 4.0	332	100	4.0
MGIT INH 0.4	33.2	100	0.4

[a] Reconstitute using 2 ml of sterile distilled and deionized water.

V. PROCEDURE *(continued)*

F. Growth control tube preparation and inoculation

1. Aseptically pipette 0.1 ml of the organism suspension into 10 ml of sterile saline to prepare the 1:100 growth control suspension.
2. Mix the growth control suspension thoroughly.
3. Inoculate 0.5 ml of the 1:100 growth control suspension into the MGIT tube labeled "GC."

G. Inoculation of drug-containing tubes

1. Aseptically pipette 0.5 ml of the organism suspension into each of the drug tubes.
2. Tightly recap the tubes. Mix tubes thoroughly by gentle inversion three or four times.
3. Enter the AST set into the MGIT 960 using the AST set entry feature (refer to the *BACTEC MGIT 960 User's Manual*, "AST Instructions").
4. Ensure that the order of the tubes in the AST set carrier conforms to the set carrier definitions selected when performing the AST set entry feature.
5. Streak 0.1 ml of the organism suspension onto a TSA blood agar plate. Enclose in a plastic bag. Incubate at 35 to 37°C.
6. Check the blood agar plate at 48 h for bacterial contamination. If the blood agar plate shows no growth, then allow AST testing to proceed. If the blood agar plate shows growth, discard the AST set (refer to the *BACTEC MGIT 960 User's Manual*, "AST Instructions") and repeat testing with pure culture.

VI. RESULTS AND INTERPRETATION

A. Susceptible: when the fluorescence in the drug-containing tube is less than that of the growth control tube, then the BACTEC MGIT 960 interprets the result as susceptible.
B. Resistant: when the fluorescence in the drug-containing tube is equal to that of the growth control tube, then the BACTEC MGIT 960 interprets the result as resistant.
C. When certain conditions occur that may affect test results, the MGIT 960 instrument reports as an error (X) and there is no susceptibility interpretation.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report "susceptible" or "resistant" based on the BACTEC MGIT 960 instrument's interpretation.
B. Do not report if the BACTEC MGIT 960 instrument interpretation result is an error (X).
C. Include test method, drug, and concentration.

VIII. LIMITATIONS

A. The BACTEC MGIT 960 susceptibility test does not interpret the degree of susceptibility of the isolate being tested. Results are reported as either S (susceptible) or R (resistant) for the drug and concentration tested.
B. The BACTEC MGIT 960 SIRE test was developed with critical concentrations for STR, INH, RIF, and EMB that are slightly lower than the critical concentrations used in the method of proportion in order to avoid false susceptibility. Testing of the higher concentrations, as recommended, will enhance the ability to detect isolates with low-level resistance.
C. The BACTEC MGIT 960 susceptibility tests can be performed only using the BACTEC MGIT 960 instrument. The AST sets cannot be read manually. Use only pure cultures of *M. tuberculosis.* Cultures that are contaminated or that may contain multiple species of mycobacteria may give erroneous results and

VIII. LIMITATIONS *(continued)*

should not be tested. Direct testing from clinical specimens is not recommended. Suspensions made from solid media must be allowed to settle for the prescribed times prior to standardization. Inoculum preparations made from solid media should be visually compared to a 0.5 McFarland turbidity standard; failure to do so may give inaccurate results or cause an AST set error.

D. Failure to use the 1:5 dilution of the organism suspension, when indicated, to inoculate the drug-containing tubes may give inaccurate results.

E. Failure to use a 1:100 dilution of the organism suspension for the inoculation of the growth control tube may give inaccurate results or cause an AST set error.

F. Failure to reconstitute the drugs with the appropriate volume of sterile distilled and deionized water may give inaccurate results.

G. Thorough mixing of inoculated tubes is important. Failure to mix the tubes adequately can lead to false-resistant results.

H. Failure to load the tubes of the AST set into the AST set carrier in the proper sequence may give inaccurate results. Failure to select the appropriate set carrier drug definition may result in invalid or inaccurate results.

I. Failure to load the AST set into the instrument correctly will result in an anonymous condition that must be resolved within 8 h. If the condition is not resolved within 8 h, the AST set must be discarded and set up again.

J. Failure to use the SIRE supplement in the AST set may give inaccurate results. *Do not* add BACTEC MGIT 960 growth supplement to the AST set.

SUPPLEMENTAL READING

Barenfanger, J. 1993. Making your lab safe against multi-drug resistant *Mycobacterium tuberculosis. Clin. Microbiol. Newsl.* **15:**76–80.

Becton Dickinson Diagnostic Systems. Data on file.

NCCLS. 1995. *Antimycobacterial Susceptibility Testing for* Mycobacterium tuberculosis. Tentative standard M24-T. NCCLS, Wayne, Pa.

NCCLS. 2000. *Susceptibility testing of Mycobacteria, Nocardia, and Other Aerobic actinomycetes,* 2nd ed., vol. 20, no. 26. Tentative standard M24-T2. NCCLS, Wayne, Pa.

7.8.6 BACTEC MGIT 960 PZA—Susceptibility Testing for *Mycobacterium tuberculosis*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The BACTEC MGIT 960 PZA kit is a 4- to 21-day qualitative test used for pyrazinamide (PZA) susceptibility testing of *Mycobacterium tuberculosis* from culture using the BACTEC MGIT 960 system. The testing for PZA requires modification from the general methods because the drug is active in vitro only at lower pH values (1). The MGIT 960 PZA medium contains a modified Middlebrook 7H9 broth at a reduced pH of 5.9.

The mycobacteria growth indicator tube (MGIT tube) contains a fluorescent compound embedded in silicone on the bottom of a 16- by 100-mm round-bottom tube. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. The initial concentration of dissolved oxygen quenches the emission of fluorescence from the compound, and little fluorescence can be detected. Later, actively respiring microorganisms consume the oxygen, which allows the compound to fluoresce. The test is based on growth of the *M. tuberculosis* isolate in a drug-containing tube compared to a drug-free tube (growth control). The BACTEC MGIT 960 instrument continually monitors tubes for increased fluorescence. Analysis of fluorescence in the drug-containing tube compared to the fluorescence of the growth control tube is used by the instrument to determine susceptibility results. The BACTEC MGIT 960 instrument automatically interprets these results using predefined algorithms (which compare growth in the drug-containing tube to that in the growth control tube), and the susceptible or resistant result is reported by the instrument.

II. SPECIMENS

Susceptibility tests are performed from pure cultures of *M. tuberculosis.*

III. MATERIALS

Include QC information on reagent container and in QC records.

A. BACTEC MGIT PZA medium (20 to 25°C)

7 ml of PZA broth in a polypropylene tube with a fluorescent indicator in the bottom

B. BACTEC MGIT 960 PZA kit (2 to 8°C)

1. two lyophilized vials of PZA

2. six vials of PZA supplement

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Upon receipt of a new shipment or lot number of BACTEC MGIT 960 PZA kits or BACTEC MGIT PZA medium, it is suggested that the control organism *M. tuberculosis* ATCC 27294 be tested.

B. Observation of the proper results within 4 to 21 days indicates that the BACTEC MGIT 960 PZA reagents are ready for use in testing patient isolates. If the proper results are not observed, repeat the test.

IV. QUALITY CONTROL
(continued)

C. The same control organism should be run as batch QC once each week when susceptibility testing is performed. If the batch QC fails, do not report patient results for the drug that failed for that testing period. Repeat the QC for the drug and patient isolates affected by the initial QC failure. If the repeat QC does not perform as expected, do not report patient results.

V. PROCEDURE

It is imperative that these cultures be handled in a biological safety cabinet.

A. Preparation of the isolate from solid media

1. Add 4 ml of Middlebrook 7H9 broth or BBL MGIT broth to a sterile tube with cap containing glass beads.
2. Scrape with a sterile loop as many colonies as possible from any solid medium. Suspend the colonies in the Middlebrook 7H9 broth. The suspension should exceed a 1.0 McFarland standard.
3. Vortex the suspension for 2 to 3 min to break up the larger clumps.
4. Let the suspension sit for 20 min.
5. Transfer the supernatant fluid to a sterile tube with cap and let sit for another 15 min.
6. Transfer the supernatant to a third tube.
7. Adjust the suspension to a 0.5 McFarland standard.
8. Dilute 1 ml of the adjusted suspension in 4 ml of sterile saline (1:5 dilution).

B. Preparation from a positive BACTEC MGIT tube

1. A positive 7-ml MGIT tube should be used the day after it first becomes positive on the BACTEC MGIT 960 instrument (day 1), up to and including the fifth day (day 5) after instrument positivity. A tube which has been positive for more than 5 days should be subcultured to a fresh 7-ml MGIT tube containing BACTEC MGIT 960 growth supplement and tested on the BACTEC MGIT 960 instrument until positive, and it should be used from 1 to 5 days following positivity.
2. If the tube is a day 1 or 2 positive, then proceed.
3. If the tube is a day 3, 4, or 5 positive, then dilute 1 ml of the positive broth in 4 ml of sterile saline (1:5 dilution) and proceed with the diluted suspension.

C. Inoculation procedure for BACTEC MGIT 960 PZA susceptibility test

1. Label two 7-ml MGlT PZA tubes for each test isolate. Label one as GC (growth control) and one as PZA. Place the tubes in the correct sequence in the two-tube AST set carrier (*see BACTEC MGIT 960 User's Manual,* "AST Procedure").
2. Aseptically add 0.8 ml of BACTEC MGlT PZA supplement to each tube.
   ◩ **NOTE:** It is important to use the correct supplement.
3. Aseptically pipette, using a micropipette, 100 µl of 8,000-µg/ml BACTEC MGlT PZA drug solution to the appropriately labeled BACTEC MGIT tube. No drug solution should be added to the MGIT growth control tube.

Drug	Concn of drug after reconstitution	Vol added to MGIT tubes for test	Final concn in MGIT tubes
MGIT PZA	8,000 µg/ml	100 µl	100 µg/ml[a]

[a] Equivalent to CDC-recommended critical drug concentrations.

D. Growth control tube preparation and inoculation

1. Aseptically pipette 0.5 ml of the organism suspension into 4.5 ml of sterile saline to prepare the 1:10 growth control suspension.
2. Mix the growth control suspension thoroughly. Inoculate 0.5 ml of the 1:10 growth control suspension into the MGIT tube labeled "GC."

V. PROCEDURE *(continued)*

E. Inoculation of drug-containing tube

1. Aseptically pipette 0.5 ml of the organism suspension into the drug tube.
2. Tightly recap the tubes and mix well.
3. Enter the AST set into the BACTEC MGIT 960 instrument using the AST set entry feature (refer to the *BACTEC MGIT 960 User's Manual*). Ensure that the growth control tube is in the first left tube position. Select PZA as the drug in the two-tube AST set carrier definition when performing the AST set entry.
4. Streak 0.1 ml of the organism suspension onto a TSA with 5% sheep blood plate. Enclose in a plastic bag. Incubate at 35 to 37°C.
5. Check the blood agar plate at 48 h for bacterial contamination. If the blood agar plate shows no growth, then allow AST testing to proceed. If the blood agar plate shows growth, discard the AST set (refer to the *BACTEC MGIT 960 User's Manual*) and repeat testing with pure culture.

VI. RESULTS AND INTERPRETATION

The BACTEC 960 instrument monitors the fluorescence in the growth control and drug-containing tubes until the test is ready to report. At this point the instrument interprets the results and reports as resistant or susceptible. Resistance is reported at the 10% proportion level, unlike SIRE interpretation, which is done at the 1% level.

The instrument will report an error (X) when certain conditions occur and results are not interpretable. In such a situation, the test should be repeated.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

Report "susceptible" or "resistant" as interpreted by the instrument.

Do not report if the instrument interprets results with the error (X).

Do not report if the culture is found not pure.

Monoresistance to PZA is rare. Verify results if found monoresistance by checking purity, identification of *M. tuberculosis*, and other parameters.

VIII. LIMITATIONS

A. The NCCLS recommends the BACTEC 460TB method for PZA susceptibility testing as a reference method (2). The nonradiometric BACTEC MGIT 960 PZA kit provides the susceptibility result in approximately the same time frame as the BACTEC 460TB system. The MGIT 960 PZA kit, like the BACTEC 460TB, uses a PZA concentration of 100 μg/ml, to achieve comparable results.

B. Growth on the solid medium should be fresh (within 14 days). Inoculum preparations made from older cultures without the use of a 0.5 McFarland turbidity standard and the appropriate dilutions may give inaccurate results.

C. Failure to use a 1:10 dilution of the isolate for the inoculation of the growth control tube may give inaccurate results.

D. Failure to use the BACTEC MGIT 960 PZA supplement in the PZA AST set may give inaccurate results. Do not add BACTEC MGIT 960 SIRE supplement or BACTEC MGIT 960 growth supplement to the PZA AST set.

REFERENCES

1. **NCCLS** 1995. *Antimycobacterial Susceptibility Testing for* Mycobacterium tuberculosis, vol. 15, no. 16. Tentative standard M24-T. NCCLS, Wayne, Pa.
2. **NCCLS** 2000. *Susceptibility Testing of Mycobacteria, Nocardia, and Other Aerobic Actinomycetes,* 2nd ed., vol. 20, no. 20. Tentative standard M24-T2. NCCLS, Wayne, Pa.

SUPPLEMENTAL READING

Butler, W. R., and J. O. Kilburn. 1982. Improved method for testing susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. *J. Clin. Microbiol.* **16:**1106–1109.

Heifets, L. B., and M. D. Iseman. 1985. Radiometeric method for testing susceptibility of *Mycobacterium tuberculosis* to pyrazinamide in 7H12 broth. *J. Clin. Microbiol.* **21:**200–204.

Kent, P. T., and G. P. Kubica. 1985. **Public Health Mycobacteriology. A Guide for the Level III Laboratory.** U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta, Ga.

Salfinger, M., L. B. Reller, B. Demchuick, and Z. T. Johnson. 1989. Rapid radiometric method for pyrazinamide susceptibility testing of *Mycobacterium tuberculosis. Res. Microbiol* **140:**301–309.

7.8.7 VersaTREK (Formerly ESP Culture System II)—Indirect Susceptibility Testing for *Mycobacterium tuberculosis*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The VersaTREK, formerly the ESP Culture System II (Trek Diagnostic Systems, Inc., Westlake, Ohio), has been adapted for the performance of antimycobacterial susceptibility testing to the first-line agents rifampin, isoniazid (INH), and ethambutol with clinical isolates of *Mycobacterium tuberculosis* (1, 2). Streptomycin and Pyrazinamide have not been Food and Drug Administration (FDA) cleared for antimycobacterial susceptibility testing with the VersaTREK as of this writing. A standardized suspension of *M. tuberculosis* is inoculated into drug-containing ESP Myco bottles and a drug-free control bottle. The bottles are placed into the ESP Culture System II instrument and monitored for growth. The test is terminated 3 days after the control bottle signals positive. Any drug-containing bottle becoming positive within that time indicates resistance of the test isolate to that drug at the tested concentration.

II. SPECIMENS

The VersaTREK system has been cleared by the FDA for use with pulmonary and extrapulmonary specimens, including blood, for indirect susceptibility testing with rifampin, INH, and ethambutol.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Materials provided by manufacturer

Storage of the following is at room temperature except when otherwise indicated.

1. Myco bottles (7H9 broth with Casitone and glycerol)
2. Myco growth supplement (oleic acid, bovine serum albumin, glucose, and catalase) (4 to 8°C in the dark)
3. Connectors
4. Lyophilized drugs or drug powder (4 to 8°C)

B. Materials required but not provided

1. Tubes with sterile saline and sterile glass beads
2. Tuberculin syringes with fixed needles
3. Middlebrook 7H11 plates
4. TSA blood agar plates
5. Sterile filter packs

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Strains

1. *M. tuberculosis* H37Rv (ATCC 27294)
2. *M. tuberculosis* ATCC 35838 (rifampin resistant)
3. *M. tuberculosis* ATCC 35837 (ethambutol resistant)
4. *M. tuberculosis* ATCC 35831 (streptomycin resistant) (optional)
5. *M. tuberculosis* ATCC 35822 (INH resistant)
6. Store stock culture suspensions at −70°C.

IV. QUALITY CONTROL *(continued)*

B. Expected results

1. *M. tuberculosis* (H37Rv) is fully susceptible to all first-line antituberculosis drugs tested.
2. Run the control weekly or each day of test, whichever is more frequent.
3. Optional but recommended: strains resistant to each of the four antituberculosis drugs (monoresistant strains) are run monthly. After preparing a new batch of drugs, both the H37Rv strain and the corresponding monoresistant strain must be run before using that batch of drug.

V. PROCEDURE

A. Rehydration of INH, rifampin, and ethambutol

1. Add 25 ml of sterile distilled water to each of the three drug-containing bottles. Swirl to dissolve the contents. Dilute each bottle 1:1 with sterile distilled water. This will result in a final volume of 50 ml. These bottles should be labeled as to their final drug concentration as outlined in Table 7.8.7–1.
2. Remove 5 ml of the rehydrated INH solution and add to a sterile tube containing 15 ml of sterile distilled water. Label as described above (0.1 μg/ml).
3. To a second lyophilized vial of ethambutol, add 40 ml of sterile distilled water. Swirl to dissolve. Dilute 1:1 with sterile distilled water. This will result in a final volume of 80 ml. Label this solution "5 μg/ml ETH."
4. Aliquot the stock drug solution into cryovials (approximately 1.1 ml each) and freeze in labeled tubes at −70°C. These tubes may be used for 6 months after rehydration.

B. Preparation of streptomycin stock solutions (optional; not FDA approved)

1. Streptomycin powder is obtained from Sigma Chemical Company (St. Louis, Mo.), catalog no. S9137.
2. Prepare 50 ml of a 1.2-mg/ml stock solution of streptomycin in distilled water according to the following formula:

$$\text{Weight (mg)} = \frac{50 \text{ ml} \times 1{,}200 \text{ μg/ml}}{\text{Assay potency (μg/mg)}}$$

   The assay potency is found on the label of each container of streptomycin powder. The powder must be weighed on an analytical balance capable of weighing milligram quantities of the antimicrobial powder.
3. Mix the stock solution until completely dissolved and sterilized by filtration through a 0.45-μm-pore-size sterile disposable filter.
4. Aseptically pipette 1.1-ml aliquots into sterile cryotubes and store at −70°C.
5. Prior to use, thaw an aliquot and prepare a 1:10 dilution in sterile distilled water. This will result in a final concentration of 8.0 μg/ml when 1 ml of this dilution is added to a Myco bottle.

Table 7.8.7–1 Drug concentrations

Drug	Vol of sterile, distilled water	Dilution with sterile, distilled water	Final concn (μg/ml) in ESP bottle
INH	25 ml	1:1	0.4
	5 ml of + 15 ml of water	1:1	0.1
Rifampin	25 ml	1:1	1.0
Ethambutol	25 ml	1:1	8.0
	40 ml	1:1	5.0

V. PROCEDURE *(continued)*

6. Take 5 ml of the 1:10 dilution and add it to 15 ml of sterile distilled water. This will result in a final concentration of 2.0 µg/ml in the Myco bottle.

C. Preparation of drug and control bottles

1. Add 1.0 ml of growth supplement to each of eight Myco bottles. This can be done up to 24 h in advance if the supplemented bottles are kept at 2 to 8°C. Prior to use, these bottles must be allowed to come to room temperature.

2. Add 1.0 ml of each of the seven drug stocks to appropriately labeled supplemented ESP Myco bottles.

3. Add 1.0 ml of sterile distilled water to the control bottle.

It is imperative that these cultures be handled in a biological safety cabinet.

D. Preparation of inoculum

1. Growth from solid media

a. Prepare a suspension of the test organism in tubes containing sterile saline and glass beads from growth obtained from slants or plates.

b. Vortex well, and allow the larger particles to settle for at least 30 min. Remove the upper half of suspension to a sterile tube and adjust, with sterile saline, to a turbidity matching that of a 1.0 McFarland standard.

c. Dilute 1:10 with sterile saline. This suspension serves as the inoculum.

2. Growth from seed bottles or primary bottles

a. Myco bottles which have been seeded with the test organism or directly from a positive ESP bottle known to contain *M. tuberculosis* may be used as the source of the inoculum.

b. A seed bottle can be prepared by inoculating a supplemented Myco bottle with a suspension of organism obtained from solid media, as may be the case with an older referred culture, or from a positive bottle.

c. For a Myco bottle to be used as the inoculum source it cannot be refrigerated and *must be* used within 72 h of it giving a positive signal. Store the bottle at room temperature prior to use as the inoculum source.

d. Vortex the bottle for 1 to 2 min.

e. Disinfect the septum with an alcohol prep pad.

f. Remove 1.0 ml of medium from the Myco bottle and add to 9.0 ml of sterile inoculum source.

g. Mix carefully to obtain a homogenous suspension. This can now serve as the inoculum source.

E. Inoculation of bottles

1. Add 0.5 ml of inoculum to each of the drug-containing and control bottles.

2. Inoculate a 7H11 agar plate with a few drops of the inoculum to serve as a purity check.

3. Invert the bottle several times to mix the contents.

4. Disinfect each bottle septum with an alcohol prep pad.

5. Place a connector onto each bottle.

F. Bottle accessioning and reading

1. Accession each bottle into the Myco system using the specimen number and the contents of the bottle, i.e., "EMB 5."

2. When the control bottle signals positive, remove bottle from the system and confirm the presence of acid-fast bacilli (AFB) by performing a Kinyoun stain.

3. Record the time, to the nearest whole day, when the control bottle becomes positive. A time to detection (TTD) of 3.4 days = 3 days; a TTD of 3.8 days = 4 days.

4. The drug-containing bottles should be left on the instrument for 3 days after the control bottle signals positive.

V. PROCEDURE *(continued)*

5. Drug-containing bottles becoming positive either before the control bottle or within 3 days of the control bottle must be removed from the system and smeared, Gram and carbol fuchsin stained, and subcultured onto a blood agar and 7H11 agar plate. The blood agar plate must be incubated at 35°C and 5% CO_2 as per standard protocol.

VI. RESULTS AND INTERPRETATION

A. Susceptible: any drug-containing bottle which fails to signal within 3 days of the control indicates that the test organism is susceptible to that drug at that concentration.

B. Resistant: any drug-containing bottle with a TTD within 3 days of the control and confirmed to have AFB and no bacterial contamination indicates that the test organism is resistant to that drug at the concentration.

C. Recommendation: any organism showing resistance to one or more drugs must have that resistance confirmed by the performance of another test method and must be tested against the second-line antitubercosis agents (New York state health code requirement).

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

Test results are reported as either susceptible (S) or resistant (R) according to the TTD of a drug-containing bottle as indicated above. Report results for each drug concentration tested.

VIII. LIMITATIONS

A. Seed bottles or primary bottles or any liquid medium used as the inoculum source must always be checked for purity.

B. The presence of contaminants or mixed mycobacterial species may lead to a false-resistant result.

REFERENCES

1. **LaBombardi, V. J., and C. Lukowski.** 1997. Antitubercular susceptibility testing using the ESP culture system II, abstr. Y75. *Abstr. Gen. Meet. Am. Soc. Microbiol.* American Society for Microbiology, Washington, D.C.

2. **Trek Diagnostic System, Inc.** 1997. ESP Culture System II. *M. tuberculosis* susceptibility testing. Clinical site protocol. Trek Diagnostic Systems, Westlake, Ohio.

7.8.8

VersaTREK (Formerly ESP Culture System II)—Indirect Susceptibility Tests with Pyrazinamide for *Mycobacterium tuberculosis*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Pyrazinamide (PZA) is now routinely used in first-line therapy for the treatment of infections with strains of *Mycobacterium tuberculosis.* Therefore, susceptibility testing of clinical isolates of *M. tuberculosis* is required. PZA-resistant strains have been observed in patients with multidrug-resistant strains but only rarely in patients with monoresistant strains of *M. tuberculosis.* PZA monoresistance in an *M. tuberculosis* complex isolate is often an indication that the isolate is actually *Mycobacterium bovis* (2). However, routine biochemical testing must be employed to confirm the identification.

The VersaTREK (ESP Culture System II) Myco system (Trek Diagnostic Systems, Inc., Westlake, Ohio) is currently utilized for testing of susceptibility of isolates of *M. tuberculosis* to the first-line antimycobacterial drugs (1). This system has the decided advantage of being able to use a primary culture bottle as the inoculum source for the test (3). This greatly reduces the time required to generate a final result. The same culture bottle can be used as the inoculum source in the PZA assay. PZA requires an acidic pH to retain activity so the assay utilizes a buffer to rehydrate the PZA and is added to the control bottle to provide an acidic environment. As with the other first-line agents, the first isolate of *M. tuberculosis* is tested against PZA and retested if *M. tuberculosis* is isolated from the same patient ≥2 months from the date of the initial isolate. The VersaTREK system has not been cleared by the Food and Drug Administration for indirect susceptibility tests with PZA for *M. tuberculosis.*

II. SPECIMEN

The VersaTREK system has not been cleared by the FDA for use with pulmonary and extrapulmonary specimens, including bloods for indirect susceptibility testing with PZA.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Materials provided by manufacturer

PZA Test kit

1. PZA, 112,500 µg
2. PZA rehydation buffer
3. Myco bottles
4. Growth supplement
5. Connectors

B. Materials required but not provided

1. 1-ml syringes with fixed needles
2. Glass slides
3. Kinyoun stain
4. Middlebrook 7H11 agar plate
5. Sheep blood agar plate
6. 1.5-ml cryotubes
7. Tubes with 9 ml of sterile isotonic saline
8. Tubes with 2 ml of sterile isotonic saline with sterile glass beads

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

M. tuberculosis H37Rv is run each day of the test as a PZA-susceptible control. It is recommended that an isolate of *M. bovis* ATCC 19210 be included each time a new batch of PZA is prepared and monthly after that.

V. PROCEDURE

A. Preparation of the drug

1. Add 25 ml of rehydration buffer to the vial of PZA. Swirl until completely dissolved and dispense 1.1-ml aliquots into the cryotubes. Freeze at −70°C.

2. For each isolate to be tested remove one cryotube from the freezer and allow to thaw completely. Mix gently before use.

B. Preparation of the Myco bottles

Use a 1-ml syringe with a fixed needle to add the following to each Myco bottle.

1. Add 1.0 ml of growth supplement to each of two Myco bottles.

2. Add 1.0 ml of rehydrated drug to the test bottle.

3. Add 1.0 ml of rehydration buffer to the control bottle.

It is imperative that these cultures be handled in a biological safety cabinet.

C. Preparation of the inoculum

1. From a primary bottle

a. A positive Myco bottle containing an isolate confirmed as belonging to the *M. tuberculosis* complex may be used as the inoculum source.

b. This bottle must be used within 72 h of the positive signal.

c. Vortex the Myco bottle for 2 to 3 min.

d. Remove a 1-ml aliquot from the Myco bottle and add to 9.0 ml of sterile isotonic saline. Mix completely.

e. Add 0.5 ml of the diluted suspension into both the control and test ESP Myco bottles.

2. From a seed bottle

If a primary Myco culture bottle is beyond the 72-h threshold, a seed bottle must be prepared from the primary bottle.

a. Vortex the ESP Myco bottle for 2 to 3 min.

b. Remove a 0.5-ml aliquot from the primary bottle and add to a Myco bottle that has been supplemented with 1.0 ml of growth supplement,

c. Place the seed bottle into the VersaTREK instrument and incubate until a positive signal is obtained. Proceed as described above.

3. From growth on solid media

a. Transfer growth from the solid medium into a tube containing sterile isotonic saline with glass beads. Vortex for 3 min. Allow to stand for 30 min for the larger particles to settle out.

b. Remove the supernatant and adjust to match that of a 1.0 McFarland standard with sterile isotonic saline.

c. Using this suspension dilute 1:10 with sterile isotonic saline. Use this as the inoculum source. Proceed as described above.

D. Purity check

An aliquot of the inoculum suspension regardless of the source must be inoculated onto a Middlebrook 7H11 plate and onto a blood agar plate to check for isolate purity.

E. Bottle placement

1. Wipe the septum of each bottle with 70% isopropyl alcohol. Place a connector onto each bottle.

2. Accession into the VersaTREK (ESP) computer system. Indicate the patient name and chart number and delineate the control from the test bottle.

3. Place into the indicated location in the VersaTREK (ESP) instrument.

VI. RESULTS AND INTERPRETATION

A. All positive bottles must be smeared and stained by the Kinyoun method to confirm the presence of mycobacteria and rule out contamination. In addition, check the blood agar purity plate for signs of contamination prior to interpreting the test results.

B. The drug-free control bottle should signal positive within 3 to 7 days of being placed into the instrument. If the bottle signals positive prior to 2.5 days, the inoculum was too heavy, or the bottle is contaminated and the test must be repeated. Any control bottle requiring more than 10 days to give a positive signal would also invalidate the test.

C. An isolate is considered resistant to PZA if the drug-containing bottle gives a positive signal within 3 days of the control bottle signaling positive.

D. An isolate is considered susceptible to PZA if the drug-containing bottle fails to give a positive signal within the 3-day period.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

The results are reported as either susceptible (S) or resistant (R) to PZA.

VIII. LIMITATIONS

A. This procedure has been validated with isolates of *M. tuberculosis* complex only.

B. When performing the test from a primary bottle or a seed bottle prepared from a primary bottle care must be taken when reporting a resistant result. Mixed mycobacterial infections can lead to false resistance. In cases of resistant results the result must be kept preliminary until the growth from the purity plate can be observed.

C. Any PZA-monoresistant strain of *M. tuberculosis* must be confirmed. The nitrate reduction and niacin accumulation tests must be performed to rule out *M. bovis.* If the strain is confirmed as *M. tuberculosis*, the PZA susceptibility test must be repeated.

REFERENCES

1. **Difco** 1996. Difco ESP Culture System II *M. tuberculosis* susceptibility testing. Clinical site protocol, phase 3. Difco, Detroit, Mich.
2. **Hannan, M. M., E. P. Desmond, G. P. Morlock, G. H. Mazwek, and J. T. Crawford.** 2001. Pyrazinamide-monoresistant *Mycobacterium tuberculosis* in the United States. *J. Clin. Microbiol.* **39:**647–650.
3. **LaBombardi, V. J.** 2002. Comparison of the ESP and Bactec systems for susceptibility testing of *Mycobacterium tuberculosis* complex to pyrazinamide. *J. Clin. Microbiol.* **40:**2238–2239.

SECTION 8

Mycology and Antifungal Susceptibility Testing

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8.1 Introduction and General Considerations

Fungi are significant, sometimes overlooked, human pathogens. Infections range in severity from merely cosmetic to life threatening. Over the last decade, in particular, the spectrum of agents responsible for infection has altered in response to changes in the susceptible population, notably, increases in immunocompromised subjects and greater use of antifungal agents. Fortunately, awareness of the role of opportunistic fungi in disease is growing, but with that awareness the clinical laboratory must be prepared to identify a range of fungal species. In addition, new antifungal agents less toxic than amphotericin B and variously administered have increased the requirement to identify potential pathogens to the species level. (It is no longer acceptable in most situations to report a yeast that does not produce germ tubes as "yeast, not *Candida albicans.*") While amphotericin B is effective against many invasive fungal pathogens, newer drugs with a narrower spectrum of efficacy are available (e.g., the echinocandins). Resistance to these drugs is sometimes a species characteristic and may also be isolate dependent for other species. This section brings together the contributions of well-respected mycologists, whose expertise should help laboratories become more adept at identifying fungi, recognizing the significance of an isolated organism, and performing susceptibility testing.

Discussion of results between the clinical mycologist and the anatomic pathologist, cytopathologist, or neuropathologist often facilitates interpretation of culture significance. In some fungal diseases (e.g., histoplasmosis and coccidioidomycoses), the histopathology services may provide the first diagnostic evidence of the disease while the clinical laboratory provides confirmation of the etiologic entity. The appearance of the fungal organism in the tissue material, along with patient history and symptomatology, provides the best guidance to the likely etiology, but this should be confirmed whenever possible by mycological analysis.

I. CLASSIFICATION

Fungal taxonomy is an evolving process. Nomenclatural changes also continue to have significant implications for understanding fungi in the biological community, fungal disease, and the treatment of disease. We have endeavored to mention developments in molecular taxonomy, but as these methods are not commercialized and are still primarily research investigation based, we have limited our presentation to more of an overview of the methods available. One problem with molecular taxonomy that leads to confusion is the assignment of new species epithets to an apparently distinct clade of an established species (e.g., *Candida africanum* for an African clade of *Candida albicans*). Once the new epithet is published, it is sometimes considered a valid and distinct species by some authors, when in fact the new epithet has no taxonomic validity until it is accepted by the International Botanical Congress. Another example of this problem is with *Pneumocystis carinii* versus the more recently coined species *Pneumocystis jirovecii*, which is associated with humans.

II. SAFETY

It is imperative that these cultures be handled in a biological safety cabinet.

Safety measures, as described elsewhere in this handbook, should always be followed in a clinical mycology laboratory. Every mould should be considered a potential pathogen and should be manipulated only in a biological safety cabinet when specimens come from regions with endemic dimorphic infections, or when the specimen is from a patient where a systemic infection is suspected. If a mould is

II. SAFETY *(continued)*

seen on a bacteriology plate, the plate should be taped closed or sealed with parafilm around the edge to prevent accidental release of infectious particles. Plates may also be sealed with Shrink-Seals (Remel), but we have found these to be cumbersome. The plate must be transported to the mycology area. Manipulation of fungal organisms requires that certain unique or less routine safety measures be followed.

III. LEARNING MORE

Medical mycology and clinical mycology continue to grow and expand. We expect that the information provided in this section will not be without omissions, and laboratorians will need to find other sources of information. A number of useful reference texts and papers are cited in this section. A source of timely information is the Internet. Some useful websites on clinical mycology are provided below.

A. http://www.doctorfungus.org
B. http://dir.yahoo.com/Science/Biology/Mycology/
C. http://www.aspergillus.org.uk
D. http://www.mycology.adelaide.edu.au/

8.2 Specimen Selection, Collection, and Transport

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Suitable specimen selection, proper specimen collection, and rapid specimen transport must be performed to ensure the successful isolation of the etiologic agent of a fungal infection. To establish or confirm the diagnosis of a suspected fungal infection, it is essential for the clinician to provide the laboratory with adequate specimens for evaluation. Also, it is essential for the laboratory to have guidelines for the clinician regarding minimum specimen volumes and appropriate specimen transport (e.g., a laboratory manual or web page). The microbiology laboratory should be notified if an unusual pathogen or an organism that can be a significant laboratory hazard is suspected, as some require special handling or special stains. Examples of unusual fungal and bacterial pathogens, respectively, include *Malassezia* spp. (some species require the addition of olive oil to culture media) and *Nocardia* spp. (which are more easily detected on a modified acid-fast stain [*see* section 6 of this handbook]). Examples of potential laboratory hazards include *Coccidioides immitis* and *Histoplasma capsulatum.* Additionally, the microbiology laboratory should be contacted prior to certain procedures, as some specimens for fungal culture may require bedside inoculation onto appropriate culture media (e.g., corneal scrapings).

Communication between the laboratory and the physician about a patient's travel history may be helpful in the determination of what specimen to collect or what test to order. The patient's travel history may suggest a likely etiologic agent, such as *Coccidioides immitis,* which is endemic in the southwestern United States; *H. capsulatum,* which is endemic in North America (tending to be associated with the major river valleys [Ohio, Mississippi, and Missouri]; and *Penicillium marneffei,* which is endemic in Southeast Asia and southern China. If there is any question about how to select, collect, or transport a specimen, the clinician should contact the microbiology laboratory for assistance.

II. TRANSPORT OF SPECIMENS FOR FUNGAL CULTURE

Observe standard precautions.

◩ **NOTE:** In general, transport media are not recommended unless the specimen can be completely retrieved from the medium. For site-specific collection guidelines, refer to Table 8.2–1.

A. General considerations

1. Collect specimens aseptically and place in sterile, leakproof containers.
2. Deliver the specimen to the laboratory within 2 h.
3. Rapid transport is critical to ensure the survival and isolation of fastidious organisms and to prevent overgrowth by more hardy bacteria.
4. Process the specimen within 2 h of receipt or store appropriately.
5. If processing is going to be delayed, incubate normally sterile specimens (e.g., blood, bone marrow, CSF, or deep-lesion material) at 37°C (or 35°C if a 37°C incubator is not available). Refrigerate at 4°C specimens that are potentially contaminated with bacterial microbiota (e.g., dermatological specimens, transtracheal aspirates, ear [internal] aspirates, and conjunctiva cultures).

   ◩ **NOTE:** *H. capsulatum* does not survive for long periods under refrigeration or on dry ice (3). Limited studies have shown significantly decreased

Table 8.2–1 Site-specific selection and collection guidelines

Specimen source	Fungal agent[a]	Acceptable collection and transport procedures[b]	Comments
Abscess/drainage/ wound	Yeasts Filamentous fungi Aerobic actinomycetes	Aspirate sample and transport in a syringe without needle. Submit in a sterile screw-cap container. Collect sample with aerobic swab transport system. Non-cotton-tip swabs are recommended. Swabs are the least preferred collection device.	Sample advancing margin of lesion. If specimen is collected at surgery, also submit a portion of the abscess wall. Several swabs, if possible, should be sent to the laboratory to allow for a Gram stain and a KOH or calcofluor white stain (*see* procedure 8.3).
Blood	*H. capsulatum* *C. neoformans* *Candida* spp. *Malassezia* spp.	Lysis-centrifugation device (Isolator [Wampole]) (1, 4) Automated blood culture systems (BACTEC [Becton Dickinson]; BacT/Alert [Organon Teknika]) 8 ml of blood in tube containing SPS (e.g., yellow Vacutainer tube no. 4960; contains 1.7 ml of 0.35% SPS; final concentration with blood, 0.05%) Biphasic vented BHI medium	Most *Candida* spp. can be recovered in bacterial blood culture systems. If using an automated blood culture system, review manufacturer's guidelines to determine which fungi are detected. Samples for cryptococcal antigen, histoplasma antigen, β-D-1,3-glucan, or galactomannan antigen testing may be useful.
Bone marrow	*H. capsulatum* *C. neoformans*	Lysis-centrifugation device (Isolator) 5 ml in a green-top (heparin) tube 5 ml of bone marrow in tube containing SPS (e.g., yellow Vacutainer tube no. 4960; contains 1.7 ml of 0.35% SPS) Bedside inoculation onto appropriate fungal medium Automated blood culture systems (BACTEC; BacT/Alert)	If using an automated blood culture system, review manufacturer's guidelines to determine which fungi are detected.
Catheter (7)	*Malassezia* spp. *Candida* spp.	Place 5 cm of the distal end into sterile screw-cap container.	A standardized method for identifying catheter-related fungal infection has not been developed.
Catheter exit site		Using aerobic swab transport system, collect sample from infected skin site surrounding the intravenous line.	Fungal blood cultures should also be submitted.
Eye (9) Corneal scrapings	*Candida albicans* *C. neoformans* Numerous filamentous fungi have been isolated in fungal keratitis. These include *Fusarium, Aspergillus, Acremonium, Paecilomyces, Penicillium, Pseudallescheria, Curvularia, Alternaria, Phialophora,* and *Bipolaris* spp.	Use direct inoculation onto appropriate fungal medium. The physician should contact the laboratory to obtain medium prior to corneal scraping procedure.	Agar plates are inoculated by lightly touching both sides of the spatula in a row of separate C streak marks.
Conjunctiva		Use bedside inoculation onto appropriate fungal medium or aerobic swab transport system.	Sample both eyes separately (even if one is uninfected) prior to applying anesthetic. The uninfected eye can act as a control to compare agents isolated from the infected eye.
Intraocular fluid		Collect in a sterile screw-cap container.	If intraocular washings, fluid should be concentrated prior to plating.

Table 8.2–1 *(continued)*

Specimen source	Fungal agent[a]	Acceptable collection and transport procedures[b]	Comments
Hair/nails	*Trichophyton* spp. *Epidermophyton* spp. *Microsporum* spp. *Candida* spp. *Trichosporon* spp.	After selecting infected area, scrape scalp and, if possible, collect at least 10 broken hairs for dermatophyte infection, scrape infected nail area, or clip infected nail. For the scalp, gently scraping with a sterile toothbrush or small hairbrush works well for culture. For piedra, cut off several hairs with nodules attached. Use bedside inoculation onto appropriate fungal medium or transport in a clean envelope or between two clean glass slides taped together (transport slides in slide carrier).	Humidity in a closed system may cause the sample to be overgrown by bacteria. With black-dot alopecia, it may be impossible to pull out broken hairs for suspected tinea capitis; scrapings are effective. To diagnose piedra, the nodules on the hair shaft must be examined.
Respiratory sites	Yeast and filamentous fungi Aerobic actinomycetes	Collect three early-morning sputa resulting from a deep cough. Collect bronchoalveolar lavage samples, transtracheal aspirates, or bronchial brushings. Induce sputum. Transport specimens in a sterile screw-cap container.	24-h sputum collections are not acceptable for fungal culture.
Skin/intertriginous areas	*Trichophyton* spp. *Epidermophyton* spp. *Microsporum* spp. *Candida* spp. *Malassezia* spp. *Sporothrix schenkii*	To reduce the likelihood of bacterial contamination, the skin surface should be disinfected with 70% alcohol. The specimen should be collected from the edge of the lesion and inoculated directly onto fungal medium or placed in a clean envelope or between two clean glass slides taped together (transport slides in a slide carrier).	Humidity in the closed system may cause the sample to be overgrown by bacteria.
Sterile fluids (CSF, pleural, pericardial, joint, peritoneal)	*H. capsulatum* *C. neoformans*	Collect a minimum of 2 ml in a sterile container. In general, the more fluid obtained for culture, the better the chance of isolation of any fungal pathogen.	If CSF, samples for cryptococcal antigen testing may be useful.
Tissues/biopsy specimens	Yeast and filamentous fungi Aerobic actinomycetes	Collect tissue and transport in sterile screw-cap container with a small amount of nonbacteriostatic saline to prevent drying. *Never transport in formalin.*	The size of the tissue specimen should approximate that of a pea.
Urine	Yeast *C. immitis* *H. capsulatum* *B. dermatitidis*	First morning clean-catch urine in sterile screw-cap cup Catheterized specimen in sterile screw-cap cup Urine collected in a sterile screw-cap cup following prostatic massage *Note:* Patients with blastomycosis or cryptococcosis may have prostatic infection.	Most *Candida* spp. will grow on bacterial culture media. 24-h urine collections and Foley catheter urine specimens are not acceptable. Samples for histoplasma and *Blastomyces* antigen testing may be useful. The significance of growth of other moulds from urine specimens should be considered cautiously.

[a] The anatomic site of the fungal infection can be an indicator of what mycotic agent to suspect, but in an immunocompromised patient population, virtually any fungus can be an opportunistic pathogen.

[b] Veterinary specimens should be collected and transported in the same manner as human specimens. SPS, sodium polyanethol sulfonate.

II. TRANSPORT OF SPECIMENS FOR FUNGAL CULTURE *(continued)*

viability for *H. capsulatum*, *C. immitis*, *Blastomyces dermatitidis*, and *Aspergillus fumigatus* stored at room temperature or on dry ice. *Rhizopus arrhizus* is also known to be difficult to recover from delayed specimens (6). Therefore, specimens suspected of containing one of these agents should be processed immediately. While it is not documented whether freezing by methods other than dry ice would reduce viability, it is likely that freeze-thaw damage associated with any method of freezing would be sufficient to cause a problem.

6. The laboratory should establish minimum volume requirement guidelines. For some fungal infections, the fungal load may be low. Inadequate amounts of specimen may yield false-negative results. In order to maximize the chance of recovering organisms in sterile fluids, it is recommended that as much specimen as possible be sent for culture. If the volume received is not adequate for the tests requested, the physician should be contacted to prioritize test requests.
7. Laboratorians should develop criteria for fungal culture frequency in collaboration with their infectious-disease consultants. Multiple specimens from the same site may not always be processed. In general, with the exception of biopsy specimens, one fungal culture per site per day, not to exceed three per week, should be adequate for the recovery of fungi. Exceptions to these guidelines should be made only after the physician of record consults the laboratory director. All biopsy specimens should be processed.
8. In general, swabs for the collection of material from open wounds or draining lesions are not recommended because these sites are frequently contaminated with environmental microorganisms.
9. When possible, to increase the chance of recovery of a fungal organism, the specimen should be collected before an antifungal agent is administered.

B. Shipping guidelines (*see* section 15 of this handbook for a more detailed discussion about shipping)

1. For off-site delivery specimen transport guidelines, refer to the International Air Transport Association (IATA) *Dangerous Goods Regulations*, 50th edition (2), and the U.S. Department of Transportation and the International Civil Aviation Organization (ICAO) regulations. The IATA and ICAO have established two categories of specimens, diagnostic and infectious substances. Both specimen types require specific packaging and handling procedures. The shipper must decide in which category specimens belong. In general, IATA considers specimens that are sent for initial and confirmatory testing for the presence of an infectious pathogen to be infectious substances. Specimens sent for routine presurgical and blood bank screens are considered diagnostic substances.
2. When preparing a specimen for transport, always check the specimen transport guidelines of the receiving laboratory. Specimen transport temperatures follow the same principle as if processing is going to be delayed. Attempt to ship clinical specimens overnight for early morning delivery when possible. Clinical specimens preferably should not be shipped over weekends or holidays. Normally sterile specimens (e.g., blood, bone marrow, CSF, and deep-lesion material) are shipped at room temperature. Overnight shipping on dry ice is the only practical alternative for refrigeration. However, this level of freezing may compromise fastidious organisms (e.g., some dermatophytes, *Malassezia* spp., and *H. capsulatum*). Thus, specimens that are potentially contaminated with bacterial microbiota (e.g., dermatological specimens, transtracheal aspirates, ear [internal] aspirates, and conjunctiva cultures) should also be shipped at room temperature. All specimens should have same-day or overnight delivery.

III. SELECTION OF SPECIMENS FOR FUNGAL CULTURES

Observe standard precautions.

A. For some organisms, ancillary antigen studies may provide information that is useful in the diagnosis and/or confirmation of systemic mycosis.

1. Cryptococcal antigen testing of CSF and serum specimens can be useful for the diagnosis of *Cryptococcus neoformans* infections. Laboratorians should substitute antigen testing for the less sensitive India ink procedure for the diagnosis of *C. neoformans* meningitis. Cryptococcal antigen titers can be useful for monitoring therapeutic efficacy; however, a decrease in titer may be delayed for more than a week following initiation of effective antifungal therapy. A better indicator of therapeutic success is mycological cure.

2. If *H. capsulatum* is suspected, histoplasma polysaccharide antigen (HPA) detection on serum or unpreserved urine can be a useful indicator of systemic infection. Additionally, there is evidence that monitoring HPA levels can help to predict relapse in patients on therapy.

◩ **NOTE:** False-positive HPA assays have occurred with patients with blastomycosis, paracoccidioidomycosis, and *P. marneffei* infections. No cross-reactivity has been found in patients with coccidioidomycosis, cryptococcosis, candidiasis, or aspergillosis (5, 8).

3. A separate *Blastomyces* antigen assay is also available. When cross-reactivity occurs between the histoplasma and the *Blastomyces* antigen assays, the higher titer is associated with the etiologic agent. Both assays can also be performed on bronchoalveolar lavage fluid, although typically higher titers are obtained with urine.

4. The sensitivity and specificity of antigen detection methods for fungi other than those tests mentioned above are still being evaluated. Two tests, the galactomannan antigen assay for the diagnosis of aspergillosis and the β-D-1,3-glucan assay for the detection of candidiasis and other fungal infections, have shown promise. Neither test is sufficient and sensitive or specific enough to provide a definitive diagnosis. Algorithms for how to employ these assays for diagnosis of fungal disease in immunocompromised individuals are under development.

B. Other guidelines for specimen selection, collection, and transport include the following.

1. The specimen must be properly labeled with the patient's name and other relevant demographic information (*see* section 2 of this handbook for information regarding specimen labeling).

2. The specimen should not be contaminated on the outside with sample debris or other potentially hazardous materials.

3. Radioactive specimens should be labeled so that proper waste disposal can be performed.

4. Specimens in syringes should not be sent to the laboratory with needles attached but should be transferred to a sterile tube. If there is little material in the syringe, the physician should draw a small amount of sterile nonbacteriostatic 0.85% NaCl or sterile broth through the syringe and then transfer the specimen to a sterile tube. Alternatively, if transferring it from the syringe will compromise the specimen, the physician should draw a small amount of sterile nonbacteriostatic 0.85% NaCl or sterile broth through the syringe, remove the needle using a method that avoids direct contact with the needle, and then cap the syringe with a sterile cap prior to transporting it to the laboratory.

REFERENCES

1. **Berenguer, J., M. Buck, F. Witebsky, F. Stock, P. Pizzo, and T. Walsh.** 1993. Lysis-centrifugation blood cultures in the detection of tissue-proven invasive candidiasis. *Diagn. Microbiol. Infect. Dis.* **17:**103–109.
2. **International Air Transport Association.** 2009. *Dangerous Goods Regulations,* 50th ed. International Air Transport Association, Miami, FL.
3. **Land, G. A., and J. Stringfellow.** 1992. Collection and transport of specimens, p. 6.1.1–6.1.5. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook,* vol. 1. American Society for Microbiology, Washington, DC.
4. **Lyons, R., and G. Woods.** 1995. Comparison of the BacT/Alert and ISOLATOR blood culture systems for recovery of fungi. *Am. J. Clin. Pathol.* **103:**660–663.
5. **Moser, S.** 1999. Laboratory diagnosis of histoplasmosis. *Clin. Microbiol. Newsl.* **21:**95–99.
6. **Thompson, D. W., W. Kaplan, and B. J. Phillips.** 1977. The effect of freezing and the influence of isolation medium on the recovery of pathogenic fungi from sputum. *Mycopathologia* **61:**105.
7. **Wey, S., and A. Colombo.** 1997. Fungal infections of catheters, p. 139–154. *In* H. Seifert, B. Jansen, and B. M. Farr (ed.), *Catheter-Related Infections.* Marcel Dekker, Inc., New York, NY.
8. **Wheat, L. J., R. B. Kohler, and R. P. Tewari.** 1986. Diagnosis of disseminated histoplasmosis by detection of *Histoplasma capsulatum* in serum and urine specimens. *N. Engl. J. Med.* **314:**83–88.
9. **Wilhelmus, K., T. J. Liesegang, M. S. Osato, and D. B. Jones.** 1994. Cumitech 13A, *Laboratory Diagnosis of Ocular Infections.* Coordinating ed., S. C. Specter. American Society for Microbiology, Washington, DC.

8.3.1 Microscopic Clinical Specimen Examination

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The microscopic examination of clinical specimens for the presence of fungi plays an important part in the laboratory diagnosis of most mycoses. Microscopic examination may provide a rapid indication of the cause of an infection, allowing the prompt initiation of appropriate antifungal therapy. It is also important to establish whether the fungus is present in the specimen prior to culture, as some organisms may also occur as laboratory contaminants. Furthermore, the results of microscopic examination may influence the choice of culture media.

An initial macroscopic examination of the specimen is also important. This will enable the selection of particular parts of the sample for microscopic examination in the case of tissue or sputa, particularly those that are obviously caseous, purulent, or necrotic. Tissue from a suspected mycetoma should be teased apart in order to check for the presence of grains. Hair specimens may be examined by Wood's lamp for fluorescence, which will indicate infection caused by some species of dermatophyte.

When the specimen is too small to allow full examination, culture usually takes precedence over microscopic examination. The exceptions are nail samples, for which there is a high rate of culture failure (ca. 30%), and cases of suspected pityriasis versicolor, for which microscopy of skin scrapings is adequate.

II. SPECIMEN PROCESSING FOR MICROSCOPIC EXAMINATION

Pretreatment of clinical specimens is often required in order to concentrate fungal cells or in order to see the fungal material more clearly. Details of the various methods of pretreatment used for different clinical materials are described in procedure 8.4.

III. MATERIALS

A. Stain reagents (*see* item V below and Appendixes 8.3.1–1 and 8.3.1–2)

B. Supplies

1. Glass slides, coverslips, and pipettes

2. Microscope equipped with low-power (×100) and high-power (×400) magnification

3. Microscope equipped with UV fluorescence for use with the calcofluor stain (excitation filter, $\lambda_{300–412\ nm}$; emission filter, $\lambda_{500\ nm}$)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Perform macroscopic and microscopic examination of all stains for the presence of contaminating organisms or artifacts.

B. Ensure that all commercially prepared reagents have not passed their expiration dates.

C. Perform visualization of yeasts stained with potassium hydroxide (KOH)-calcofluor by fluorescence microscopy to control for calcofluor activity.

V. PROCEDURES

Observe standard precautions.

Staining for direct microscopic examination

A. KOH-calcofluor mount

KOH dissolves proteinaceous tissues, including keratin, and renders them transparent. This enables fungi to be visualized more easily. Calcofluor binds to the chitinous cell walls of fungi and fluoresces under UV light. This allows small amounts of fungus to be easily detected, aids visualization of microscopic morphology, and may also facilitate microscopic examination of specimens. It may also assist rapid screening of specimens by inexperienced operators. Most specimens are suitable for this method. A KOH-calcofluor wet mount is essential for skin, hair, nail, and tissue specimens and may be useful for the examination of mucous membrane swabs, urine, liquified sputa, and other respiratory specimens. Brain biopsy specimens are best evaluated with Gram stain, as KOH produces misleading artifacts. As high concentrations of KOH are caustic, hand protection is recommended.

1. This reagent is typically 10 to 30% (wt/vol) potassium hydroxide and 0.1% calcofluor.
2. Place the material to be examined on a glass slide, add a drop of KOH, and place a coverslip on top. Warming the slide on a heating block or over the pilot flame of a Bunsen burner or incubating it at 30°C for 20 min can accelerate digestion of tissue by the KOH (do not boil!). Keratinous tissue specimens should be left at room temperature for 20 to 30 min to allow digestion and "clearing" of the keratin.
3. Prior to examination, squash the preparation firmly, for example, with a mounting needle or covered marker pen, and remove excess KOH with a paper tissue. This enhances viewing, particularly in the case of nail specimens; however, hair specimens should also be viewed prior to being squashed to aid differentiation of endothrix and ectothrix infections.
4. Examine with fluorescent microscope using UV light source and appropriate filters. With emission filters, fungi appear yellow-green or blue-green (depending on the filters used); without filters, fungi fluoresce bright white. Examine first at ×100 magnification, focusing on possible fungal cells at ×400. Dermatophytes in skin or nail are seen as branching hyphae or arthrospores. Most hyphae will be parallel sided and around 2 μm in width. Yeasts are present as budding cells, pseudohyphae, or yeast mycelium. Artifacts, such as fibers, may be distinguished from hyphae by the lack of septa, tapering ends, and size differences.
5. KOH-calcofluor mounts may be stored overnight, preferably in moist chambers at 4°C, but eventually even fungal hyphae are degraded by KOH, and immediate observation is advised.

B. KOH and KOH-DMSO mount

Where a fluorescence microscope is not available, a simple KOH mount can be used. Dimethyl sulfoxide (DMSO) is sometimes added to the KOH reagent in order to enhance clearing of specimens without heating or incubation. This is usually required only for keratinous or fibrous tissue.

1. Place specimen on slide, add a drop of KOH or KOH-DMSO, add coverslip, and squash preparation. Heat KOH preparations as described above.
2. Examine immediately, particularly KOH-DMSO mounts, as these specimens degrade quickly.

C. India ink method

The capsule of *Cryptococcus neoformans,* while virtually invisible by normal microscopy, will exclude ink particles, resulting in a halo-like appearance around the cells. Preparations of CSF in India ink will allow the presumptive

V. PROCEDURES *(continued)*

identification of encapsulated cryptococcal cells. This stain is suitable to use on specimens of body fluids, CSF, and urine. If the stain is prepared using formalin, hand protection and adequate ventilation are recommended. Physicians should be informed that the India ink method is less sensitive than antigen testing for the diagnosis of cryptococcal meningitis.

1. Addition of a drop of detergent may aid maintenance of dispersed ink particles in this reagent. The India ink should be replaced regularly, because moulds can grow in the solution. Alternatively, 10% (wt/vol) nigrosine in 10% formalin, heated to dissolve the nigrosine and filtered, can be used.
2. After centrifugation, place a drop of sediment (CSF, urine, or body fluid) onto a slide, add a small drop of ink, and mix with the sediment, using the coverslip. Place a coverslip on top.
3. Examine by light microscopy. If the preparation is too dark, add a drop of water on one side of the coverslip and draw across by applying blotting paper to the other side of the coverslip, forming an ink gradient.

   ◩ Cryptococcal cells can be differentiated from white blood cells present in CSF by the presence of a halo and by budding in some of the yeast cells. Care must be exercised, as some strains of cryptococci can be nonencapsulated; therefore, it is important to ensure that the yeast cell is in focus when interpreting the presence or absence of a capsule.

D. Giemsa stain for *Histoplasma capsulatum*

The small yeast cells of *H. capsulatum* can be highlighted by Giemsa staining of peripheral blood or bone marrow smears.

1. Giemsa stain solution (commercial preparation) should be diluted as directed by the manufacturer prior to use, typically 1:10 or 1:20 in 65 mM potassium phosphate buffer or distilled water.
2. Fix smear in 100% methanol for 1 min, drain alcohol, and dry.
3. Flood slide with diluted Giemsa stain and stain for 5 min; wash slide with copious water and air dry (do not blot).
4. Examine slide by light microscopy. Phagocytosed yeast cells stain light to dark blue and will be surrounded by a clear halo.

E. Microscopic morphologies seen in specimens

Figure 8.3.1–1 lists the different clinical specimens, the fungal morphologies that may be present in these materials, and the possible etiologic agents.

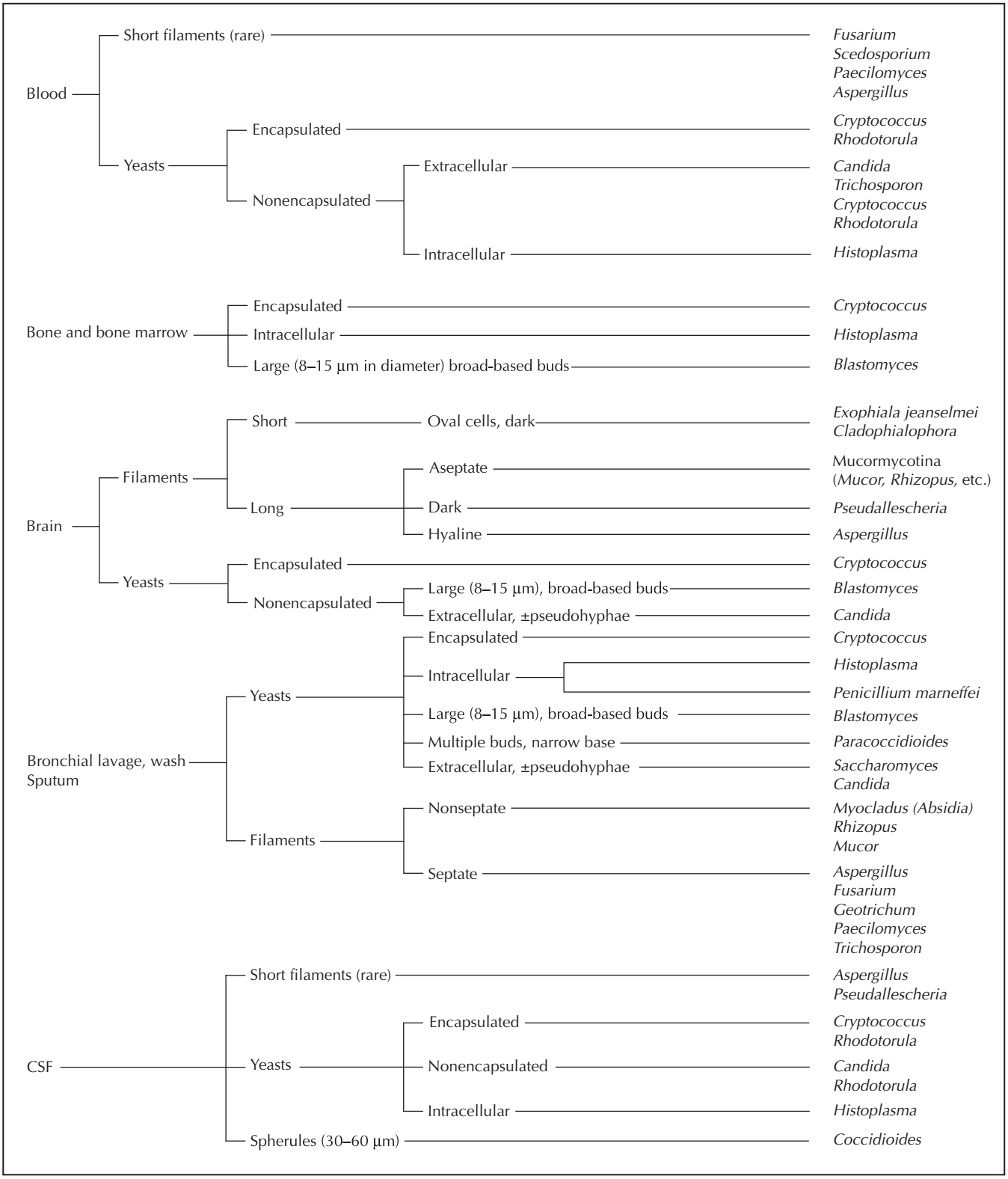


Figure 8.3–1 Most common possible fungal etiologies and morphologies seen in specimens (assuming appropriate stain was used to visualize organism [e.g., calcofluor white] or the specimen was left unstained to see pigment) *(continued).*

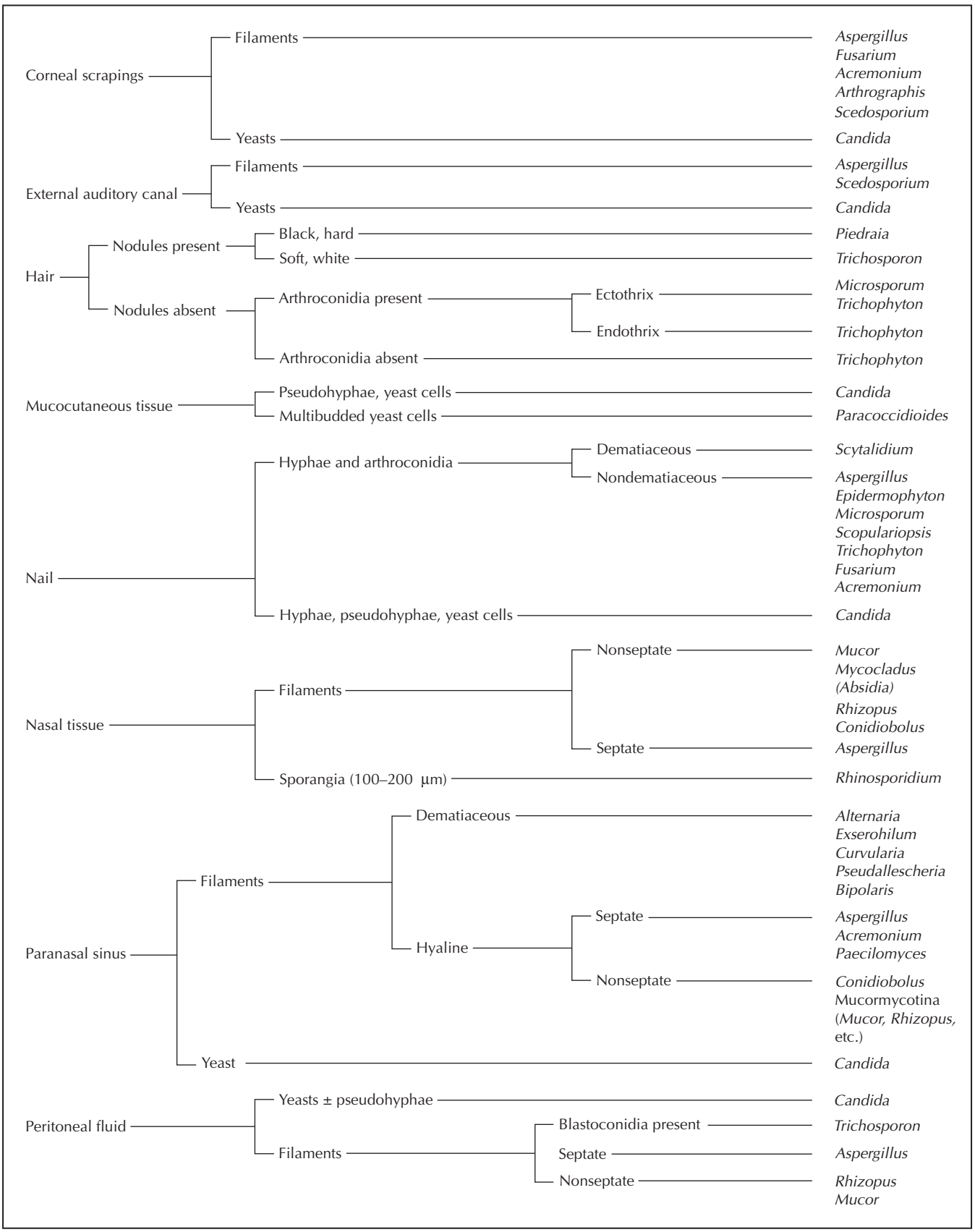


Figure 8.3–1 *(continued)*

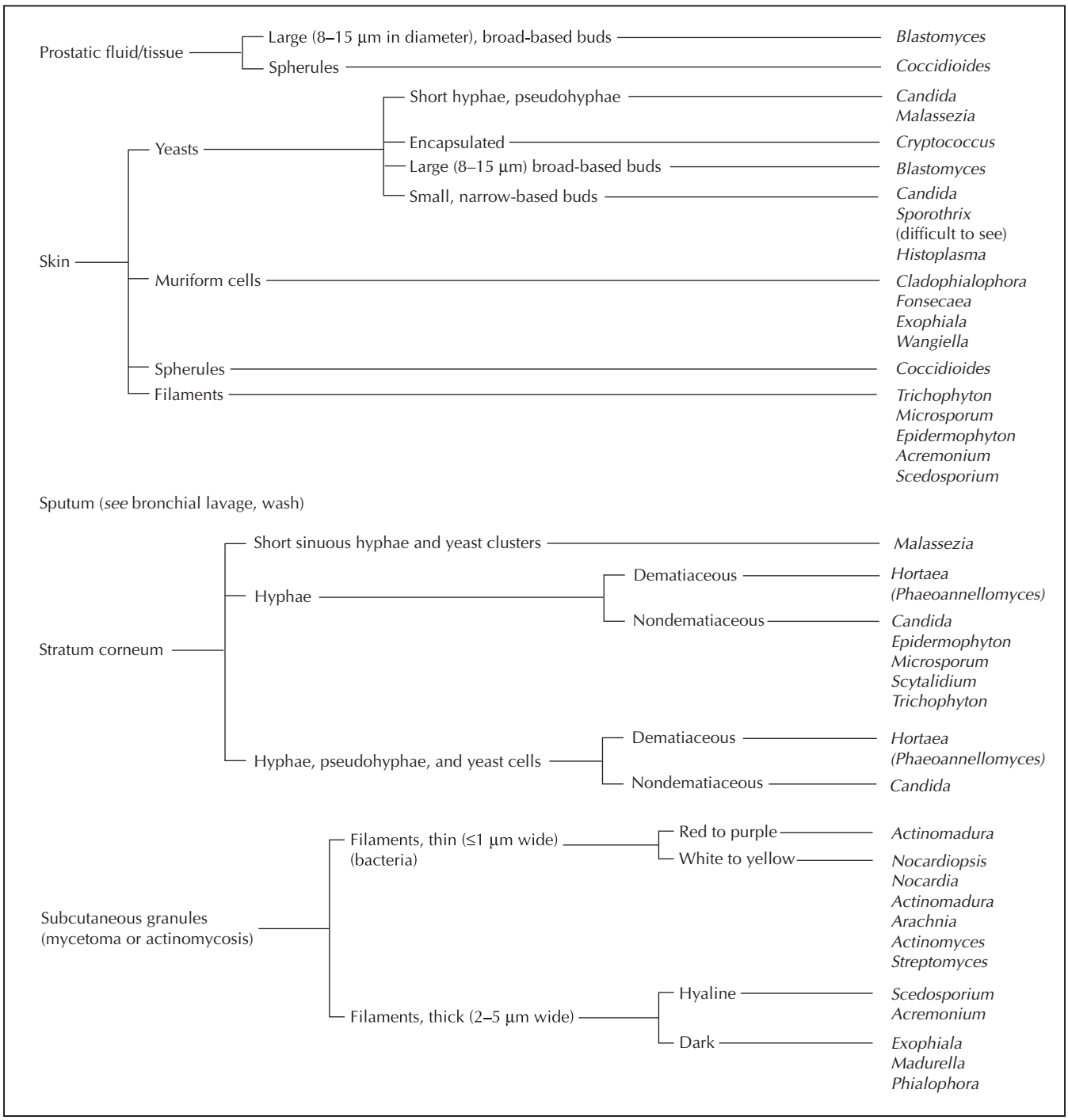


Figure 8.3–1 *(continued)*

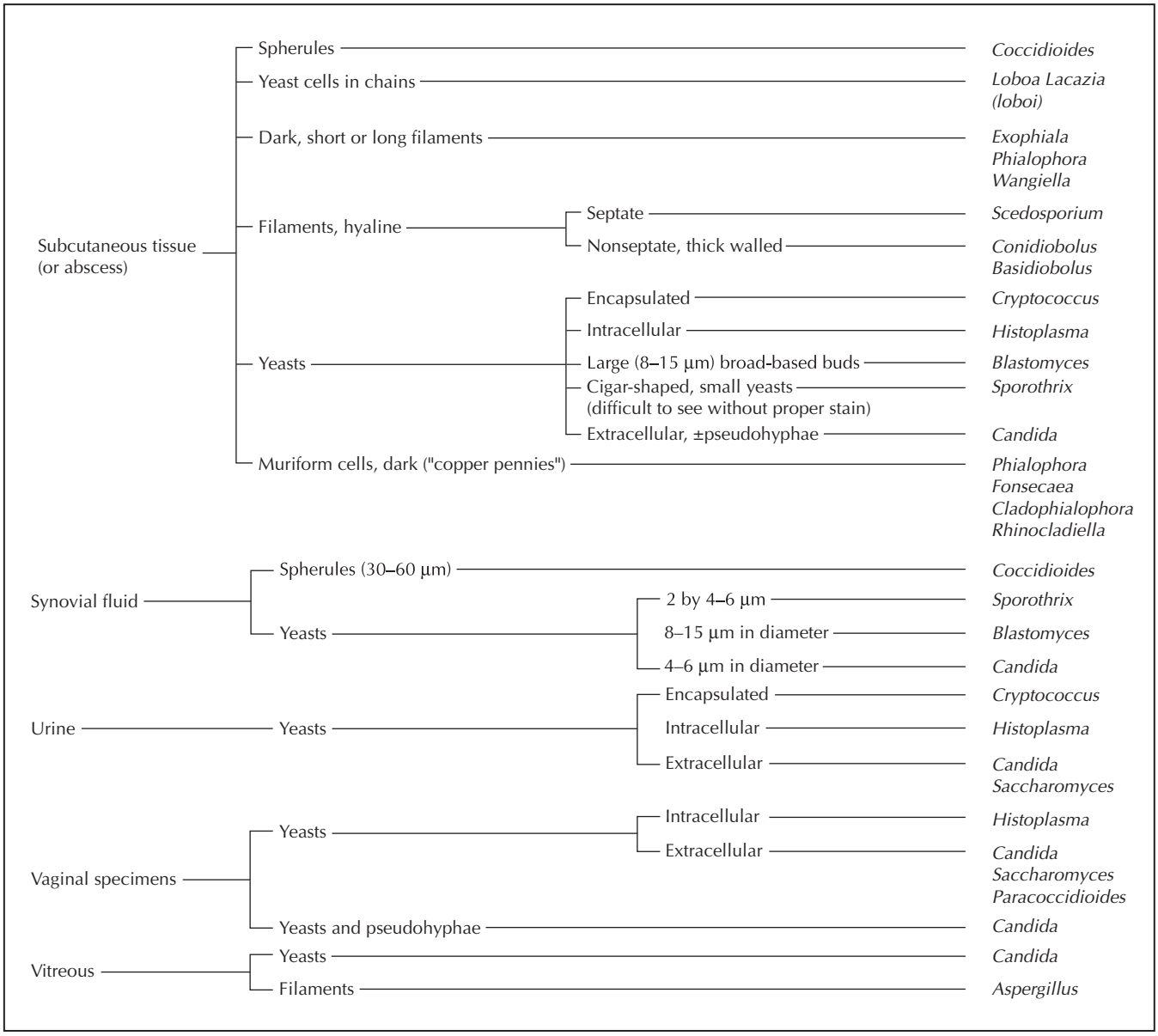


Figure 8.3–1 *(continued)*

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. General considerations

Prompt reporting of the results of microscopic examination for fungi is often important for the speedy diagnosis of infections. Results from cultures may not be known for several days, and visualization of tissue invasion by fungal structures may provide a more reliable result than culture in some cases. Experienced laboratory personnel will have confidence in the differentiation of true fungal cells from artifacts and, in some cases, may be able to determine the type of fungus.

The following reports are suggested. Note that microscopy reports will usually be generic, as a specific etiology cannot usually be definitively determined based on morphological criteria alone. The one notable exception to this is that

VI. REPORTING RESULTS *(continued)*

diagnosis of pityriasis versicolor can be reliably made on the basis of the characteristic combination of round yeasts and short hyphae of *Malassezia* species, the so-called "spaghetti and meatballs" appearance. In rare cases in which *Aspergillus* sporulating heads are seen, this is worth indicating in the report.

However, in most cases, indicating a possible specific etiology prior to culture results becoming available may influence physicians to treat, perhaps ineffectively, for the named etiologic agent and not for other infectious fungi.

B. Slide preparations

The following are suggested reports based on smear evaluation.

1. "No fungus seen"
2. "Yeast cells seen"
3. "Yeasts with pseudohyphae seen"
4. "Encapsulated yeasts seen"
5. "Mycelium seen"
6. "Ribbon-like nonseptate mycelium seen" (Lack of septation should be reported only if sufficiently long and abundant hyphal elements are present. If unsure about the presence of septations, report "mycelium seen.")
7. "Septate fungal mycelium seen"
8. "Negative for pityriasis versicolor"
9. "Positive for pityriasis versicolor"
10. "Septate hyphae with reproductive structures suggesting *Aspergillus* spp." *or*
11. Provide a description of the fungal elements if they are different from the most common findings, listed in items VI.B.1 to VI.B.10.

C. Dermatological specimens

Due to the slow growth of dermatophyte fungi and the sensitivity and specificity of microscopy, treatment for dermatophyte infections is frequently initiated on the preliminary report of the microscopic examination. Reporting the quantity of fungal material seen is unnecessary, and the presence or absence of fungal mycelium or arthroconidia is usually all that is required. The reporting of scalp infections as endothrix or ectothrix hair lesions is often useful, since it may be predictive of the causative species and can help in management of the infection.

The presence of yeast cells and pseudohyphae in skin and nail specimens should be reported, though the assessment of their clinical significance is less straightforward.

D. Respiratory and sterile body specimens

The examination of sputa and bronchial and alveolar washings may reveal fungal mycelium, yeast cells, or pseudohyphae. The presence of fungal mycelium is important in the diagnosis of aspergillosis and other pulmonary infections caused by moulds and must be reported urgently for immunocompromised patients. The presence of yeasts, pseudohyphae, or yeast mycelium is usually a reflection of oropharyngeal candidiasis (candidosis), since pulmonary candidiasis is rare.

The presence of any fungal cells in sterile fluids, such as blood, CSF, peritoneal dialysate, or vitreous humor, and in biopsy specimens from deep tissues should be reported rapidly to clinicians as clear evidence of a fungal infection.

SUPPLEMENTAL READING

Kwon-Chung, K.-J., and J. E. Bennett. 1992. *Medical Mycology.* Lea and Febiger, Philadelphia, PA.

Larone, D. L. 2002. *Medically Important Fungi: a Guide to Identification,* 4th ed. ASM Press, Washington, DC.

APPENDIX 8.3.1–1

Reagent Preparation

I. KOH

KOH is prepared as 10 to 30% (wt/vol) potassium hydroxide in water or in 20% (vol/vol) glycerol, which helps prevent the KOH mount from drying out.

II. KOH-DMSO

KOH-DMSO is prepared as 20% (wt/vol) KOH in 40% (vol/vol) DMSO in distilled water.

III. CALCOFLUOR WHITE

Calcofluor white consists of 0.1% (wt/vol) calcofluor white, either M2R (Polysciences Inc.; also sold as Blankophor BA [Bayer]) or Fluorescent Brightener 28 (Sigma), in distilled water or with 0.05% Evans blue as a counterstain. Commercial ready-to-use preparations are also available. It is often convenient to make up a 20 to 40% KOH solution and daily make up a working solution by mixing this 1:1 with 0.1% calcofluor. This can be filtered beforehand (Whatman no. 1) to remove calcofluor crystals.

APPENDIX 8.3.1–2

Reagent Suppliers

Polysciences, Inc.
400 Valley Rd.
Warrington, PA 18976

Calcofluor white (M2R)

Bayer Corp.
511 Benedict Ave.
Tarrytown, NY 10591

Calcofluor white (Blankophor BA)

Remel
12076 Santa Fe Dr.
Lenexa, KS 66215

Calcofluor White Stain Kit

Sigma-Aldrich Corp.
3050 Spruce St.
St. Louis, MO 63103

Calcofluor white (Fluorescent Brightener 28)
Evans blue
Nigrosine
Giemsa stain

Becton Dickinson
1 Becton Dr.
Franklin Lakes, NJ 07417

India ink

8.3.2

Induced Sputum: Stained Preparations for Detection of *Pneumocystis carinii*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Use concentrated stained preparations of induced sputa to detect *Pneumocystis carinii* and differentiate trophozoite and cyst forms from other possible causes of pneumonia. Organisms must be differentiated from fungi such as *Candida* spp. and *Histoplasma capsulatum.* (For detection of other organisms in sputum specimens, see procedure 9.7.1. Other organisms detected may be reported if control materials for their identification are included.)

The current valid name for the etiologic agent of human pneumocystis disease is *P. carinii.* The epithet *Pneumocystis jiroveci* (more correctly, *jirovecii*) has been recommended, but it has not been officially validated.

II. SPECIMEN

Observe standard precautions.

A. Induced sputa are collected by pulmonary or respiration therapy staff after patients have used appropriate cleansing procedures to reduce oral contamination.

B. Nebulizing procedures are determined by the staff collecting specimens. The induction protocol is critical for the success of the procedure. Patients with *Pneumocystis* sp. pneumonia usually have dry, nonproductive coughs. Organisms are rarely detected in expectorated sputa.

C. The laboratory processing specimens must establish a protocol for this diagnostic procedure in cooperation with the pulmonary staff. Induced sputum specimens are most useful for detection of *P. carinii* in human immunodeficiency virus-infected individuals, as others have fewer, less readily detected organisms (9).

III. MATERIALS

A. Reagents (*see* Appendix 8.3.2–1)

B. Supplies

1. Pasteur pipettes
2. 15- and 50-ml conical centrifuge tubes
3. 1- and 10-ml pipettes
4. Glass slides (1 by 3 in.)
5. Stain rack
6. Glass Coplin jars
7. 1 plastic Coplin jar with lid for silver stain
8. Graduated cylinder
9. Bleach
10. Protective gloves

C. Equipment

1. Microwave oven or water bath (80°C) and thermometer
2. Centrifuge with carriers for 50- and 15-ml tubes with safety caps
3. Binocular microscope with 40× or 50× oil and 100× oil immersion objectives and 10× oculars
4. Fluorescence microscope with filters to obtain appropriate wavelength of light if immunospecific stains with fluorescent labels are used.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Incorporate control slides in all stain procedures.
- **1.** Yeast-containing material may be used as a positive control for silver staining.
- **2.** Material containing RBCs and WBCs may be used as a control for Giemsa staining (*see* procedure 9.8.5).
- **3.** Both *P. carinii*-positive and *P. carinii*-negative but yeast-positive material should be used for immunospecific staining (check with suppliers).
- **4.** Stains cannot be evaluated if controls do not stain appropriately. Stain is not within acceptable results when the following occur.
  - **a.** *P. carinii* does not stain or stains black without delineation of "parentheses."
  - **b.** Fungi and actinomycetes do not stain.

B. Mucolytic agent must be free of contamination, as determined by clear appearance. Replace working solution after 48 h.

C. Clean and check centrifuge for speed, and clean brushes once a year.

D. Maintain temperature of refrigerator where mucolytic agent working solution is stored at 4°C (range, 2 to 8°C).

E. Keep stock stain solutions in area away from light, buffers, and other reagents; discard (prior to expiration date) if cloudy or apparently contaminated.

F. Check each new lot of stain for staining quality. Check Giemsa with a specimen containing blood to see that RBCs stain grayish, WBC nuclei stain red-purple, and WBC cytoplasm stains bluish (*see* procedure 9.8.5).

G. Fluorescence microscope can be evaluated for correct light wavelength for label with commercially available QC slides.

H. Record all QC results.

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. Treat specimens that appear to contain mucus with a mucolytic agent by adding a volume of agent equal to or two-thirds of the volume of the specimen (usually 2 or 3 ml) and incubating at room temperature for 15 min. Large-volume specimens often are thin and watery. For these, larger amounts (up to 20 to 25 ml) may be concentrated by centrifugation prior to the addition of a mucolytic agent.

C. Centrifuge the specimen at 1,300 $\times$ *g* for 5 min, making sure the safety caps are in place on the centrifuge tube carrier.

D. Decant supernatants into a disinfectant solution such as a 1:10 dilution of bleach.

E. If the sediment appears to contain significant blood, a portion (one-half to one-third) should be treated with an RBC lytic agent such as saponin or Lyse at one-half to one-third the volume of the sediment, left at room temperature for 5 min, and recentrifuged to repack sediment.

F. Decant supernatants from treated specimens.

G. Use Pasteur pipettes to resuspend sediments in material remaining after decanting.

H. Prepare smears on glass slides (1 by 3 in.) by placing drops of sediment in centers of slides. For specimens treated to lyse RBCs, prepare two smears: use a drop of sediment before lytic treatment for one smear and a drop of lysed sediment for the other.

I. Drops may be spread with the pipette so that they are thin and even.

V. PROCEDURE *(continued)*

J. Air dry slides, and fix smears for Giemsa or silver staining by dipping in methanol. Slides for immunospecific staining must be fixed according to the package insert directions.

K. Rapid Giemsa stain (Diff-Quik or Giemsa Plus)

1. Stain solutions should be kept in dropper bottles. Place 1 or 2 drops of red stain solution 1 on specimen smear and control slide (normal blood film), hold for 10 s, and drain.

2. Add 1 or 2 drops of blue solution 2, hold for 10 s, drain, and rinse very briefly with deionized water.

3. Stand slides on end to drain and air dry.

4. Slides must be examined with oil or mounted with mounting medium.

L. Giemsa stain

Make Giemsa working solution fresh each day. Discard and make new solution after 10 slides have been stained in a Coplin jar.

1. Place 2 ml of Giemsa (azure B) stain in Coplin jar. Remove stain from stock stain in bottle with clean, dry pipette.

2. Add 40 ml of phosphate buffer, pH 7.0 to 7.2, containing 0.01% Triton X-100.

3. Place fixed specimen smears and control smears in Giemsa stain for 30 min.

4. Remove slides, dip in phosphate buffer, stand slides on end, and allow to drain and air dry.

5. Examine with oil or mount slides.

M. Silver stain (3)

1. Place specimen smear slides and control slides on stain rack.

2. Add 10% chromic acid to cover smears, and let stand for 10 min.

3. During this period, prepare working methenamine-silver nitrate by placing the following in this order in a plastic Coplin jar: 20 ml of 3% methenamine, 1 ml of 5% silver nitrate, 1.5 ml of 5% sodium borate, and 17 ml of distilled or deionized water.

4. Wash slides with distilled or deionized water.

5. Cover with 1% sodium metabisulfate for 1 min.

6. Wash with water, place slides in plastic Coplin jar containing methenamine-silver nitrate, and cover with cap.

7. Place in microwave oven at 50% power for approximately 35 s, rotate 90°, heat for another 35 s, and then leave slides in hot liquid for 2 min. Solution should turn brown to black.

8. If a water bath is used, heat water bath to 80°C. Place plastic Coplin jar containing stain reagents in bath for 6 min prior to placing slides in jar, add slides, and leave jar in bath for an additional 5 min. Solution should turn brown to black.

9. Remove slides, and wash in distilled, deionized water.

10. Dip slides up and down in 1% gold chloride, wash in distilled or deionized water, and place on rack again.

11. Cover slides with 5% sodium thiosulfate for 1 min.

12. Wash in distilled, deionized water.

13. Cover with 0.2% fast green in acetic acid counterstain for 1 min.

14. Wash with distilled, deionized water, and stand slides on end to drain and dry.

15. Examine with oil immersion or mount slides.

16. Fill Coplin jar used for staining with bleach (full strength), and let stand for at least 1 h.

V. PROCEDURE *(continued)*

N. Immunospecific staining
- **1.** Follow package directions (by the manufacturer) exactly.
- **2.** If negative control slide containing yeast cells exhibits fluorescence or if specimen slide is at all equivocal, do not report specimen as positive for *P. carinii* on the basis of this stain.
- **3.** Perform cyst wall and organism stains.

O. For all stains
- **1.** *Examine control slide for each stain prior to examination of specimen stains.* Stain intensity of controls will be a guide to stained appearance of organisms in specimens.
- **2.** Examine stained specimen smears by systematically moving from field to field until the majority of the smear has been covered (*cover total area for silver stain*).
- **3.** In Giemsa stains, clumps of trophozoites of various sizes may be detected. In large clumps, it may be difficult to differentiate individual organisms. *Look at organisms at the edges of clumps, and look for small, more dispersed clumps.*
  - **a.** Yeastlike fungi will have blue cytoplasm and purple-red nuclei.
  - **b.** Filamentous fungi stain purple-blue; their nuclei usually do not stain.
  - **c.** Actinomycetes stain purple-blue.
- **4.** In silver- or other cyst wall-stained smears, look for the various cyst forms, including those that show dark centers, are cup shaped, or show foldlike lines. If dark-staining organisms appear more oval, look carefully for budding forms that indicate that the organisms are yeasts.
  - **a.** *P. carinii* cysts
    For these, 70% should have delicately stained walls, usually brown or grey. They will appear rather transparent, with structures described as "parentheses" staining black.
  - **b.** Fungi and actinomycetes stain gray to black.
  - **c.** Glycogen, mucin, and RBCs stain rose taupe to gray.
  - **d.** Background is pale green.
- **5.** Detecting *P. carinii* in specimen smears of one stain should prompt a careful examination of the other stain to confirm the identification.
- **6.** *Retain all positive-stained specimen slides and the control slides from the procedure for reference.*

VI. RESULTS

P. carinii trophozoites and/or cysts may be recovered with these techniques. Other fungi may also be recovered.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report *P. carinii* from any specimen in which organisms are detected (magnification of $\times$1,000).
Examples: No *Pneumocystis carinii* seen.
Pneumocystis carinii seen. (No quantitation should be included.)

B. Report fungi that may be present if positive control specimens contain both yeastlike and filamentous fungi and have been stained appropriately.
- **1.** Fungi ($\times$400)
  Examples: No mycotic elements seen.
  Budding yeast, budding yeast cells and pseudohyphae resembling *Candida* species (quantitated as few, moderate, or many)

VII. REPORTING RESULTS *(continued)*

2. Hyphae are reported with no quantitation.
Examples: Septate hyphae seen.
Nonseptate hyphae seen.

C. Report actinomycetes (oil immersion, ×1,000) if positive control slides are included and exhibit proper staining characteristics.
Examples: No filamentous branching bacteria seen.
Filamentous branching bacteria seen. (No quantitation.)

VIII. PROCEDURE NOTES

A. It is advisable to use at least two stains for detection and identification of *P. carinii.* With traditional histochemical stains, a trophozoite stain such as Giemsa and a cyst wall stain such as methenamine-silver nitrate have been described.

B. There are many cyst wall stains in addition to the ones described here (2, 4, 6, 17), and there are other modifications of the silver stain (7, 10, 12, 15).

C. Reports of usefulness of stains are variable (14). Other counterstains may be used. A counterstain with Giemsa is useful if one referral slide is to be examined (20).

D. In selecting a cyst wall stain, consider access to quality stains, stability of reagents, and frequency of testing. Dye lots of toluidine blue O stains vary (21), and stability of sulfation reagents also varies (18). In the modified procedure (6), the sulfation reagent made of glacial acetic acid and concentrated sulfuric acid presents a problem of disposal. Good Gram-Weigert stains depend on decolorization with aniline-xylene reagent, which requires a specific aniline oil.

E. The rapid silver stain described above is a modification of the stain described by Brinn (3). Heating the chromic acid causes too much nonspecific staining, making the background dark, staining the granules of cells, and slowing examination. With the silver stain described above, it was found that buildup of silver on the stain container interfered with staining. For that reason, bleach should be added to the stain jar after staining. Periodically, jars should be cleaned with a brush.

1. Residual silver plating on glassware from previous use (not applicable if the plastic Coplin jar is used)
Inspect glassware before use to be sure it is not cracked and does not have residual silver plating.

2. Always prepare fresh reagents and rinse slides with distilled, deionized water.

3. If methenamine-silver nitrate is made according to the method described above, the solution will be clear. If it is cloudy or opaque, the glassware may have been dirty or some reagent may have been incorrectly made. Discard the cloudy solution. Check your distilled, deionized water source and prepare fresh working solution. Distilled, deionized water must be used throughout the procedure.

F. If slides are mounted with mounting medium and coverslips, the stained preparations must be dehydrated in xylene prior to mounting. If mounted slides look opaque, the dehydration and clearing with xylene (or xylene substitute) was not adequate. Soak slides in xylene to remove the coverslips. Repeat dehydration steps with fresh ethanol and xylene.

G. Occasionally, degenerating PMNs may resemble *P. carinii.*

H. Monoclonal antibodies specific for human strain *P. carinii* have been described elsewhere (5); immunospecific staining is becoming more widely used in routine laboratory evaluations. The commercial systems vary; some are indirect stains, and some are direct. Reports with all systems have been variable (1, 8, 13, 19).

VIII. PROCEDURE NOTES *(continued)*

I. It is best to select an organism stain and a cyst wall stain or immunospecific stain and gain experience with the stains. Using a pair of stains will help avoid both false-negative and false-positive reporting.

J. Cytocentrifuge preparations of sputum have been described elsewhere (11), and this procedure is considered helpful by some for determining cell populations for further evaluation of patients. This is a consideration other than detection of organisms.

IX. LIMITATIONS OF THE PROCEDURE

Although induced sputum specimens have been used successfully in diagnosis of *P. carinii* in some institutions (1), they have not been useful at others (16). This may be due, in part, to careful adherence to specimen rejection criteria. If the clinical evaluation of a patient suggests *P. carinii* pneumonia and the induced sputum specimen is negative, a bronchoalveolar lavage specimen should be evaluated by using the stains presented in this protocol.

Nucleic acid amplification of pneumocystis-specific sequences and subsequent oligonucleotide hybridization have been used to detect *P. carinii.* Also, a number of procedures have been developed using PCR or nested PCR; these methods have been very useful, particularly for AIDS patients. Direct-fluorescent-antibody reagents have also been used; these tests are sensitive and specific, and the slides are easy for an experienced reader to review. However, a fluorescent microscope is required.

REFERENCES

1. **Blumenfeld, W., and J. A. Kovacs.** 1988. Use of a monoclonal antibody to detect *Pneumocystis carinii* in induced sputum and bronchoalveolar lavage fluid by immunoperoxidase staining. *Arch. Pathol. Lab. Med.* **112:**1233–1236.
2. **Bowling, M. D., I. M. Smith, and S. L. Wescott.** 1973. A rapid staining procedure of *Pneumocystis carinii. Am. J. Med. Technol.* **39:**267–268.
3. **Brinn, N. T.** 1983. Rapid metallic histological staining using the microwave oven. *J. Histotechnol.* **6:**125–129.
4. **Chalvardjian, A. W., and L. A. Grawe.** 1963. A new procedure for the identification of *Pneumocystis carinii* cysts in tissue sections and smears. *J. Clin. Pathol.* **16:**383–384.
5. **Gill, V., G. Evans, F. Stock, J. Parillo, H. Masur, and J. Kovacs.** 1987. Detection of *Pneumocystis carinii* by fluorescent-antibody stain using a combination of three monoclonal antibodies. *J. Clin. Microbiol.* **25:**1837–1840.
6. **Gosey, L. L., R. M. Howard, F. G. Witebsky, F. P. Ognibene, T. C. Web, V. J. Gill, and J. D. MacLowry.** 1985. Advantages of a modified toluidine blue O stain and bronchoalveolar lavage for the diagnosis of *Pneumocystis carinii* pneumonia. *J. Clin. Microbiol.* **22:**803–809.
7. **Hinds, I.** 1988. A rapid and reliable silver impregnation method for *Pneumocystis carinii* and fungi. *J. Histotechnol.* **11:**27–29.
8. **Koch, M., and W. Heizmann.** 1990. Problems in the detection of *Pneumocystis carinii* by indirect immunofluorescence. *Eur. J. Clin. Microbiol. Infect. Dis.* **9:**58–59.
9. **Limper, A. H., K. P. Offord, T. F. Smith, and W. J. Martin II.** 1989. Differences in lung parasite number and inflammation in patients with and without AIDS. *Am. Rev. Respir. Dis.* **140:**1204–1209.
10. **Mahan, C. T., and G. E. Sale.** 1978. Rapid methenamine silver stain for Pneumocystis and fungi. *Arch. Pathol. Lab. Med.* **102:**351–352.
11. **Martin, W. J., II, T. F. Smith, D. R. Sanderson, W. M. Brutinel, F. R. Cockerill III, and W. W. Douglas.** 1989. Role of bronchoalveolar lavage in the assessment of opportunistic pulmonary infection: utility and complication. *Mayo Clin. Proc.* **62:**549–557.
12. **Musto, L., M. Flanigan, and A. Elbadawi.** 1982. Ten-minute silver stain for *Pneumocystis carinii* and fungi in tissue sections. *Arch. Pathol. Lab. Med.* **106:**292–294.
13. **Ng, V. L., D. M. Yajko, L. W. McPhaul, I. Gartner, B. Byford, C. D. Goodman, P. S. Nassos, C. A. Sanders, E. L. Howes, G. Leough, P. C. Hopewell, and W. K. Hadley.** 1990. Evaluation of an indirect fluorescent-antibody stain for detection of *Pneumocystis carinii* in respiratory specimens. *J. Clin. Microbiol.* **28:**975–979.
14. **Paradis, I. L., C. Ross, A. Dekker, and J. Dauber.** 1990. A comparison of modified methenamine silver and toluidine blue stains for detection of *Pneumocystis carinii* in bronchoalveolar lavage specimens from immunosuppressed patients. *Acta Cytol.* **34:**513–516.
15. **Pintozzi, R. L.** 1978. Technical methods: modified Grocott's methenamine silver nitrate method for quick staining of *Pneumocystis carinii. Am. J. Clin. Pathol.* **31:**803–805.
16. **Rolsten, K. V. I., S. Rodriguez, L. McRoy, G. Uribe-Botero, R. Morice, and P. W. A. Mansell.** 1988. Diagnostic value of induced sputum in patients with the acquired immunodeficiency syndrome. *Am. J. Med.* **85:**269.

REFERENCES *(continued)*

17. **Rosen, P., D. Armstrong, and C. Ramos.** 1972. *Pneumocystis carinii* pneumonia. *Am. J. Med.* **53:**428.
18. **Settnes, O. S., and P. Larsen.** 1979. Inhibition of toluidine blue O stain for *Pneumocystis carinii* by additives in the diethyl ether. *Am. J. Clin. Pathol.* **72:**493–494.
19. **Stratton, N., J. Hryniewicki, S. L. Aarnaes, G. Tan, L. M. de la Maza, and E. M. Peterson.** 1991. Comparison of monoclonal antibody and calcofluor white stains for the detection of *Pneumocystis carinii* from respiratory specimens. *J. Clin. Microbiol.* **29:**645–647.
20. **Walker, J., G. Conner, J. Ho, C. Hunt, and L. Peckering.** 1989. Giemsa staining for cysts and trophozoites of *Pneumocystis carinii*. *J. Clin. Pathol.* **42:**432–434.
21. **Witebsky, F. G., J. W. B. Andrews, V. J. Gill, and J. D. MacLowry.** 1988. Modified toluidine blue O stain for *Pneumocystis carinii:* further evaluation of some technical factors. *J. Clin. Microbiol.* **26:**774–775.

SUPPLEMENTAL READING

Baselski, V. S., M. K. Robinson, L. W. Pifer, and D. R. Woods. 1990. Rapid detection of *Pneumocystis carinii* in bronchoalveolar lavage samples by using Cellufluor staining. *J. Clin. Microbiol.* **28:**393–394.

Baughman, R. P., S. S. Strohofer, S. A. Clinton, A. D. Nichol, and P. T. Frome. 1989. The use of an indirect fluorescent antibody test for detecting *Pneumocystis carinii*. *Arch. Pathol. Lab. Med.* **113:**1062–1065.

Cohen, R. J., and Z. Hoffajee. 1990. Diagnosis of *Pneumocystis carinii* in sputum samples using a modified toluidine blue O method. *Acta Cytol.* **34:**583–585.

Garcia, L. S. 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.

Genaw, C. 1989. Use of cresyl echt violet for the staining of *Pneumocystis carinii* as compared to Grocott's (GMS) and Giemsa methods. *J. Histotechnol.* **12:**39–40.

Holten-Anderson, W., and H. J. Kolmos. 1989. Comparison of methenamine silver nitrate and Giemsa stain for detection of *Pneumocystis carinii* in bronchoalveolar lavage specimens from HIV infected patients. *Acta Pathol. Microbiol. Immunol. Scand.* **97:**745–747.

Ognibene, F. P., V. J. Gill, P. A. Pizzo, J. A. Kovacs, C. Godwin, A. F. Suffredini, H. D. Shelhamer, J. E. Parillo, and H. Masur. 1989. Induced sputum to diagnose *Pneumocystis carinii* pneumonia in immunosuppressed pediatric patients. *J. Pediatr.* **115:**430–433.

Tuncer, S., S. Erguven, S. Kocagoz, and S. Unal. 1998. Comparison of cytochemical staining, immunofluorescence and PCR for diagnosis of *Pneumocystis carinii* on sputum samples. *Scand. J. Infect. Dis.* **30:**125–128.

APPENDIX 8.3.2–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. Mucolytic agent such as Sputolysin Stat-Pack dithiothreitol solution (Behring Diagnostics, Inc.) and RBC lytic agent such as saponin (Aldrich, Milwaukee, Wis.; ICN Biochemicals, Costa Mesa, Calif.), Lyse (Curtin Matheson Scientific, Inc.), or Hematall LA-Hgb reagent (Fisher Scientific)

B. Rapid Giemsa stain such as Diff-Quik (Baxter Scientific Products) or Giemsa Plus (Trend Scientific). Follow manufacturer's instructions or use Giemsa stain.

C. Giemsa stain, azure B alcoholic stock (Harleco, Philadelphia, Pa.), diluted 1 to 20 with phosphate buffer containing 0.01% Triton X-100 (*see* procedure 9.8.4).
   1. Buffers prepared from stock buffers
      a. Alkaline buffer ($NaHPO_4$ solution, 0.067 M) is 9.5 g of Na_2HPO_4 dissolved in 1 liter of distilled water.
      b. Acid buffer ($NaHPO_4$, 0.067 M) is 9.2 g of $Na_2HPO_4 \cdot H_2O$ dissolved in 1 liter of distilled water.
   2. These buffers can be kept for a long time (12 months).
   3. To prepare buffered water for stain, add 39 ml of acid buffer to 61 ml of alkaline buffer, and add that mixture to 900 ml of water.

D. Cyst wall stain such as rapid methenamine-silver nitrate (3)
   1. Chromic acid (10% solution)

      chromic acid (CrO_3) ……100.0 g
      distilled deionized water ……1,000.0 ml

      Solution is usable for up to 1 year.

APPENDIX 8.3.2–1 *(continued)*

2. Methenamine (3% solution)

   hexamethylenetetramine, USP
   [$(CH_2)_6N_4$] ..... 12.0 g
   distilled deionized water .....400.0 ml

   Solution is usable for up to 6 months.
3. Silver nitrate (5% solution)

   silver nitrate ($AgNO_3$) .....5.0 g
   distilled deionized water .....100.0 ml

   Solution is usable for up to 1 month at 4°C.
4. Sodium borate (Borax) (5% solution)

   sodium borate ($Na_2B_4O_7 \cdot 10H_2O$) .....5.0 g
   distilled deionized water .....100.0 ml

   Solution is usable for up to 1 year.
5. Sodium bisulfite (1% solution)

   sodium bisulfite ($NaHSO_3$) ..... 10.0 g
   distilled deionized water .....100.0 ml

   Solution is usable for up to 1 year.
6. Gold chloride (1% solution)

   gold chloride .....5.0 g
   distilled deionized water .....500.0 ml

   Solution is usable for up to 1 year.
7. Sodium thiosulfate (5% solution)

   sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) ..... 50.0 g
   distilled deionized water .....1,000.0 ml

   Solution is usable for up to 1 year.
8. Stock light green

   light green, S.F. yellowish C.I.
   no. 42095 .....0.2 g
   distilled deionized water .....100.0 ml
   glacial acetic acid ($CH_3 \cdot COOH$) .....0.2 ml

   Solution is usable for up to 1 year.
9. Working light green

   stock light green ..... 10.0 ml
   distilled deionized water ..... 40.0 ml

   Solution is usable for up to 1 month.
10. Ethanol, 100% (absolute) and 95%
11. Methanol, 100% (absolute)
12. Xylene (or xylene substitute)

    All reagents can be stored at 25°C except the silver nitrate, which must be refrigerated. Silver nitrate should be discarded and replaced after storage for 1 month.

E. Immunospecific stain
   Commercially available kits (*see* Appendix 9.10.6–1 at the end of section 9)

F. Positive control slides are commercially available (Hardy Diagnostics, Santa Maria, CA). These slides are not appropriate for immunospecific stains. For positive control slides for immunostaining, one possibility is to make multiple slides with a human-derived specimen that has a high concentration of organisms.

8.4 Processing Specimens for Fungal Culture

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

When a specimen is suspected to contain a fungal etiologic agent, it should be processed for fungal culture, regardless of direct microscopic findings. Recovery of fungal pathogens in culture provides definitive diagnosis of mycotic disease, identifies the etiologic agent of infection, and allows evaluation of in vitro susceptibility to antifungal agents. In the event that there is insufficient material for both microscopy and culture, all of the specimen should be used for culture, since this is the more sensitive procedure for detection of fungi. Methods of specimen processing and culture are designed to retain the viability of the fungus and to obtain the maximum yield of organisms from clinical specimens. The choice of media for the isolation of fungi from clinical material is based primarily on the most likely species to be found in a particular site or under a recognized clinical condition. Selective media are included when other microorganisms, particularly bacteria, might also be present in the specimen. Specimens should be processed as soon as possible after receipt. Some specimens may require pretreatment prior to culture.

Molecular methods are under development for the detection and identification of fungi directly from patient specimens. As these methods become part of the normal approach for laboratory diagnosis of mycoses, laboratorians will need to be mindful of whether the specimen should also be cultured to allow subsequent antifungal susceptibility testing.

II. SPECIMENS

A. Specimens should be submitted in closed containers without preservatives.

B. All specimens submitted to the laboratory are initially examined macroscopically, and representative parts are selected for further investigation. Specimens such as sputum and tissue are examined for any obvious signs of purulence, caseation, blood, or necrosis, and these areas are selected for microscopy and culture.

C. When specimens are submitted to the laboratory, the following information should be obtained in addition to pertinent demographic information.

1. Source and/or anatomical site
2. How and when it was collected
3. Formulation of transport medium (if any)
4. How it was transported
5. How long it was in transit to the laboratory
6. How long after receipt by the laboratory the specimen was processed
7. How the specimen was maintained by the laboratory after receipt

D. Communication with the clinician about details of the patient's area of residence, travel history, contact with animals, and previous therapy with antibacterial, antifungal, or immunosuppressive cytotoxic agents may also help guide the laboratory toward processing the specimen for a particular fungal pathogen. Such information is often not easily obtained from the submitting physician. It is best if a request for this information is made at the time the specimen is submitted, perhaps on the (paper or electronic) request form.

II. SPECIMENS *(continued)*

E. Specimens may be obtained from both sterile and nonsterile body sites or sources.

1. Examples of specimens collected from sources usually considered sterile include the following.

a. CSF
b. Blood
c. Bone marrow
d. Surgical tissue
e. Body fluids
f. Catheterized urine

2. Examples of specimens obtained from sources considered nonsterile include the following.

a. Sputum
b. Tracheal aspirate
c. Bronchial wash and brush
d. Midstream urine
e. Gastric lavage fluid
f. Hair, skin, and nail samples
g. Superficial wound swabs
h. Throat, mouth, or nasopharynx mucocutaneous materials
i. Ear material
j. Vaginal or cervical material
k. Feces

F. Specimens received on swabs are inferior to samples of tissue or fluid, but they should not be rejected for culture unless the swab is dry. If circumstances allow, communicate with the patient's physician that the laboratory prefers a better specimen. Rejection criteria for other specimens are described in sections 2 and 3 of this handbook. Other unsuitable specimens include material received in fixative, 24-h urine, specimens received in leaking containers, and specimens comprised of insufficient material. In these circumstances, the laboratory should communicate with the physician and ask that another specimen be collected if possible.

III. MATERIALS

A. General supplies and equipment

1. Sterile plastic pipettes
2. Sterile cotton swabs
3. Sterile scalpel blades and forceps
4. Microscope slides and coverslips
a. With etched center well
b. With frosted ends
5. Class II biosafety cabinet
6. Bacticinerator
7. Inoculating loops
8. Latex gloves and mask
9. Puncture-resistant biohazard discard container
10. Potassium hydroxide (10% for soft tissues and 20% for nails and hair)
11. India ink

B. Media for fungal culture

1. The selection of suitable media for the recovery of fungi is primarily a function of the specimen type and suspected fungal pathogen(s). See Fig. 8.3–1 for specimen type and microscopic indications of potential etiologic agents. A combination of several different media is used for culturing most specimens. Media are dispensed in either screw-cap tubes (25 by 150 mm) or 100-mm-diameter petri dishes. Plated media are useful because of the large surface area available. However, because plated media are vented to the atmosphere, the layer of poured medium must be thick to resist dehydration during prolonged incubation. Lids should be secured with gas-permeable tape or Shrink-Seals to prevent exogenous contamination and to protect personnel from pathogenic moulds. Tubed media have a reduced surface area for fungal isolation but provide maximum safety and resistance to dehydration and contamination.

III. MATERIALS *(continued)*

2. In general, there are two categories of media employed for the recovery of fungi from clinical specimens. General-purpose media, such as BHI agar, Sabhi agar, and inhibitory mould agar with antimicrobial agents (IMAA), support the growth of most fungi found in clinical material and are used as primary isolation media. Selective agents, such as chloramphenicol, gentamicin, and cycloheximide, may be incorporated into these media to inhibit the growth of bacteria or saprobic fungi that may be found in nonsterile specimens. For recovery of suspected *Malassezia* spp., an additional plate or tube of primary medium overlaid with sterile olive oil should be included. The second category of media is used for subculture and identification of fungi. On these media, particular growth characteristics or sporulation patterns are encouraged. Examples include cornmeal agar, Czapek-Dox agar for *Aspergillus* spp. and *Penicillium* spp., Sabouraud dextrose (glucose) agar (SDA) for dermatophytes, and potato flake agar. Laboratories that use fungal identification atlases to help with identification of unusual moulds should know what medium is commonly used by the author(s) because descriptions of growth and culture characteristics are based on that medium. One common medium is malt extract agar. Some features of commonly used fungal media are listed in Table 8.4–1.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Ensure that media to be used are within their expiration period.
B. Follow laboratory procedure or manufacturer's instructions for preparation of inocula using automated equipment (e.g., lysis centrifugation).
C. Inspect calibrated loops regularly to ensure that they remain in good condition and free from damage.

V. PRETREATMENT OF SPECIMENS FOR MYCOLOGICAL EVALUATION

It is imperative that these cultures be handled in a biological safety cabinet.

This topic concerns only deviations from treatments described in section 2 of this handbook or with issues specific to mycology.

A. Liquefaction or homogenization of sputum
The use of mucolytic agents, such as *N*-acetyl-L-cysteine or Sputolysin, on viscous samples of sputum is of unproven value for the recovery of most fungi. However, these agents should be used, along with centrifugation, on sputa submitted for detection of *Pneumocystis carinii.*

B. Mincing of tissues
Tissue samples are minced with a sterile scalpel blade and forceps. The procedure may be performed in a sterile petri dish to which a few drops of sterile distilled water have been added. Do not use mechanical tissue homogenizers or a mortar and pestle, as these can adversely affect the recovery of fungi such as zygomycetes because of damage to fragile hyphal structures.

VI. INOCULATION OF MEDIA

It is imperative that these cultures be handled in a biological safety cabinet.

A. Transfer a maximum amount of the clinical specimen to culture medium, but retain some for preparation of smears for direct microscopic examination. Refer to procedure 8.2 for refrigeration and freezing of specimens.
B. The number of media inoculated may be dependent on the specimen type and amount. Both nonselective (Sabhi and BHI agars) and selective (inhibitory mould agar [IMA]/IMAA, Mycosel, and Mycobactin) media should be included, and specialized media may be required for specimens in which difficult-to-grow etiologic agents (*see* Fig. 8.3–1) are suspected. Selective media, with the exception of IMA/IMAA, may be omitted if the specimen is free of contaminating

Table 8.4–1 Media for the isolation and identification of fungi[a]

Medium	Intended use	Remarks
SDA	Originally formulated for dermatophyte growth and pigment production. It is inferior to Sabhi agar as a primary medium.	Emmons modification contains 2% glucose and is slightly acidic (pH 6.9). Overlay with olive oil for growth of *Malassezia furfur.*
BHI agar	Enriched medium for recovery of yeasts, especially *Cryptococcus neoformans* Can be used for mould-yeast conversion in some dimorphic fungi	Addition of sheep erythrocytes and/or antimicrobial agents optional
Sabhi agar	Enriched medium for recovery of yeasts	Addition of sheep erythrocytes and/or antimicrobial agents optional
Mycosel or mycobiotic agar (Becton Dickinson)	Commercial selective medium for primary isolation of fungi. Inhibits bacteria and saprobic fungi.	SDA with chloramphenicol and cycloheximide Will inhibit some pathogenic fungi. Note that chloramphenicol-resistant bacteria will grow on this medium.
IMA/IMAA	Enriched selective medium	Contains chloramphenicol (IMA). IMAA also has gentamicin.
Potato dextrose agar and potato flake agar	Promotes sporulation of moulds	Ideal medium for slide culture preparation
Malt extract agar	May enhance recovery of zygomycetes	
Yeast extract phosphate medium (Remel)	Selective medium for dimorphic fungi	Must add concentrated ammonium hydroxide to surface
Cornmeal agar with Tween 80 Rice agar with Tween 80 Cornmeal glucose agar	Chlamydoconidium formation in *Candida albicans;* pigment production in *Trichophyton rubrum* (cornmeal glucose agar is superior to the other two)	
Dermatophyte test medium	Selective recovery and presumptive identification of dermatophytes	Dermatophyte turns medium pink to red. Bacteria and saprobic fungi turn medium yellow. *Caution:* Some nondermatophytes also turn the medium red.
Czapek-Dox agar	Reference medium for identification of *Aspergillus* spp.	
Caffeic acid agar or birdseed (niger seed) agar	For detection of *C. neoformans*	
Candida ID (bioMérieux), CHROMagar	Selective and differential medium for *Candida* spp.	Characteristic color development identifies some species.

[a] With few exceptions, or unless specifically noted, all of the media listed in this table are available from BD Biosciences, Hardy Diagnostics, and Remel. Czapek-Dox and malt extract agar plates and slants are not available from BD Biosciences. Formulations for the nonproprietary media are provided in other procedures within section 8, as well as in the *Handbook of Media for Clinical Microbiology* (1).

VI. INOCULATION OF MEDIA *(continued)*

microbiota. See Table 8.4–1 for the selection of media for isolation and identification of fungi.

C. Specific specimen types

1. Blood

a. Automated blood culture systems may provide specialized media for the recovery of fungi from blood. These media can be incorporated into testing protocols as indicated by the manufacturer.

b. Lysis centrifugation (Isolator; Wampole) has proven to be a superior method for the recovery of some fungi from blood, especially *Histoplasma capsulatum.* The blood sample is processed according to the manufacturer's instructions, and 3 to 5 drops of sediment are inoculated and streaked onto enriched medium. If some of the sediment remains after all desired media have been inoculated, it can be distributed onto additional plates of medium to ensure that the entire concentration is cultured.

2. Body fluids (CSF; urine; pericardial, pleural, and peritoneal fluids; ascites)

a. Centrifuge quantities of >2 ml.

b. Up to 0.5 ml may be inoculated and streaked onto each type of medium.

c. Urine may be inoculated and streaked with a calibrated loop if quantitation is desired.

d. Inoculate CSF onto media free of cycloheximide (inhibitory to *Cryptococcus neoformans*). Supernatant from the sample preparation may be aseptically removed and used for antigen testing.

3. Bone marrow aspirates

a. Inoculate and streak 3 to 5 drops onto each plate of medium.

b. Lysis centrifugation using the pediatric (1.5-ml) Isolator may enhance recovery.

4. Bronchial brushings

a. Vortex brush in sterile distilled water.

b. Inoculate 3 to 5 drops onto each plate of medium.

c. Place brush on surface of selective medium.

5. Tissue

Mince and place several pieces on each plate of medium. Include inhibitory medium if bacterial contamination is suspected. Scratch the surface and embed a portion of tissue into the scratch (this method provides an oxygen tension gradient which may help initiate fungal growth).

6. Exudates, pus, and drainage specimens

Exudates, pus, and drainage specimens should be carefully examined with a dissecting microscope for the presence of granules. If found, granules should be teased out with a dissecting needle and crushed between two glass slides. The slides may be examined microscopically for the presence of fungal elements.

7. Specimens on swabs

Swab specimens may be eluted into a small volume of sterile water, and the water may be used to inoculate media. Swabs may also be directly applied to the surface of the agar.

8. Hair, skin, and nails

a. Strands of hair are cut into 1-mm lengths with a scalpel or sterile scissors and then applied with sterile forceps to selective medium.

b. Nail and skin samples are cut into small pieces with a scalpel or sterile scissors and placed directly on the agar surface using sterile forceps. They should be pressed into the agar slightly.

VII. INCUBATION AND EXAMINATION OF FUNGAL CULTURES

It is imperative that these cultures be handled in a biological safety cabinet.

A. Fungal cultures are incubated at 30°C for a minimum of 4 weeks. Plates should be examined daily for the first 7 days and at least twice per week thereafter.
B. Specimens from genital sites or mucosal surfaces submitted for isolation of *Candida* spp. may be discarded after 7 days.
C. When *H. capsulatum* or *Blastomyces dermatitidis* is suspected, 6 to 8 weeks of incubation may be required.

VIII. SAFETY CONSIDERATIONS

It is imperative that these cultures be handled in a biological safety cabinet.

Personnel working in the mycology laboratory should be aware of the potential dangers when working with fungal organisms and exercise appropriate safety measures.

A. Processing of specimens for fungal culture is done in a BSL 2 facility using standard precautions for protection against infectious agents.
B. All clinical material is processed under a class II biosafety cabinet that is monitored and maintained regularly for proper function. The cabinet blower should be turned on at least 10 min prior to work and left on for 10 min after completion of work and surface disinfection.
C. Disposable gloves and protective clothing are worn during processing. Gloves should be wiped with dilute bleach between specimens.
D. When *H. capsulatum* or *Coccidioides immitis* is suspected, all isolates should be maintained on slants to prevent aerosolization when cultures are manipulated.
E. Seal all plated media with gas-permeable tape or Shrink-Seal before removing them from the biosafety cabinet.
F. All excess clinical materials are discarded in biohazard bags secured with tape and placed in containers designated for disposal of biomedical waste.
G. All laboratory surfaces that come in contact with specimens or cultures should be decontaminated with 10% bleach that is made fresh each day.

REFERENCE

1. **Snyder, J. W., and R. H. Atlas.** 2006. *Handbook of Media for Clinical Microbiology*, 2nd ed. CRC Press, Boca Raton, FL.

SUPPLEMENTAL READING

Evans, E. G. V., and M. D. Richardson. 1989. *Medical Mycology—a Practical Approach.* IRL Press at Oxford University Press, Oxford, United Kingdom.

LaRocco, M. T. 2007. Reagents, stains, and media: mycology, p. 1737–1744. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.

Robinson, B. E., and A. A. Padhye. 1988. Collection and transport of clinical specimens, p. 11–32. *In* B. B. Wentworth (ed.), *Diagnostic Procedures for Mycotic and Parasitic Diseases*, 7th ed. American Public Health Association Inc., Washington, DC.

Sutton, D. A. 2007. Specimen collection, transport, and processing: mycology, p. 1728–1736. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.

8.5 Examination and Evaluation of Primary Cultures

PREANALYTICAL CONSIDERATIONS

I. EXAMINATION OF FUNGAL GROWTH ON PRIMARY MEDIA

It is imperative that these cultures be handled in a biological safety cabinet.

NOTE: Manipulate cultures in a biosafety cabinet.

Primary plates are read daily for the first week, every other day for the second week, and twice weekly for the remaining 2 weeks. The use of 4 weeks of incubation has been challenged by some groups because few new positive cultures develop in the fourth week. I have noted that clinically significant positive cultures are sometimes seen in the fourth week, suggesting the need to retain this incubation period. In areas of endemicity of systemic dimorphic pathogens, incubation for 5 weeks should be considered, as occasional isolates of *Histoplasma capsulatum* and *Blastomyces dermatitidis* may require that much time to form evident colonies. In cases of eumycetoma, the etiologic agent may not be evident on culture until the fifth or sixth week. When growth appears, differentiate between yeast and filamentous forms (moulds) that may require microscopic examination. Use wet mounts or stain with lactophenol cotton blue (LPCB) (*see* item V.B below). If the isolate suggests an actinomycete, examine with Gram stain and with a modified acid-fast stain. (Procedures for the identification of aerobic actinomycetes are given in procedure 6.1, items V.D.1 and V.D.2.)

Several key characteristics of a fungus may be obtained from primary cultures. For the yeasts, these include the presence of a capsule, budding characteristics (single, multiple, or catenated), size, morphology, and colony color. If CHROMagar or another chromogenic medium was used in the primary setup, a presumptive identification of a yeast may be possible (*see* item VI.A below) (1). For moulds, helpful features include colony characteristics (obverse and reverse), sporulation characteristics, the presence of septa, and hyphal or conidial color (e.g., phaeoid moulds). For the moulds, see procedures 8.7 and 8.9.

Blood cultures or fluid cultures incubated in broth which have fungal elements upon Gram staining (*see* section 3) should be subcultured onto appropriate media. If yeasts are seen, then the subculture medium battery should include CHROMagar or a similar differential medium, and it should also include an India ink stain if yeast morphology is suspicious for *Cryptococcus* spp.

II. PREPARATION OF CULTURE MATERIAL FOR MICROSCOPIC EXAMINATION

Once colony formation has occurred, the organism should be viewed microscopically. For some fungi (e.g., zygomycetes), intact colonies should be viewed initially with a dissecting microscope to obtain an indication of growth characteristics. The following list includes the general methods for colony preparation and staining.

A. Yeast
- **1.** Wet prep (with or without Tween 20)
- **2.** Stains
  - **a.** LPCB
  - **b.** India ink

B. Moulds
- **1.** Wet prep (with Tween 20)
- **2.** Scotch tape (or pinworm paddle)
- **3.** Tease prep
- **4.** Slide culture
- **5.** Stains
  - **a.** LPCB
  - **b.** Ascospore

III. MATERIALS

A. General
Slides, coverslips, dissecting needles, loops, pipettes, and cellotape

B. Stains and reagents
- **1.** 0.05% Tween 80 in sterile distilled water (50 µl of Tween 80 in 99.95 ml of water; sterilize by autoclaving)
- **2.** LPCB (available from Remel and other suppliers)
- **3.** India ink (available from Remel and other suppliers)

C. Media
- **1.** Nonselective, fungus-specific agar medium
- **2.** CHROMagar or a similar chromogenic medium
- **3.** Other agars suitable for ascospore production (*see* procedures 8.4 and 8.8)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Stains
- **1.** Each lot of reagent should be tested to ensure that it stains adequately. For example, a tease preparation of *Penicillium* sp. mould in LPCB should stain the cell walls blue. There is no specific strain of *Penicillium* for QC. Laboratorians should, however, select a strain and use it for all subsequent QC evaluations of LPCB. QC for India ink is described in item IV of procedure 8.3.
- **2.** Check stain for the presence of extraneous material (fungal elements, etc.).

B. Organisms
Different batches of media can result in variation in the colony characteristics of organisms. Therefore, inoculate a control plate with organisms known to display specific colony features. For CHROMagar, *Candida albicans* ATCC 10231, *Candida krusei* ATCC 14243, and *Escherichia coli* ATCC 25922 are recommended by the manufacturer.

C. Media
Ensure that all media are used before their expiration date. See section 14 of this handbook for a general discussion of QC.

V. MICROSCOPIC EXAMINATION OF YEASTS AND MOULDS

It is imperative that these cultures be handled in a biological safety cabinet.

A. Yeast colony examination with 0.05% Tween 80 wetting agent

The addition of Tween 80 helps reduce bubble formation and prevent infectious conidia from escaping. The procedure is as follows.

1. Place a drop of 0.05% Tween 80 onto a slide.
2. With a loop, touch the surface of the suspicious colony, being sure that some fungal material is on the loop. Gently rub the material in the drop of Tween.
3. Place a coverslip on top of the drop and examine with a microscope.

B. LPCB solution

LPCB is an excellent stain for examination of fungal material. The phenol kills the fungi, and the lactic acid increases preservation. Cotton (china) blue stains chitin and cellulose. The procedure is as follows.

1. Place a drop of LPCB onto a slide.
2. Remove a piece of fungal material, using a dissecting needle, and place into the drop of stain.
3. Place a coverslip on top and examine microscopically.

C. India ink

The India ink test helps to demonstrate the presence of a capsule on an organism (*see* item V.D of procedure 8.3).

D. Gram stain

See procedure 3.2.1.

POSTANALYTICAL CONSIDERATIONS

VI. TESTS USEFUL FOR YEAST IDENTIFICATION FROM PRIMARY CULTURES

A. CHROMagar

CHROMagar contains enzymatic substrates that are linked to chromogenic compounds. When specific enzymes cleave the substrates, the chromogenic substrates produce color. The action of different enzymes produced by yeast species results in color variation useful for the presumptive identification of some yeasts. Colony variation is seen for some species (1). The medium is best suited for *C. krusei*, *C. albicans*, *Candida tropicalis*, and *Trichosporon* spp. The medium should not be used alone to make a definitive identification of a yeast. The medium contains chloramphenicol to inhibit bacterial contamination in primary specimens.

1. Applications

   CHROMagar is useful for the detection of mixed infections (especially in wounds and normally sterile body site specimens) and as an additional test to resolve difficult identifications.
2. Procedure
   - **a.** Primary specimens should be plated by procedures typical for the specimen.
   - **b.** Subcultures should be streaked for isolation.
   - **c.** Incubate at 35°C in the dark for 48 to 72 h (not less than 48 h). Exposure to higher or lower temperatures for a prolonged period will alter the final color production and lead to possible incorrect presumptive identification of some *Candida* spp.
   - **d.** *Use only isolated colonies* to determine the organism's characteristics for purposes of presumptive identification.
   - **e.** *Do not use the isolated colonies to perform subsequent screening tests for identification or for biochemical identification systems.* Subculture onto a nonselective medium before performing additional identification tests on the isolate.

VI. TESTS USEFUL FOR YEAST IDENTIFICATION FROM PRIMARY CULTURES *(continued)*

Table 8.5–1 General list of yeast species or genera based on colony color[a]

White, cream, or tan	Brown or black	Salmon, pink, or red
Blastoschizomyces	*Aureobasidium*	*Rhodotorula*
Candida	*Phaeoannellomyces elegans*	*Sporobolomyces*
Cryptococcus	*Hortaea werneckii*[b]	
Geotrichum[d]	*Phaeococcomyces exophialae*[c]	
Hansenula	*Ustilago*	
Kloeckera		
Malassezia		
Prototheca[d]		
Saccharomyces		
Trichosporon		
Ustilago		
Teleomorphs[e]		
Debaryomyces		
Kluyveromyces		
Pichia		

[a] Based on growth on Sabouraud glucose agar (Emmon's modification), potato dextrose agar, malt extract agar, or BHI agar. Note that all *Malassezia* spp. except *Malessezia pachydermatis* require exogenously supplied lipid (e.g., olive oil) for growth.

[b] Previously *Exophiala werneckii.*

[c] A second anamorphic (asexual) form (i.e., synanamorph) of *Exophiala (Wangiella) dermatitidis.*

[d] Not a yeast but produces yeastlike colonies on mycological media.

[e] Sexual genera associated with some species of the anamorphic genera.

B. Other tests

Only a few tests are available to identify a yeast when it is first isolated. Colony color (pigment) (Table 8.5–1) and microscopic morphology are helpful. The tests described below provide only presumptive identification (an exception is the Accu-Probe for *Cryptococcus neoformans* [Gen-Probe], which is described in procedure 8.9). Subculture of the yeast colony onto appropriate media will lead to yeast identification (*see* procedure 8.8). Isolates from medically significant specimens must be identified to the species level. Stopping at the presumptive identification step could lead to inappropriate therapy.

VII. SUBSEQUENT TESTING

Based on the type of organism and special characteristics, several tests may be performed to obtain an identification of a primary culture (Table 8.5–2). If the fungal colonies are not well separated from bacterial colonies or there is insufficient material for testing, subculture the organism (*see* procedures 8.4 and 8.7). The identification schemes shown in Fig. 8.5–1 and 8.5–2 utilize pigment (Table 8.5–1), microscopic morphology (*see* Table 8.3–1), and presumptive test results (Table 8.5–2; described in procedure 8.6).

VIII. INTERPRETATION

CHROMagar

A. *C. albicans*

A medium-size (2- to 3-mm diameter), green, smooth, matte colony with a very slight green halo in the surrounding medium. Note that *Candida dubliniensis* produces a similar colony, but it is typically darker green.

B. *C. tropicalis*

A medium-size, smooth, matte colony which is blue to blue-gray with a paler pink edge. The colony may have a dark-brown to purple halo which diffuses into the agar.

Table 8.5–2 Summary of subsequent tests

Primary growth	Key characteristic	Further tests[a]
Yeast	Capsulated	Caffeic acid disk, urease, rapid nitrate, serology
	Nonencapsulated	Urease, germ tubes, *C. albicans* screen, rapid trehalose, serology
	CHROMagar pigmentation or	Color chart Determine mixed infection May allow presumptive identification of *C. krusei*, *Candida glabrata*, *C. albicans*
	Other chromogenic media	See manufacturer's information for organism identification and limitations.
Mould	Nonseptate hyphae	Likely zygomycete; subculture to potato dextrose agar
	Septate hyphae	Evaluate reproductive and hyphal characteristics. Subculture to one or more media (*see* procedure 8.9). Accu-Probe if suspect systemic pathogen *(Histoplasma, Blastomyces, Coccidioides)*

[a] These tests are described in procedures 8.6 and 8.7.

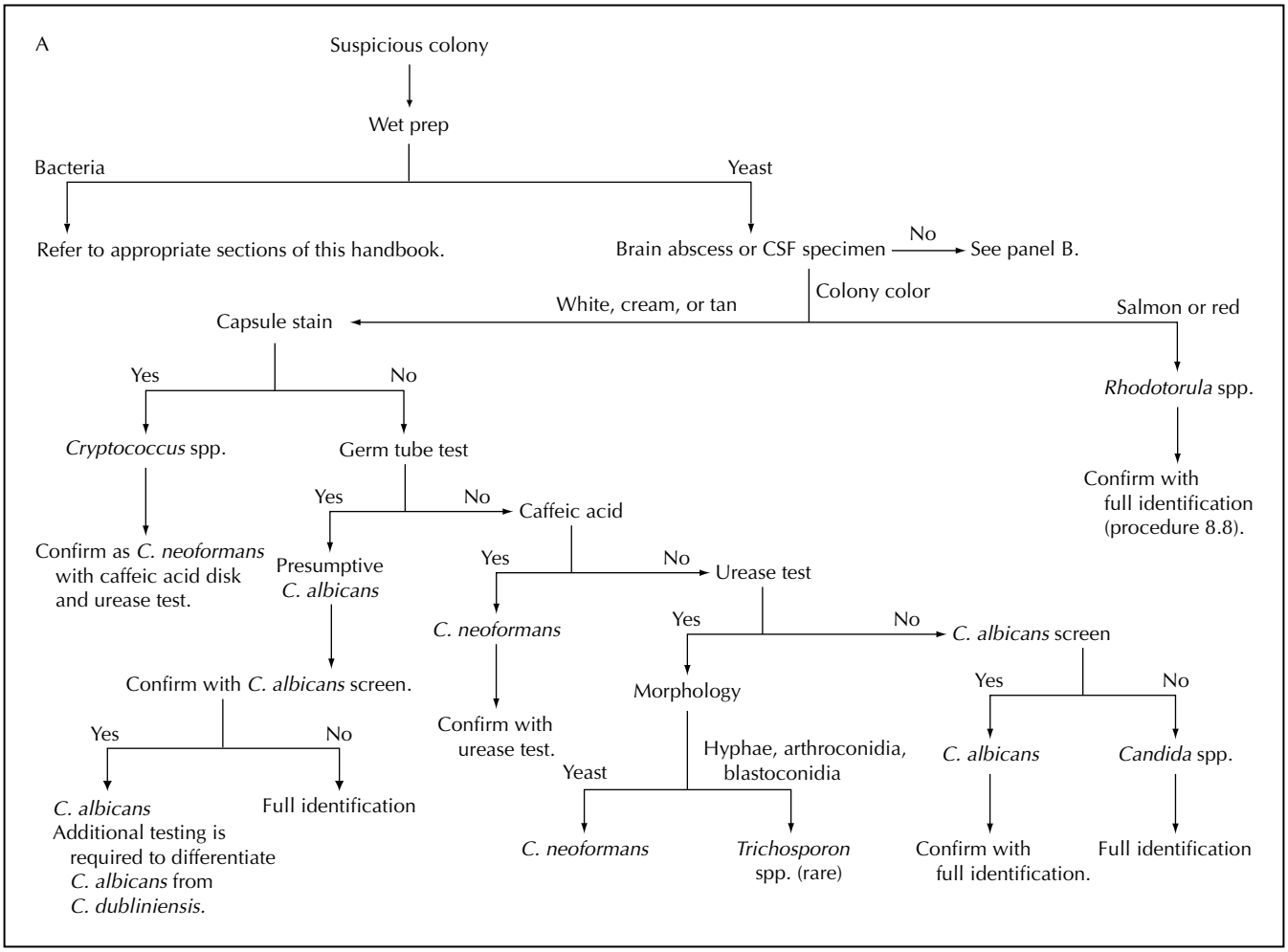


Figure 8.5–1 Flowchart for evaluating a possible yeast species on primary culture. (A) Identification procedure when yeast cells are found in brain abscess or CSF specimen. See Fig. 8.3–1 for common yeast agents isolated from different body sites. (B) Identification procedure for yeasts when the specimen is not brain abscess or CSF *(continued).*

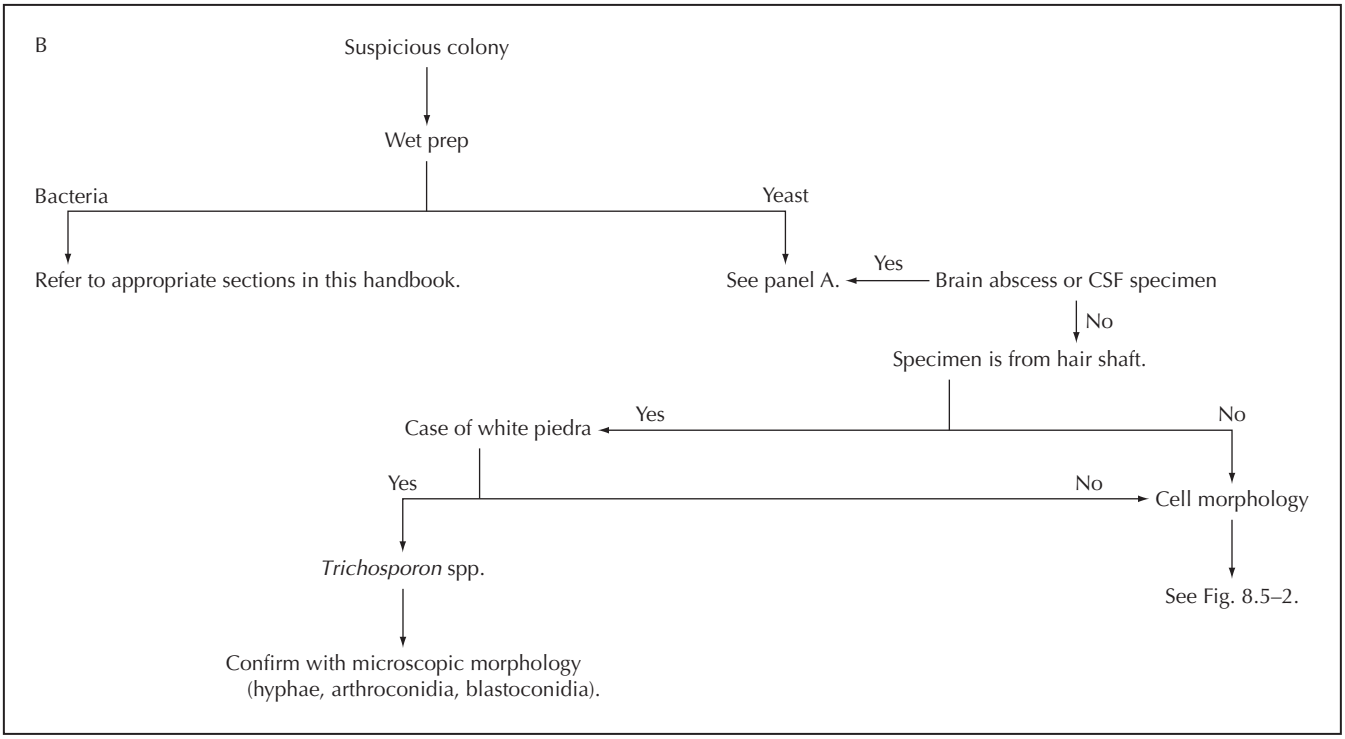


Figure 8.5–1 *(continued)*

VIII. INTERPRETATION *(continued)*

C. *C. krusei*
A large (≥4-mm diameter), spreading, rough, pink colony with a pale pink to white edge

D. Other yeasts produce colony characteristics that are useful to obtain a suggestive identification. According to Odds and Bernaerts (1), *Trichosporon* spp. produce a distinctive blue-green colony, particularly with prolonged incubation, when it becomes rough and crenated. The manufacturer does not include *Trichosporon* in its list of organisms that can be presumptively identified. Further testing is required to identify *Trichosporon* spp.

E. If the test is used to determine mixed infection in primary specimens, additional testing must be performed. If the test is used to help resolve a difficult identification, further tests may not be required.

IX. LIMITATIONS

A. CHROMagar

1. The CHROMagar test provides only presumptive identification of four yeasts *(C. albicans, C. tropicalis, C. krusei,* and *C. dubliniensis)*. Individual clinical laboratories should test at least five isolates of each of the four species to become aware of the color variation that may occur. Strict adherence to the manufacturer's incubation conditions is imperative. Enzymatic reactivity is sensitive to temperature. Review the manufacturer's instructions before performing this test.

2. Other chromogenic media (e.g., Albicans ID [bioMérieux, Marcy l'Étoile, France] and CandiSelect [Sanofi Diagnostics, Marnes-la-Coquette, France]),

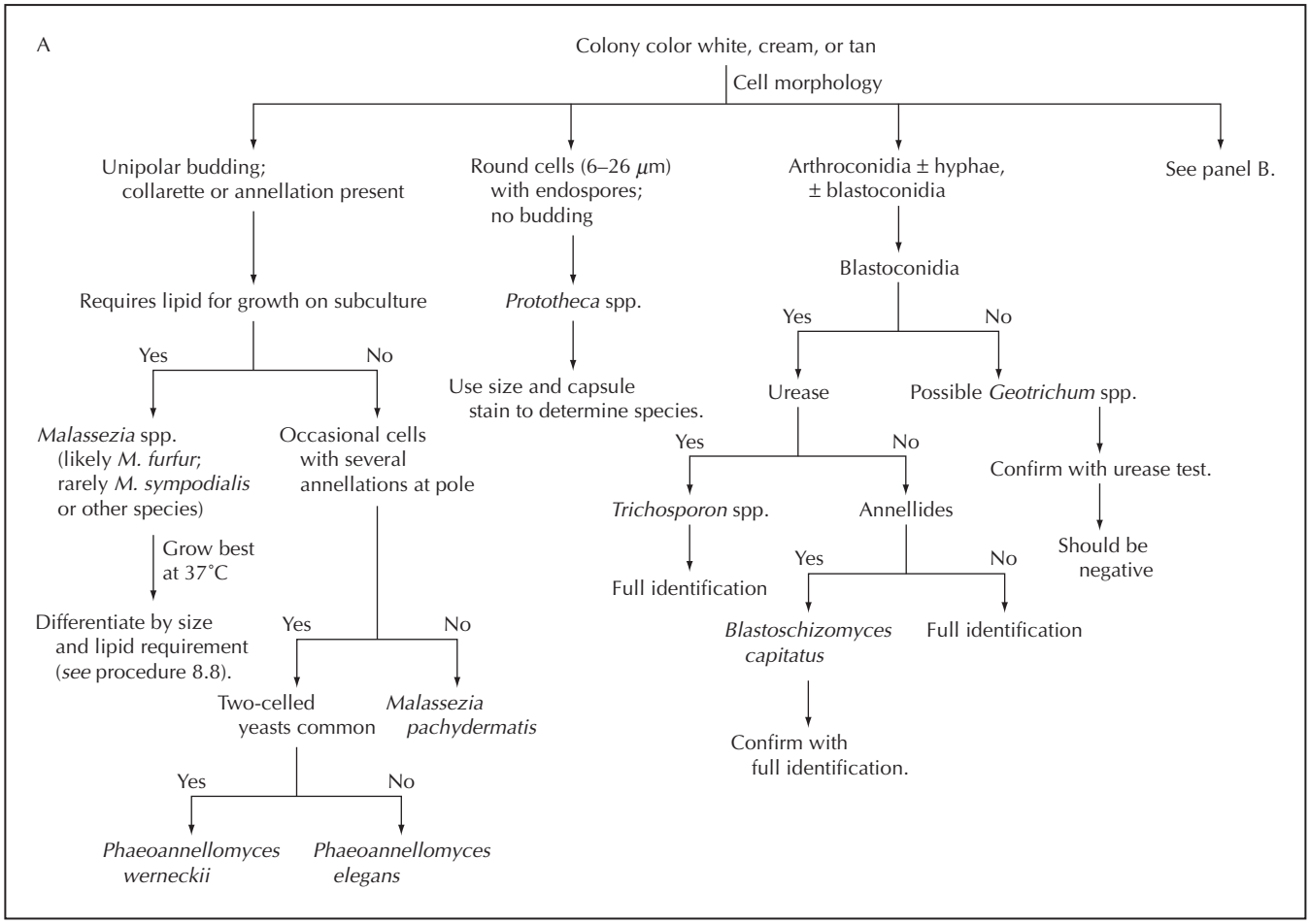


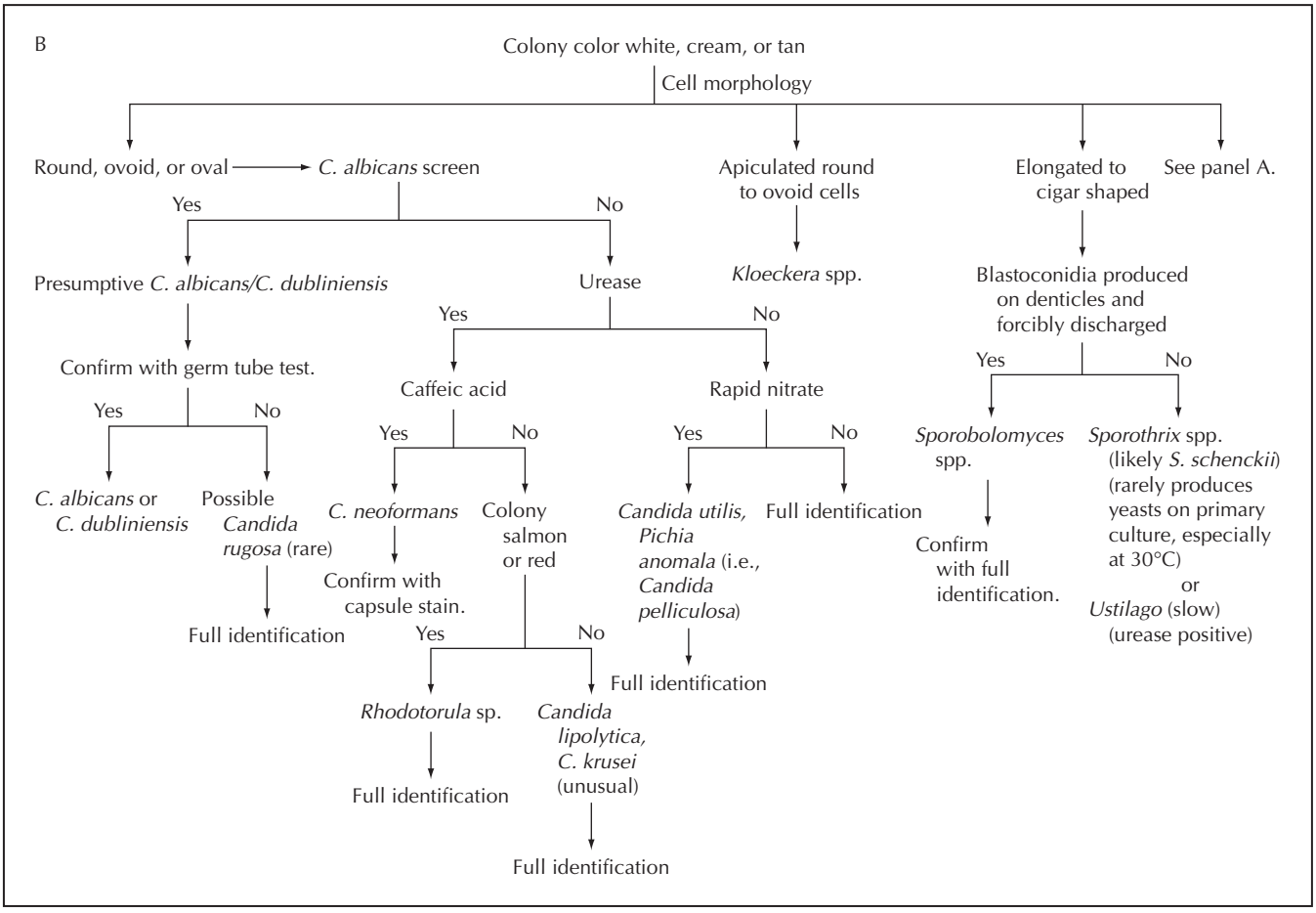
Figure 8.5–2 Simple tests to determine genera of yeast cells seen on primary culture. (A and B) Identification procedures when colony color is white, cream, or tan. (C) Identification procedure when colony color is salmon, pink, or red. (D) Identification procedure when colony color is brown or black *(continued).*

IX. LIMITATIONS *(continued)*

as well as fluorogenic media (e.g., Fluoroplate Candida [Merck, Darmstadt, Germany] and SDCA-MUAG Candida agar [Biolife, Milan, Italy]), have been developed to provide presumptive identification of *C. albicans*, but these media could also be used to help detect mixed infections. The media will also produce positive color or fluorescence development with *C. dubliniensis*. Fluorogenic media have the additional problem that the fluorophore can diffuse into the medium and make non-*C. albicans* colonies appear positive. Until extensive studies have been performed, no chromogenic or fluorogenic medium should be used as the sole determinant of species identification.

B. India ink

When cryptococci are cultured, capsule production is generally reduced compared to what is seen with organisms in a specimen. Therefore, the India ink test may not provide much help to identify *C. neoformans* from a primary culture. Size, shape, and budding characteristics are usually more helpful and should suggest to the laboratorian the need to perform a rapid presumptive test, such as the caffeic acid test (*see* procedure 8.6).



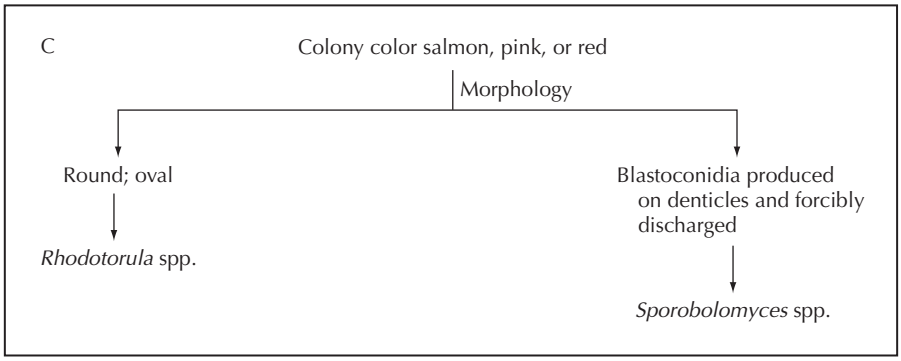


Figure 8.5–2 *(continued)*

X. REPORTING

General

The tests described in this procedure are intended to provide preliminary or presumptive information (such as mould, yeast, dematiaceous, or encapsulated) about the identity of an organism recovered from a specimen. Consequently, reporting should indicate that the species identification information is not definitive and that additional testing is needed to obtain more definitive identification information (e.g.,

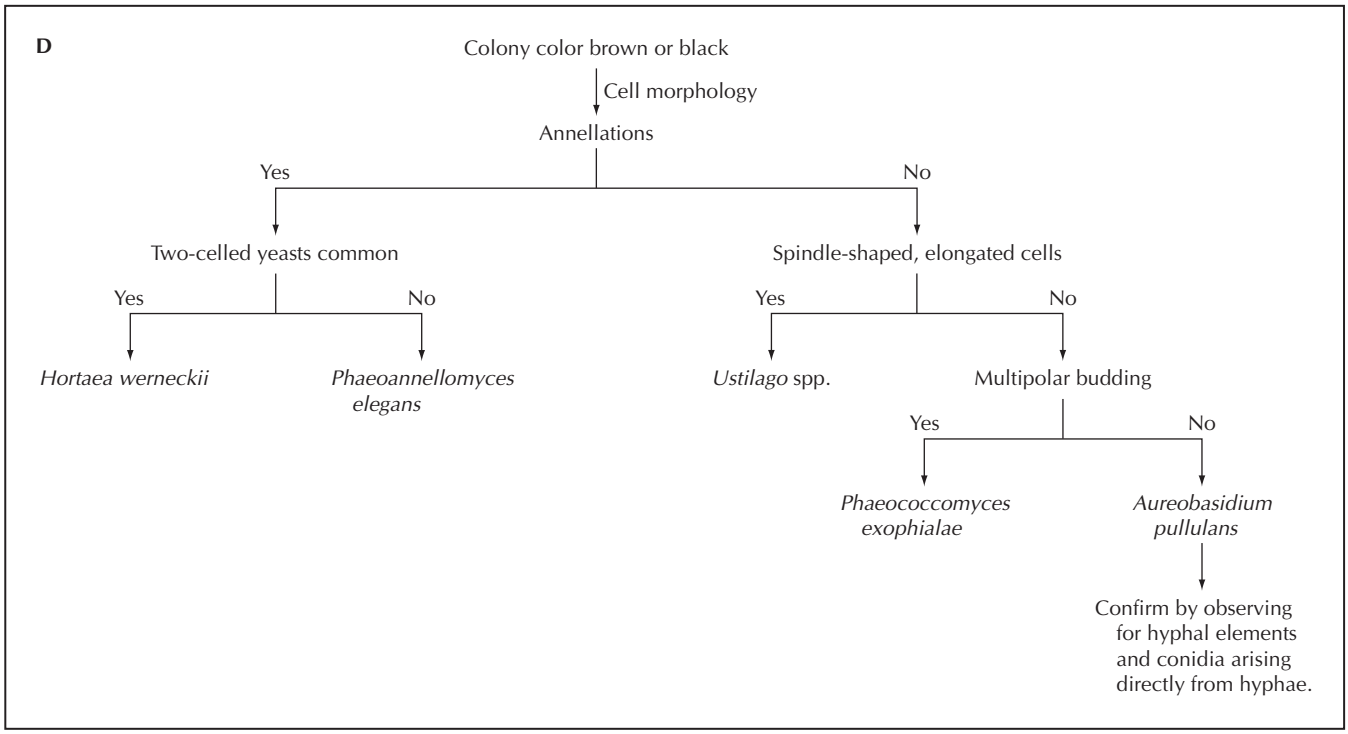


Figure 8.5–2 *(continued)*

X. REPORTING *(continued)*

"presumptive [species]; further testing needed for definitive identification"). Significant isolates should always be identified to the species level. When the significance is in doubt, the presumptive report should be sufficient. It is possible to identify some moulds (such as some dermatophytes and aspergilli) to the species level from primary cultures. When this occurs, there is no need to assign any preliminary nature to the identification. Whether all significant moulds should be identified to the species level is controversial. However, with the advent of new antifungals and the different susceptibilities of species within a genus to different antifungals (within a class and among classes), identification to the species level should be strongly considered.

One of the more controversial issues regarding primary cultures is quantitation. While for yeasts, quantitation is generally reported using criteria similar to those used in bacteriology for deciding clinical significance, the situation is different for moulds because they grow as contiguous cells which may break off into fragments. Such fragmentation could lead to the appearance of a higher fungal burden than is actually present in the tissue. Similarly, the recovery of a single colony of a potentially pathogenic mould does not necessarily mean that the fungal burden in the specimen is low (i.e., that the single colony represents a contaminant). Sample bias, sample preparation, and other factors can contribute to the recovery of so few colonies from an infected site. When available, it is helpful to correlate what was recovered on primary culture with what was seen with histopathology.

◩ One problem with the bacteriological quantitation approach for yeasts is that yeast cell volume is much greater than the volume of bacteria, so the space occupied by a given number of yeast calls will typically exceed >10-fold the space occupied by the same number of bacteria. This raises the issue that 2+ of yeasts on a primary isolation plate is not equivalent to 2+ of bacteria (that is, the yeast is occupying more tissue space).

REFERENCE

1. **Odds, F. C., and R. Bernaerts.** 1994. CHROMagar Candida, a new differential isolation medium for presumptive identification of clinically important *Candida* species. *J. Clin. Microbiol.* **32:**1923–1929.

SUPPLEMENTAL READING

Freydiere, A.-M., R. Guinet, and P. Boiron. 2001. Yeast identification in the clinical laboratory: phenotypic methods. *Med. Mycol.* **39:**9–33.

Haley, L. 1978. *Laboratory Methods in Medical Mycology.* U.S. Department of Health, Education, and Welfare, CDC publication no. 78-8361, p. 94. U.S. Department of Health, Education, and Welfare, Washington, DC.

Haley, L. D., and C. S. Callaway. 1978. *Laboratory Methods in Medical Mycology.* U.S. Department of Health, Education, and Welfare, CDC publication no. 78-8361, p. 30. U.S. Department of Health, Education, and Welfare, Washington, DC.

APPENDIX 8.5–1

Medium and Reagent Preparation

A. Yeast colony examination using water and Tween 80

Into a 125-ml flask, place the following.
distilled water ..... 100 ml
Tween 80 ..... 0.05 ml

Autoclave at 121°C for 15 min. Store at room temperature for 3 months.

B. Lactophenol cotton blue

Available from several sources (for example, Remel no. 40-028, lactophenol aniline blue). Store at room temperature for up to 6 months.

C. CHROMagar

Source: Hardy Diagnostics (catalog no. G34)

8.6 Presumptive Identification Tests for Yeasts Isolated on Primary Culture

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

All of the tests described in this procedure (Table 8.6–1) are considered presumptive because they do not test a characteristic of a species that is unique to that species. Some of the tests do have high specificity values, which would make the test sufficient for the purpose of medical management of some clinical situations (e.g., intertrigenous candidiasis due to *Candida albicans*) but insufficient for others (e.g., fungemia due to *C. albicans*). Presumptive tests also are generally restricted in the range of species they identify. The results of two different physiological tests with high specificity for a particular species may be appropriate for identifying the species presumptively. However, there are instances when two different species elicit identical positive reactions in both tests. Mycologists and clinical microbiologists must be aware of these obfuscations. For example, *C. albicans*, by far the most frequently encountered *Candida* sp. in the clinical setting, and *Candida dubliniensis* are both germ tube positive and positive for the enzymes β-galactosaminidase and L-proline aminopeptidase (3).

As with more definitive tests, care must be taken to be sure that the tests are performed on yeasts cultured on the appropriate media and under the appropriate conditions. Results obtained with a presumptive test must be consistent with other information regarding the yeast (e.g., colony color and cell morphology) and the clinical specimen from which the yeast was isolated. Thus, presumptive tests should be performed only on isolates that have morphological and cultural characteristics consistent with the use of a given test. For a review of current presumptive tests, see reference 2. It is important to remember that mixed yeast infections do occur, such as in fungemia. The presumptive tests described below assume that yeast inocula are from pure cultures or from single colonies. Tests such as the *C. albicans* screen and the *Candida* check require multiple colonies in the preparation of the inoculum. Mixed cultures will therefore cause erroneous results.

Table 8.6–1 Presumptive identification tests for yeasts on primary culture

Test	Item no.[a]	Organism
CHROMagar	8.5.VI.A	*C. krusei, C. albicans, C. tropicalis, Trichosporon* spp.
Germ tube	8.6.IV.A	*C. albicans* complex[b]
C. albicans screen	8.6.IV.B	*C. albicans, C. dubliniensis*
Rapid urease	8.6.IV.C	*Cryptococcus* spp., *Rhodotorula* spp., *Trichosporon* spp. (variable), *C. krusei* (variable), *Malassezia pachydermatis, Candida lipolytica* (i.e., basidiomycetous yeasts)
Rapid nitrate reductase	8.6.IV.D	*C. albidus* (+), *C. neoformans* (−), *C. terreus* (+)
Caffeic acid disk	8.6.IV.E	*C. neoformans*
Rapid trehalose assimilation	8.6.IV.F	*C. glabrata*
India ink	8.3.V.D	*Cryptococcus* spp.

[a] In this handbook.

[b] This complex consists of *C. albicans, C. dubliniensis, C. stellatoidea* (obsolete), and *Candida africanum* sp. nom. inval.

II. MATERIALS

A. General
1. Sterile applicator sticks
2. Heat block
3. Microscope slides
4. Sterile Pasteur pipettes
5. Pipettes (50 µl)
6. Coverslips
7. Sterile tubes, 16 by 125 mm
8. Transfer loops
9. Sterile screw-cap 16- by 120-mm black-top tubes
10. Sterile saline
11. Distilled water

B. Germ tube test
0.5-ml aliquots of sterile fetal bovine serum

C. Preformed-enzyme test
C. albicans screen or CA-50

D. Urease disk test
Urea differentiation disks

E. Nitrate swab rapid test
1. Rapid nitrate swab (0.2% nitrate)
2. Nitrate reagent A (0.08% sulfanilic acid in acetic acid)
3. Nitrate reagent B (0.06% *N*-dimethyl-1-naphthylamine)

F. Caffeic acid disk screen
1. Caffeic acid disks
2. Cornmeal agar plates

G. Rapid trehalose test
Rapid trehalose assimilation broth

H. Serologic identification of *Candida* spp.
1. *Candida* check kit
2. GYEP agar (*see* Appendix 8.6–1)

ANALYTICAL CONSIDERATIONS

III. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Germ tube test
The following yeasts should be included each time the test is performed.
1. Germ tube positive, *C. albicans* ATCC 14053
2. Germ tube negative, *Candida tropicalis* ATCC 66029

B. Preformed-enzyme test (*C. albicans* screen or CA-50)
Randomly select two tubes from each lot of kits received and conduct the test using *C. albicans* ATCC 14053 (positive for β-galactosaminidase and L-proline aminopeptidase) and *Cryptococcus laurentii* ATCC 18803 (negative for both enzymes).

C. Urease disk test
Test each new numbered lot using the following controls.
1. Positive control, *Cryptococcus neoformans* ATCC 66031
2. Negative control, *C. albicans* ATCC 60193
3. Reagent control, uninoculated

D. Nitrate swab rapid test
The medium is tested on receipt, using control strains, and each time a test is conducted. Use *C. neoformans* (ATCC 66031) as a negative control and *Cryptococcus albidus* (ATCC 10231) as a positive control.

E. Caffeic acid disk test for *C. neoformans* screen
Each new lot of disks should be tested and should yield a brown pigment with *C. neoformans* (positive) and no pigment with *C. albicans* (negative). The disks should be white; discard if discolored.

F. Rapid trehalose test
Each test should include the positive control, *Candida glabrata* ATCC 2001, producing a color change to yellow, and a negative control, *C. albicans* ATCC 10231, which produces a color change to blue or green.

G. Serologic identification of *Candida* and *Cryptococcus* spp.
There are no established control organisms for the serologic identification kit, but it is recommended that the following species be used for the *Candida* check kit to demonstrate positive and negative reactions for each factor serum.
1. *Candida parapsilosis*, factor sera 1, 5, 13, and 13b positive
2. *Candida guilliermondii*, factor sera 1, 4, and 9 positive
3. *Candida kefyr*, factor sera 1 and 8 positive
4. *C. glabrata*, factor sera 1, 4, 6, and 34 positive

IV. PROCEDURES

See Appendix 8.6–1 for reagents.

A. Germ tube test

The germ tube test provides one of the most rapid approaches to the *presumptive* identification of *C. albicans* and *C. dubliniensis* (Fig. 8.6–1).

Procedure

1. Lightly touch a yeast colony with a wooden applicator stick.

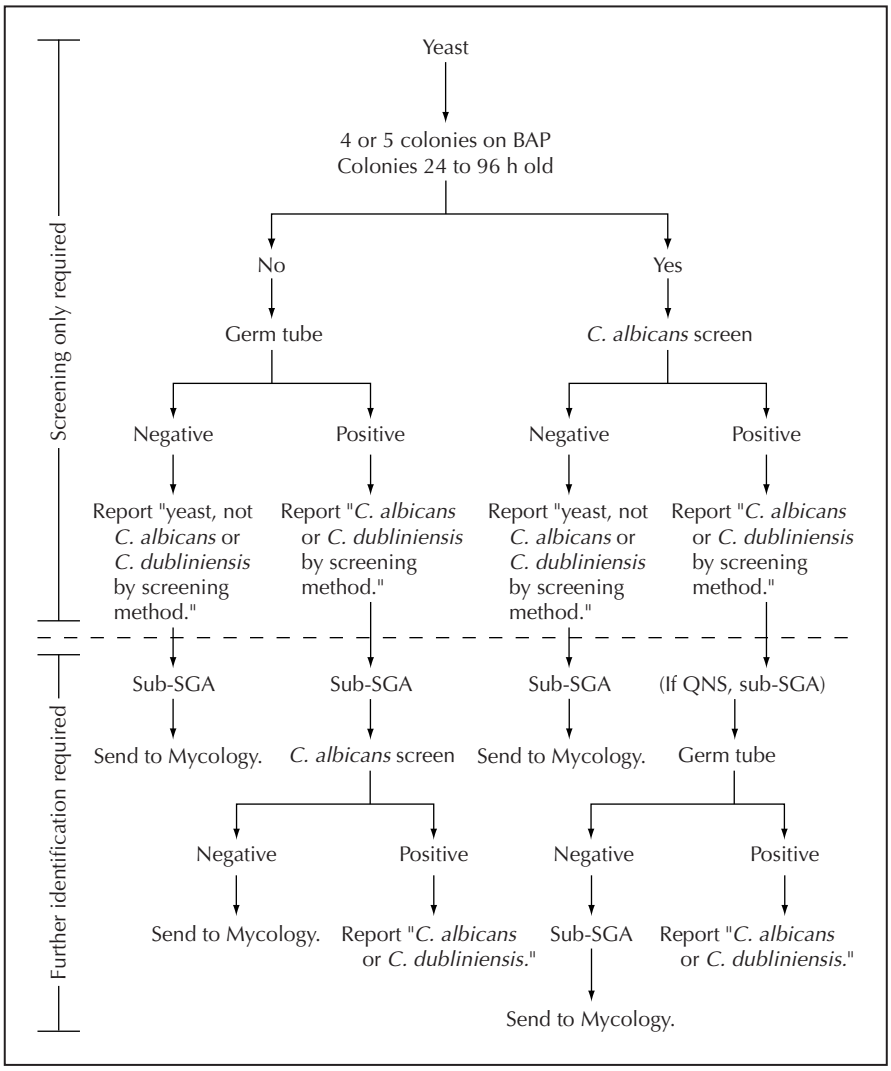


Figure 8.6–1 Algorithm for identification of *C. albicans* on primary culture. Follow specimen guidelines for significance for specimens submitted for routine bacteriology cultures. When a protocol requires screening tests or identification for yeasts, proceed as outlined in this diagram. If the yeast is from a sterile body fluid or blood, two tests must be positive to identify *C. albicans* or *C. dubliniensis* (e.g., if the *C. albicans* screen was positive, perform a germ tube test). Note that *C. dubliniensis* is a rare to occasional bloodstream pathogen (usually associated with catheters) depending on its endemic density. To discriminate between *C. albicans* and *C. dubliniensis*, use growth at 45°C (presumptive, as not all isolates of *C. albicans* tolerate this temperature and not all isolates of *C. dubliniensis* are inhibited by it) or, more definitively, assimilation tests (*see* procedure 8.8). If yeasts are seen in a blood culture bottle, subculture the bottles to CHROMagar to check for purity. SGA, Sabouraud glucose agar; QNS, quantity not sufficient.

IV. PROCEDURES *(continued)*

2. Suspend the yeast cells in an appropriately labeled tube of fetal bovine serum.
3. Incubate at 37 ± 1°C for 2.5 to 3 h.
4. Place a drop of the suspension on a microscope slide.
5. Place a coverslip over the suspension.
6. Examine under high power for the presence or absence of germ tubes. A germ tube appears as a short lateral extension from the yeast cell and does not have a constriction (septum) where it meets the yeast cell or any constriction at the septum along the tube (if the tube is sufficiently long to produce a septum). In a true germ tube, the first septum produced by a germinating cell is located along the tube near the point of juncture with the parent cell but not at the juncture. A constriction where the lateral extension meets the yeast cell is produced by pseudohyphae or budding cells. A minimum of five germ tubes should be observed before the isolate is called positive.

B. Preformed-enzyme tests

C. albicans produces two exoenzymes, L-proline aminopeptidase and β-galactosaminidase, when grown on appropriate media. The detection of these enzymes with a 30-min chromogen-coupled substrate-impregnated disk affords *presumptive* identification of *C. albicans.* A commercial kit, the *C. albicans* Screen (Remel; also sold by Murex as CA-50), which utilizes the impregnated disk is available. Presumptive identification of *C. albicans* based on only one of the two preformed enzymes mentioned above or on β-D-glucosidase leads to an unacceptably low level of sensitivity. This test, in combination with the germ tube test, has been used to confirm an isolate as *C. albicans* (3). However, *C. dubliniensis* will produce the same results. Laboratorians should be mindful that *C. dubliniensis* may be obtained from blood, urine, vaginal, and other specimens, especially when the patient is immunocompromised or appears refractory to azole therapy. Rare isolates of other *Candida* spp., especially *C. tropicalis,* can produce germ tubes in the germ tube assay, although few germ tubes are present and they are more constricted at the bases. The enzyme test will help distinguish these isolates from *C. albicans.* This test can also be used in combination with CHROMagar and the rapid trehalose assimilation assay to obtain presumptive identification of common species associated with bloodstream infections (Fig. 8.6–2).

1. Procedure
   - **a.** Use only pure 18- to 24-h cultures of the control organisms grown on Sabouraud dextrose (glucose) agar and only pure cultures of the test organisms grown on blood agar, Sabouraud dextrose agar, Mycosel, or potato dextrose agar.
   - **b.** Remove a disk from the vial and place it in one of the tubes provided. Add 1 drop of sterile demineralized (deionized) water.
   - **c.** With an applicator stick, pick up a heavy visible "paste" of the yeast and rub it onto the disk. Incubate at 35°C for 30 min.
   - **d.** After incubation, add 1 drop of 0.3% NaOH and examine against a white background for a distinct yellow color. No color is considered negative.
   - **e.** Add 1 drop of cinnamaldehyde reagent. Development of a pink to red color within 1 min is a positive test. No color or a slight yellow color is a negative test.
2. Results

   The development of a distinct yellow color upon the addition of 0.3% NaOH is a positive test for β-galactosaminidase. No yellow color indicates the absence of the enzyme. The development of a red color within 1 min after adding cinnamaldehyde reagent is a positive test for L-proline aminopeptidase.

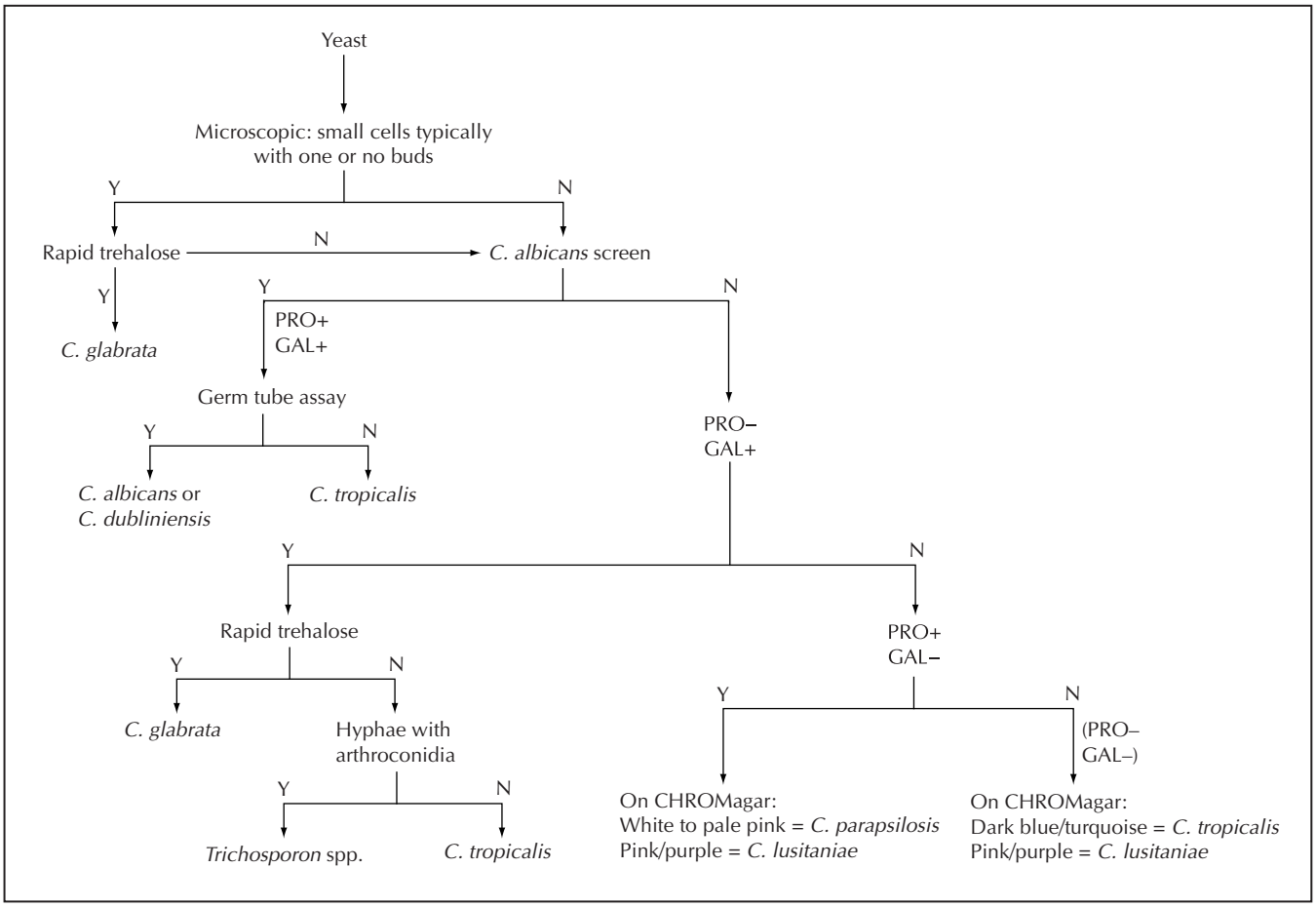


Figure 8.6–2 Identification of the most common clinically important *Candida* species isolated from sterile body specimens. Algorithm assumes that CHROMagar *Candida* was included in the primary set up media. Y, yes; N, no.

IV. PROCEDURES *(continued)*

C. Urease disk test for the differentiation of yeasts

The urease disk test is a rapid detection test to distinguish basidiomycetous yeasts (e.g., *Cryptococcus*) from ascomycetous yeasts. Urease splits urea to form ammonia and carbon dioxide, which raises the pH and causes a color shift in the phenol red indicator from amber to pinkish red. When an organism appears urease negative but there is a strong suspicion that the organism should be urease positive, a more definitive urease test should be performed (e.g., urea agar slants).

1. Procedure
   - **a.** Emulsify a colony of the yeast in 0.5 ml of sterile distilled water in a 16- by 125-mm tube.
   - **b.** Add one urea disk.
   - **c.** Incubate at 28°C.
     - **NOTE:** Room temperature may not be 28°C and therefore should not be used.
   - **d.** Check at 4 h and daily up to 72 h.

2. Results
   - **a.** Positive, turns to pink to red
   - **b.** Negative, no color change

IV. PROCEDURES *(continued)*

c. Urease activity by species

C. albicans	−
C. glabrata	−
C. guillermondii	−
C. kefyr	−
Candida krusei	+[a]
C. parapsilosis	−
C. tropicalis	−
All *Cryptococcus* spp.	+ (strong)
Geotrichum spp.	−
Saccharomyces spp.	−
Rhodotorula spp.	+
Trichosporon spp.	+[a]
Malassezia spp.	+

[a] Variable depending on strain

D. Nitrate swab rapid test for yeast nitrate reduction

Nitrate reduction can be used to differentiate species of *Cryptococcus. C. neoformans* does not reduce inorganic nitrate. *C. albidus* and *Cryptoccus terreus* are nitrate positive. Organisms that reduce nitrate do not necessarily assimilate nitrate; however, the opposite is true (if they assimilate nitrate, they must reduce it).

Cotton swabs impregnated with KNO_3-Zephiran are used to test yeasts for the ability to form nitrite from nitrate (the presence of nitrate reductase). The formation of nitrite is detected by the addition of a color reagent. The reaction (color changes) occurs on the cotton swab.

Not all nitrate reductase-positive organisms listed in Table 8.8–4 will produce a positive result, presumably due to insufficient enzyme production. However, the rapid test can sometimes provide laboratorians with an indication of an isolate's identity. The test is also useful when species discrimination cannot be made based on more elaborate tests (such as standard yeast identification panels). The organisms which produce a positive result with the rapid nitrate test are not frequently isolated in the laboratory, so incorporation of this test into the laboratory's test menu may not be cost-effective.

1. Procedure

a. Positive and negative controls are run each time the test is done.

b. Sweep the KNO_3-Zephiran-impregnated swab across several (four or five) colonies. Swirl the swab against the inside wall of a sterile empty tube to ensure good contact of the organisms with the substrate.

c. Incubate the swab in the tube at 45°C for 10 min or at 35°C for 20 to 30 min.

d. Remove the tubes from the incubator; place the swab in a tube containing 2 drops each of nitrate reagents A and B.

e. Observe the swab for the development of a pink to red color, indicating a positive test. A positive test result should be visible within 3 to 5 min.

2. Results

a. Positive test

The development of a pink to red color on the swab indicates that nitrate has been reduced to nitrite.

b. Negative test

No color development on the swab indicates a negative test result. Zinc powder is not added to confirm that the result is a true negative, as residual nitrate is nearly always present.

E. Caffeic acid disk test for *C. neoformans* screen

Caffeic acid and ferric citrate are the substrates for the phenol oxidase enzyme, produced only by *C. neoformans.* This enzymatic reaction produces melanin, which is absorbed by the yeast cell wall, resulting in a brown pigmentation.

IV. PROCEDURES *(continued)*

1. Procedure
   a. Place the disk on the cornmeal agar plate for rehydration. (Do not use a medium containing glucose; it will inhibit the phenoloxidase activity.)
   b. Inoculate the disk with several yeast colonies. A heavy inoculum is critical.
   c. Inoculate the plate for yeast morphology.
   d. Replace the lid, and incubate the plate at 35 to 37°C in ambient air.
   e. Examine at 30-min intervals for up to 4 h for the production of a brown pigment on the disk, indicating a positive test result.
2. Results
   a. The production of a brown pigment after incubation at 35°C indicates phenoloxidase activity in the yeast cell wall.
      (1) If a brown pigment is observed, report "presumptive for *Cryptococcus neoformans*" and proceed to a full yeast identification procedure (*see* procedure 8.8).
      (2) If there is no brown pigment but *C. neoformans* is strongly suspected, proceed with yeast identification workup. Include a birdseed (niger seed or thistle seed) agar plate.
   b. After 48 h, read the plate for yeast morphology and record results.

F. Rapid trehalose test for *C. glabrata*

When heavily inoculated and incubated at 42°C, rapid trehalose assimilation broth detects in 3 h the trehalose assimilation associated with *C. glabrata*. This reaction is used for the presumptive identification of *C. glabrata*. Isolates which are germ tube and pseudohypha negative and are microscopically small (compared to organisms such as *C. albicans*) may be *C. glabrata*, and therefore this rapid test provides presumptive identification. It is important to note that this test should not be used in place of standard methods to detect trehalose assimilation of other yeast species. Other tests which specifically evaluate trehalose assimilation or fermentation and can be used to presumptively identify *C. glabrata* are available (e.g., Hardy Diagnostics Trehalose Fermentation with Durham Tube and Remel Yeast Fermentation Broth with Durham Tube). As with the Remel Rapid Trehalose Assimilation Broth test, incubation occurs at 42°C, as this provides better selectivity for *C. glabrata* versus other species. However, the two fermentation tests require prolonged incubation (24 h) and therefore compromise the ability to provide useful information rapidly to physicians. False-positive results occur with each of the trehalose tests (1), but confusion can be reduced by correlating the trehalose test results with other characteristics of the yeast isolate, as mentioned above (i.e., lack of germ tube formation and small size).

1. Procedure
   a. Allow the number of tubes (stored at 2 to 8°C) needed for testing to come to room temperature.
   b. Prepare a cloudy suspension of the yeast isolate in an appropriately labeled tube of broth.
   c. Incubate the tubes at 42°C.
   d. Monitor the tubes for up to 3 h for a color change to yellow.
      Caution: *Incubation beyond 3 h may result in false-positive results.*
2. Results
   a. Positive test
      A color change to yellow within 3 h of incubation
   b. Negative test
      Blue, blue-green, or green color after 3 h of incubation

VI. PROCEDURES *(continued)*

It is imperative that these cultures be handled in a biological safety cabinet.

G. Serologic identification of *Candida* spp.

Candida spp. produce polysaccharide antigens that are exposed on the cell surface and that vary among serotypes and species. Specific agglutination antisera that have been produced in rabbits and adsorbed to remove nonspecific antibodies can be used to identify serotypes and species of *Candida*. Host immune status appears to be the most critical determinant for disease development and progression. Therefore, only the serologic test for *Candida* spp. is described.

1. Procedure

- **a.** Culture yeast isolate on GYEP (*see* Appendix 8.6–1, item J.3) agar for 48 h at 25°C.
- **b.** With a loop or applicator stick, transfer a small amount of the yeast colony onto the agglutination test tray.
- **c.** Add ~50 μl of specific factor serum or, for negative-control purposes, sterile saline.
- **d.** Roll the test tray to stir the cells and serum together for 1 to 2 min.
- **e.** Agglutination reactions are either negative, weak positive, moderately positive, or strong positive as indicated in the manufacturer's directions.
- **f.** No agglutination should occur with the negative control.
- **g.** Isolates that are positive for only factor sera 1, 4, 5, and 6 are either *C. albicans* serotype A or *C. tropicalis.* These can be differentiated by sucrose fermentation as described by the manufacturer. *C. tropicalis* ferments sucrose, while *C. albicans* serotype A does not.

2. Results

Isolates are identified to the species level based on which factor sera give positive agglutination. Refer to the manufacturer's package insert for reactive patterns.

POSTANALYTICAL CONSIDERATIONS

V. INTERPRETATION

Preformed-enzyme test

A positive test result, i.e., both enzymes present, is presumptive identification of *C. albicans* or *C. dubliniensis.* Rare isolates of *Candida rugosa* and *C. tropicalis* may be positive. Rule out these possibilities by performing a germ tube test.

VI. LIMITATIONS

A. Germ tube test

Sabouraud dextrose agar is the best medium to isolate yeasts for germ tube production; sheep blood agar is an acceptable substitute (my unpublished observations). Some *C. tropicalis* isolates produce pseudohyphae, and some also produce germ tubes that require careful observation to discriminate from those of *C. albicans.* A good rule is to see at least five germ tubes before calling an organism positive, as rare tubes may be produced by other species. Frequently, isolates of *C. albicans* produce colonies described as "yeasts with feet" in recognition of a peripheral fringe. This occurs most commonly on serum-enriched media, such as sheep blood agar. These colonies should not be used for the germ tube test, as cells within the colony have produced pseudohyphae and germ tubes.

B. Preformed-enzyme test

The preformed-enzyme test requires several colonies to produce the needed inoculum paste. A mixed culture could cause a false-negative result. A heavy paste of the organism on the disk is necessary. Insufficient inoculum may result in a negative test result.

VI. LIMITATIONS *(continued)*

C. Nitrate swab rapid test

1. If pink to red yeasts are tested by this method, the upper part of the swab should be observed for the color change (to distinguish it from the yeast inoculum in the lower part of the swab).

2. Inoculate the swab with a heavy inoculum to ensure a clear-cut reaction.

D. Caffeic acid disk test

The test described here is for rapid detection of phenoloxidase activity. If a false-negative result is suspected, the isolate could be subcultured onto agar medium containing caffeic acid or niger seed (thistle seed) extract. Detection of melanin production on such media depends on colony development; thus, the time to a positive result may be 24 to 72 h.

E. Serologic identification of *Candida* spp.

C. dubliniensis and *C. albicans* serotype A cannot be differentiated by the serologic method.

VII. REPORTING

General

An algorithm for reporting germ tube tests is provided in Fig. 8.6–1. If a combination of at least two of the tests described above does not provide a presumptive identification of *C. albicans*, *C. dubliniensis*, *C. glabrata*, or *C. neoformans*, then a full identification should be performed; these procedures are outlined in procedure 8.8.

The tests described in this procedure are designed to provide a presumptive identification, or at least information to guide the choice of subsequent identification tests. If a single presumptive test is used for preliminary reporting purposes, then the report should indicate that the identification is presumptive; for example, "yeast, presumptive *Cryptococcus neoformans*." It may also be useful to provide the basis of the presumptive identification.

Example: Yeast, presumptive *Cryptococcus neoformans* based on capsule production.

Rare isolates of other yeast species may show capsule production.

REFERENCES

1. **Fenn, J. P., E. Billetdeaux, H. Segal, L. Skodack-Jones, P. E. Padilla, M. Bale, and K. Carroll.** 1999. Comparison of four methodologies for rapid and cost-effective identification of *Candida glabrata*. *J. Clin. Microbiol.* **37:**3387–3389.
2. **Freydière, A.-M., R. Guinet, and P. Boiron.** 2001. Yeast identification in the clinical microbiology laboratory: phenotypical methods. *Med. Mycol.* **39:**9–33.
3. **Spicer, A. D., and K. C. Hazen.** 1992. Rapid confirmation of *Candida albicans* identification by combination of two presumptive tests. *Med. Microbiol. Lett.* **1:**284–289.

SUPPLEMENTAL READING

Carr-Scarborough Microbiologicals, Inc. 1998. Package insert no. 1423-1290. Carr-Scarborough Microbiologicals, Inc., Decatur, GA (*C. albicans* screen).

Hopfer, R. L., and D. Gröschel. 1975. Six-hour pigmentation test for the identification of *Cryptococcus neoformans*. *J. Clin. Microbiol.* **2:**96–98.

Iatron Laboratories. Serologic identification of Candida and Cryptococcus. Package insert. Candida Check, RM302-K. Iatron Laboratories, Inc., Tokyo, Japan.

Remel. 1998. Technical information bulletin TI no. 21128. Remel, Lenexa, KS (Caffeic acid screen).

Remel. 1997. Package insert. Rapid trehalose assimilation broth, TI no. 64856. Remel, Lenexa, KS.

APPENDIX 8.6–1

Medium Preparation, Storage, and Sources

A. Sterile calf serum
1. Purchased from BioWhittaker (catalog no. 14-401E)
2. Aliquots of 0.5 ml are stored at −10 to −30°C.
3. The outdate is 6 months from the date the serum is aliquoted.
4. Racks of aliquots in use are stored at 2 to 8°C.

B. Carr-Scarborough (*C. albicans* screen)

C. Murex (CA-50)

D. Differentiation Disks Urea
1. Purchased from Difco (catalog no. DF1625-33)
2. Expiration date established by manufacturer

E. Rapid nitrate swab (0.2% nitrate)
1. Purchased from Remel (catalog no. 20-355). If unavailable, cotton swabs impregnated with KNO_3-Zephiran chloride may be used; however, performance of the test will be less satisfactory than with the commercial swabs. The KNO_3-Zephiran chloride solution is described by Hopkins and Land (1).
2. Stored at 2 to 8°C
3. Expiration date established by manufacturer

F. Nitrate reagent A (0.08% sulfanilic acid in acetic acid)
1. Purchased from Remel (catalog no. 21-239)
2. Stored at 2 to 8°C
3. Expiration date established by manufacturer

G. Nitrate reagent B (0.06% *N*-dimethyl-1-naphthyl amine)
1. Purchased from Remel (catalog no. 21-242)
2. Stored at 2 to 8°C
3. Expiration date established by manufacturer

H. Caffeic acid disk for *C. neoformans* screen
1. Remel caffeic acid disk (catalog no. 21-128)
2. Stored at 2 to 8°C
3. Use manufacturer's expiration date.
4. Do not use if the color has changed. The disks should be white.
5. Remel cornmeal agar plates (catalog no. 01-330). Stored at 2 to 8°C. Use manufacturer's outdate.
6. Remel birdseed agar (catalog no. 01-192). Stored at 2 to 8°C. Use manufacturer's outdate.

I. Rapid trehalose assimilation broth tubes
1. Purchased from Remel (catalog no. 06-4856)
2. Stored in the original container at 2 to 8°C

J. *Candida* check kit
1. Purchased from Iatron Laboratories, Inc., Tokyo, Japan
2. Stored in the original container at 2 to 8°C
3. GYEP agar

glucose	20 g
yeast extract	0.5 g
peptone	10 g
agar	15 g
distilled water	1 liter

Mix, autoclave for sterilization (typically at 121°C for 15 min), and pour plates.

Reference

1. **Hopkins, J. M., and G. A. Land.** 1977. Simple method for determining nitrate utilization by yeasts. *J. Clin. Microbiol.* **5:**497–500.

8.7 Identification of Moulds on Primary Culture

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

A pure culture is a prerequisite for the identification of a fungus. Fungi may be polymorphic, and any visible structures present must be demonstrated to be produced by one fungus, not several. Bacteria may also interfere with the development of key characteristics.

Characteristics of any mould, such as whether the hyphae are septate or aseptate, hyaline or dematiaceous; the color and topography of the colony; and production of reproductive structures, all provide information that helps with the identification of the isolate. Although the structures produced by the sexual state are the predominant features for fungal classification and give the fungus the teleomorph name that takes precedence under the *International Code of Botanical Nomenclature*, they are not usually produced in the diagnostic-mycology laboratory. It is usually the vegetative state of the fungus that is isolated from clinical specimens. Moreover, moulds on primary culture are predominantly heterothallic and are therefore in the anamorph, or asexual, state when encountered in the diagnostic-mycology laboratory. It is the architecture of the spore-bearing apparatus of the asexual state that often provides the most important taxonomic features. Filamentous fungi isolated in the laboratory must be accurately identified, and their significance must be assessed, before being reported to the clinician. The fungi isolated may be regarded as contaminants; however, they must be seen as potential pathogens in the correct patient setting. It is therefore imperative to implement procedures that allow the isolate to be assessed as a potential pathogen and differentiated from a contaminant acquired during collection of the specimen, transport to the laboratory, or processing of the specimen in the laboratory or during incubation.

II. CULTURE EXAMINATION

It is imperative that these cultures be handled in a biological safety cabinet.

The initial growth of some fungi might require up to 4 weeks of incubation. Occasional isolates of dimorphic, systemic pathogens require 5 to 6 weeks. Plates should be examined frequently during the incubation period. Cultures from specimens from deep sites should be examined daily for the first week and twice weekly for the subsequent 3 weeks. If a dimorphic, systemic pathogen is highly suspected, laboratorians may want to consider examining plates daily for 2 weeks. It should be borne in mind that prolonged incubation increases the risk of contamination, and control plates should be incubated concurrently. When growth appears, it should be examined both macroscopically and microscopically; however, the structures required for genus or species identification might not manifest until the organism has been subcultured onto a suitable medium.

■ *All mould cultures with the possible exception of cultures from clear-cut cases of dermatophytosis should be handled in a class II biological safety cabinet! Mounting reagents for microscopy should contain a disinfectant.*

A. Colony characteristics

The most commonly used agar media in the mycology laboratory are Czapek-Dox agar, malt agar, and glucose peptone agar (Sabouraud glucose agar is one variation of glucose peptone agar). However, the original sources of agar, peptone, and other basic ingredients can subtly alter the appearance of some fungi on different media. It is preferable to subculture a referred organism onto an

II. CULTURE EXAMINATION
(continued)

agar with which you have become familiar to demonstrate the culture appearance of the fungus under the conditions of your laboratory. Several features should be taken into account when investigating the gross morphology of the culture. The macromorphology features for consideration and examination are the texture, topography, and color of the surface and reverse of the colony.

1. Colony texture
The range of textures of the colony can be described as fluffy (cotton wool-like), some aerial mycelium (velvety), flat (suede-like), and growing into the agar (submerged hyphae). The description of the topography as smooth, flat, roughened, raised, or folded is worth noting; other attributes which should be considered are the appearance of concentric rings of growth and the delineation of the margins of the colony, e.g., smooth or feathered edges.

2. Incubation temperature
Incubation temperature is crucial for the dimorphic fungi that are in the filamentous phase at 28 to 30°C but demonstrate yeast phase growth at higher temperatures akin to those found in human and animal tissue. Growth temperature can also be an aid to identification in other groups of fungi, for example, the zygomycetes.

3. Growth rate
The rate of growth of a mould may not be reliable on initial culture, but after subculture onto standardized medium, it may be a useful characteristic, if the colony diameter is recorded.

4. Color
The color of the surface of the colony, the uniformity of that color, any color seen on the reverse of the colony, and the presence of diffusible pigments in the medium aid the description of the mould and are useful in the identification of the isolate.

B. Microscopic morphology

The presence of large structures, such as pycnidia, sclerotia or ascocarps, and synnemata (these terms are defined in procedure 8.9), are useful in identification, and their presence can be more easily detected by the use of a low-power dissecting microscope. Also, this microscope can be used to visualize larger structures, such as rhizoids or stolons, to examine the method of spore production, and it is useful for seeing the wetness of the spores (whether they are held in globules of liquid), which can often be difficult to see on microscopical examination and which are too small to be seen by the naked eye.

If fungal isolate from an unknown site or origin is sent to the laboratory, especially from an area of the world where dimorphic fungi are endemic or from a patient who has visited such an area, then the isolate should be handled within the safety cabinet. Such information is typically not known to the laboratorian. Therefore, all moulds should be considered possible dimorphic pathogens until otherwise demonstrated. Mounts and subcultures of such fungi should also be prepared within the safety cabinet. The use of lactophenol as a mountant will kill the fungus and allow the preparations to be handled outside a safety cabinet.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Media
See appendixes in procedures 8.4, 8.7, and 8.9 for medium sources, components, and preparation.

B. Reagents and stains
See the individual procedures and Appendix 8.5–1 for sources and preparation.

C. Other supplies
Slides, coverslips, cellophane tape, laboratory loops, inoculation wire, and mounted needles

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Ensure that all media used are within their expiration dates.

B. Examine all reagents for the presence of contamination or extraneous material.

C. To ensure that media support growth, color development, and fruiting structures, it is worthwhile to test new lots of media with appropriate control organisms or to compare the characteristics of moulds obtained from clinical specimens against those presented in illustrated texts (or guides) such as those of St. Germain and Summerbell (3), Beneke and Rogers (1), and Ellis et al. (2). However, one should be mindful that some variation in mould characteristics from strain to strain and from one lot of medium to another is likely; thus, organisms will not appear exactly as presented in those illustrated texts. There are several websites that are extremely well illustrated and informative, but use caution and check the provenance of the authors of the sites.

V. PROCEDURES

It is imperative that these cultures be handled in a biological safety cabinet.

A. Cellophane tape mount

Most types of transparent sticky tape work for the cellophane tape mount method, but they are not suitable for permanent mounts, as the tape will disintegrate with time. This method usually maintains the original distribution of fungal structures, so it is extremely useful and may eliminate the need to set up slide cultures. However, it is also only suitable for plate cultures. This method should not be used on an original, unrepeatable culture of which there is no duplicate. It is recommended that there be more than one culture, as the tape may contaminate the plate when the preparation is made.

1. A drop of lactophenol cotton blue or lactofuchsin is placed on a clean slide.

2. A short piece of tape is cut, and the middle of gummed side is applied to the surface of the mould using forceps.

3. The loop of tape is then smoothed onto the slide with the area that has been in contact with the mould uppermost. Another drop of mountant is applied.

4. A coverslip is placed over the tape to try to remove any air bubbles. This ensures the mould is viewed not through the tape but through the coverslip.

5. The slide is examined microscopically, with structures such as hyphae, conidia, spores, or fruiting structures being noted.

It is imperative that these cultures be handled in a biological safety cabinet.

B. Tease mount

The tease mount is probably the most common technique used for microscopic examination of fungi; it is a good simple technique but requires practice to obtain optimal results. Do not just drag the wire over the surface, or only spores will be removed. The idea is to see how the spores are attached and any other structures that are present. Poorly sporing isolates may require the preparation of several mounts. Carbol fuchsin or lactofuchsin is preferred to lactophenol cotton blue by some workers. If identification cannot be made from the tease preparation, it might be necessary to use a slide culture.

1. A drop of alcohol is placed on a clean glass slide.

2. Using an inoculation wire, a fragment is taken from the culture approximately midway between the culture edge and center and placed in the alcohol. The fungus is gently teased apart using the wire and a dissecting (mounting) needle, endeavoring not to destroy any fragile structures. The idea is to separate the tangle of hyphae to allow the paggage of light and the sufficient segregation of any characteristic structures to allow them to be studied. The alcohol serves as a wetting agent and helps to drive out the air bubbles.

V. PROCEDURES *(continued)*

3. A drop of lactophenol cotton blue is then placed on the fungus, and a coverslip is added by laying it along the edge of the liquid and lowering it with the tip of the dissecting needle to ensure that there are no air bubbles.
4. After removal of any excess mountant with a tissue, the coverslip can be sealed with a proprietary sealant or nail varnish to preserve the preparation.
5. The sample is examined microscopically, with hyphae, conidia, spores, or fruiting structures being noted.

It is imperative that these cultures be handled in a biological safety cabinet.

C. Slide cultures

The slide culture technique is an invaluable weapon in the diagnostic armory. Its greatest benefit for identification is that it allows the mycologist to examine delicate fungal structures that are often disrupted during the preparation of a cellophane tape mount or a tease mount. The object of the method is to allow the mould to grow out from an agar block onto the glass surface of a slide or coverslip, where the mould features can be viewed with no or minimal disruption. This method gives two slide preparations for examination from one slide culture. On completion, slide culture preparations can be sealed and preserved as a record of the isolate.

◩ **NOTE:** Slide cultures should not be prepared with isolates suspected to be *Blastomyces, Coccidioides, Histoplasma, Paracoccidioides,* or *Cladophialophora (Xylohypha) bantiana,* as the risk of infection is too great. The agar selected should induce maximum spore production in the mould group to which the isolate is thought to belong. Two slide cultures should be prepared to allow examination after different incubation times. They should be prepared in a biological safety cabinet.

1. Slide culture dishes are prepared by placing a cleaned, grease-free glass slide supported by a glass rod bent into a V shape in a glass petri dish. The dishes and contents are then sterilized. Another option is placing previously sterilized glass supports and slides in a plastic petri dish.
2. Using aseptic techniques, a block of agar about 0.5 cm square should be cut from an unused agar culture plate and placed on the slide.
3. A little sterile distilled water is poured into the base of the dish, but not so much as to wet the agar cube; alternatively, a damp piece of sterile filter paper may be used. The water should be topped up regularly to prevent the agar from drying out, but it should not be kept so humid that excessive condensation forms.
4. Inoculate around the edge of the cube with the tip of inoculation wire, cover with a cleaned sterile coverslip, and incubate. The temperature of incubation is selected by the state of the fungus desired and may be isolate dependent. It is imperative that the cube of agar be smaller than the coverslip, as hyphae grow onto the glass. The diagnostic structures are likely to be formed around the edges of the block.
5. Check regularly during incubation, and when growth is visible, carefully remove the coverslip with fine sterile forceps and place a drop of lactophenol cotton blue or chosen mountant on the slide. Invert the coverslip; place onto one edge first and then gently lower using a needle to ensure that air bubbles are driven out. Care should be taken not to disturb the delicate fungal structures. Remove the block from the slide, drop on some lactophenol cotton blue, and then place on a new coverslip.
6. Before examination, wipe the underside and edges of the slide with disinfectant. Note the type of spores and how they are attached and the structure of any spore-bearing bodies.
7. There are variations of the slide culture technique which are also suitable. The important concerns are that there is a glass surface over which the hyphal elements may grow while receiving nutrients from the agar block and that

V. PROCEDURES *(continued)*

It is imperative that these cultures be handled in a biological safety cabinet.

the resulting mould growth on the glass surface can be covered with disinfectant in the mountant, and can be easily viewed by microscopy.

D. Subculture, medium selection, and incubation conditions

If the organism cannot be identified from the primary culture or there is too much contamination for reliable identification, colonies of interest should be subcultured for purity and to induce characteristic features.

1. Subculture

Stab inoculation. Using a bent inoculating wire, remove some of the fungus and stab it into the center of an agar plate. Incubate the plate at the appropriate temperature until there is sufficient growth for identification. If there is a mixture of fungi or contamination with fungi or bacteria, a streak inoculum of the fungal growth will aid separation from the contaminants for subsequent subculture for purification. A dissecting microscope is often useful for this.

2. Medium selection

The standard laboratory media for subcultures are those that have been used for colony descriptions, such as Czapek-Dox medium for *Aspergillus.* Other common laboratory media used by mycologists include malt agar, glucose peptone agar, and potato dextrose agar. In general, moulds should be subcultured onto nutritionally limited medium, such as potato dextrose agar, and onto a rich medium. Common mycological media and media that are particularly useful for specific groups of fungi, such as dermatophytes, black yeasts, coelomycetes, and dematiaceous moulds, are described in procedure 8.9.

There is a vast range of more specialized media developed for particular genera of fungi. Water agar is a minimal medium used to induce sporulation in zygomcetes such as *Apophysomyces* or *Saksenaea.* Dermatophyte test medium is useful in nonspecialized laboratories, as it turns red when a dermatophyte is cultured; lactritmel agar may induce dermatophyte sporulation, and nutrient agar is recommended for the isolation of *Trichophyton verrucosum.* Further details of specialized media are given in procedure 8.9 and its appendixes.

3. Incubation temperatures

Most fungi will grow well if incubated at between 28 and 32°C. However, some species can tolerate higher growth temperatures, and this can be another useful aid to mould identification.

Two subcultures are made from the parent colony, labeled, dated, and incubated, one at 28 to 30°C and one at the higher test temperature. For the test to be valid, there has to be growth of the mould at the lower temperature.

Aspergillus fumigatus is the only *Aspergillus* sp. that will tolerate up to 50°C and will grow well at 45°C; *T. verrucosum* will grow faster at 35 to 37°C than at 28°C, and *Trichophyton mentagrophytes* will tolerate 37°C. Studies of the zygomycete fungi show that *Rhizomucor pusillus* will tolerate 50 to 55°C and that *Rhizopus* spp. will grow at 45°C, whereas *Mucor* (with the exception of *Mucor indicus*) has a maximum growth temperature of 36 to 37°C.

4. Cycloheximide resistance

Cycloheximide resistance can be an indicator for identification, as several fungi, including *Aspergillus* and the zygomycete moulds *Rhizopus, Absidia,* and *Mucor,* are sensitive to it. The inclusion of cycloheximide in agars used to isolate dermatophytes is useful, as most dermatophyte species and some of the dimorphic primary pathogens, *Blastomyces dermatitidis, Coccidioides immitis, Histoplasma capsulatum, Paracoccidioides brasiliensis,* and *Sporothrix schenckii,* are resistant to this antimicrobial agent. There have been

V. PROCEDURES *(continued)*

It is imperative that these cultures be handled in a biological safety cabinet.

occasional problems with the supply of cycloheximide, and alternatives, e.g., primaricin, have not been evaluated with all these genera. More detail can be found in Table 8.9–6.

Inoculate plates of medium with and without cycloheximide and incubate under identical conditions. Commercial plates typically contain 500 µg of cycloheximide/ml. Growth will be roughly equivalent on both plates if the organism is resistant and vastly reduced or lacking on the cycloheximide plate if the organism is sensitive.

E. Molecular methodologies

There has been an explosion in the recent literature of molecular methods published for the identification of unknown moulds. While it is likely that these methods will continue to improve and be introduced more generally, some of the basic mycological principles will still hold true. The interpretation of the significance of isolation will continue to depend on multifactorial mycological judgments. As many of the moulds implicated in human disease are ubiquitous in the environment, the quality and the potential contamination of any kits, reagents, and consumables used in these practices must be rigorously monitored and defined. There is little doubt that the sequencing of some mould isolates will achieve their identification more rapidly than traditional methods and, it is hoped, accelerate the subsequent treatment of patients.

The methods all involve the extraction of the fungal DNA (which is often the most problematic part of the procedures), PCR amplification of the extracted DNA, and then sequencing of the product and comparison to a library. The selection of the search tool used for identification is critical; it is imperative that it be from a reputable, knowledgeable source with robust input.

POSTANALYTICAL CONSIDERATIONS

VI. INTERPRETATION AND REPORTING

A. General considerations

Whether a mould should be identified to the genus or species level depends on the clinical status of the patient and the site from which the organism was isolated. The isolation of moulds from clinical specimens (*see* item VI.B below) should be considered significant unless there is clear evidence that the mould is a contaminant. The most common fungal etiologies from a range of clinical specimens are shown in Fig. 8.3–1. The correlation of any morphological information from the direct microscopic examination of the specimen with the characteristics of the fungus isolated in culture can aid identification and provide an indication of whether the isolate is likely to be a contaminant.

The reporting of any moulds isolated should, when appropriate, reflect the mycologist's opinion as to whether the mould is likely to be of clinical significance. Reports may include wording such as "possible (or probable) contaminant." The quantities of fungus grown may be reported with a comment added regarding the mycologist's interpretation of the results, considering such factors as (i) the sample site and specimen type, (ii) the time in transit from the patient to the laboratory, (iii) the processes performed on the specimen, (iv) any potential for contamination of the sample or the culture medium, and (v) the identification of all fungi isolated.

Fungal hyphae can fragment during some processing procedures, so the fragments do not accurately reflect the organism burden in the specimen. This is often the case in ground tissue samples. Thus, a single colony can, in a particular clinical situation, have as much significance as multiple colonies. Similarly, when multiple plates are inoculated with specimen but only one plate gives rise to a colony, the significance of that one colony is not lessened. Multiple factors,

VI. INTERPRETATION AND REPORTING *(continued)*

such as the specimen sampling error and growth requirements, can influence the isolation of a fungus.

B. Dermatological specimens

Dermatophytes would be reported as significant if grown from hair, skin, or nail specimens. Moreover, other filamentous fungi may be significant if grown from nail specimens that were positive by direct microscopy or if from a patient who is immunosuppressed or immunocompromised or has some other underlying problem. In these instances, the clinician ought to be aware that the presence of a mould such as *Acremonium* or *Fusarium* could be suspicious, and repeat specimens should be taken and evaluated. The isolation of *Scytalidium dimidiatum* from such susceptible patients could be important, as this fungus does not respond well to azole antifungals.

Evaluation of the fungal load recovered from scalp samples taken using soft, single-use toothbrushes or small, circular, soft hairbrushes is potentially useful when screening families with children for whom scalp ringworm is suspected. Children with infection tend to yield significantly greater counts of dermatophytes when the brushes are directly inoculated into the growth medium.

C. Respiratory and sterile body site specimens

Any fungus grown from a sterile site is potentially significant. The type of patient, specimen, result of direct examination, antigen tests (if appropriate), and fungus grown need to be considered, and the possibility of contamination during collection, transport, processing, or incubation must be excluded. The addition of QC uninoculated spacer plates adjacent to specimen culture plates and the establishment of a defined inoculation pattern should help determine the presence of contaminating moulds and therefore the significance of any mould isolations. If the fungus is of dubious importance, repeat specimens should be requested with additional serologic screening when possible.

REFERENCES

1. **Beneke, E. S., and A. L. Rogers.** 1996. *Medical Mycology and Human Mycoses.* Star Publishing Co., Belmont, CA.
2. **Ellis, D., S. Davis, H. Alexiou, R. Handke, and R. Bartley.** 2007. *Descriptions of Medical Fungi*, 2nd ed. Adelaide Medical Centre for Women and Children, North Adelaide, Australia.
3. **St. Germain, G., and R. Summerbell.** 1996. *Identifying Filamentous Fungi.* Star Publishing Co., Belmont, CA.

8.8 Full Identification of Yeasts

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

In an era of increasing resistance of yeast species to antifungal agents and a widening range of species capable of causing diseases previously the domain of *Candida albicans*, there is almost no situation in which identification to species level is not warranted. This is especially true given the growth in the number of immunocompromised patients in our society, which has provided more opportunities for yeast infections to occur and to complicate and prolong the recovery period. Molecular methods to identify yeasts directly in specimens and after growth in culture are under development. Since the last edition of this handbook, peptide nucleic acid fluorescence in situ hybridization (PNA FISH) technology (AdvanDx, Woburn, MA) has been developed, which allows identification of several yeasts directly from blood cultures. However, no commercially available method which identifies a broad range of species has been introduced. In this procedure, methods which take advantage of physiological characteristics of various yeast species are presented. Molecular methods are discussed in general terms.

Carbon and nitrogen source differences are the primary characteristics used to discriminate yeast species, along with cell morphology and sexual reproductive structures when applicable. A variety of semiautomated and automated devices are available to perform carbon and nitrogen assimilation and carbohydrate fermentation tests. However, not all of these provide 100% agreement with the traditional method of Wickerham and Burton (50). In particular, less commonly isolated species tend to show poorer agreement. This problem can be offset by testing a large number of isolates of the unusual species for inclusion in any database information provided by the manufacturer of commercial yeast identification systems. Other commercial systems take advantage of preformed enzymes alone or in conjunction with carbon and nitrogen source assimilation and fermentation. When preformed enzymes are the only basis of the identification system, final results can be obtained in <8 h for many commonly encountered yeasts. The issue of database potency is also a consideration with these enzymatically based systems.

Sexual reproductive structures (e.g., asci and ascospores) may be formed by some yeasts on specialized media. As a result of the evidence of sexual reproduction, yeasts with species epithets in the form-phylum Deuteromycota, which was reserved for organisms for which no sexual form had been identified, were invalid and the organisms were reassigned to one of the other phyla (typically Ascomycota) and taxonomically identified with a new genus-species binomial (teleomorph). While it is technically correct to call such an organism by this new binomial, the invalid asexual (anamorph) taxon is commonly used by physicians and microbiologists. It is unlikely that the anamorph name will disappear from use in the near future, and therefore, we have provided a listing of the anamorph-teleomorph binomials for the most commonly isolated yeasts in the clinical laboratory (Table 8.8–1). Molecular methods have also influenced the taxonomy of asexual yeasts, as it is possible to determine how related a particular species is to species within the sexual phyla. One significant consequence is that *Candida* species lacking a sexual form have been reassigned to the phylum Ascomycota, subphylum Saccharomycotina, class Saccharomycetes, order (mitosporic) Saccharomycetales, family Candidaceae. This allows retention of the *Candida* species epithets without violation of taxonomic rules.

The methods discussed below are designed to provide identification information for most of the commonly encountered pathogenic yeasts (Tables 8.8–2, 8.8–3, 8.8–4, and 8.8–5). The following several points concern yeast identification.

A. Figure 8.5–1 in procedure 8.5 provides an approach based on colony and microscopic characteristics. The initial screening tests are very useful and may suffice for the extent of identification required for a particular specimen and quantity of organisms.

B. A common practice for some specimens is to report a yeast isolate as either "*Candida albicans*" or "not *Candida albicans*" based on a germ tube test. While the germ tube test is an excellent screening test, the identification is presumptive because *Candida dubliniensis* can also form germ tubes and *Candida tropicalis* can produce pseudohyphae that closely resemble germ tubes except for the location of the septum, which can be difficult to see without high magnification. Clinical situations in which this report may be sufficient include the initial occurrence of either oropharyngeal or vaginal candidiasis, sputum isolates when deemed

Table 8.8–1 Anamorph-teleomorph binomials of commonly encountered yeasts[a]

Anamorphic sp. or obsolete name	Previous synonym(s) (alternative species)	Teleomorph(s)
Blastoschizomyces capitatus (Trichosporon capitatum)	*Geotrichum capitatum*	*Clavispora capitatus*
Candida ciferrii		*Stephanoascus ciferrii*
Candida famata	*Torulopsis candida*	*Debaryomyces hansenii*
Candida guilliermondii (two varieties) *(Yamadazyma guillermondii)*		*Pichia guilliermondii, Pichia ohmeri*
Candida kefyr (Candida macedoniensis)	*Candida pseudotropicalis* var. *marxianus*	*Kluyveromyces marxianus*
Candida krusei		*Issatchenkia orientalis*
Candida lipolytica		*Yarrowia lipolytica*
Candida lusitaniae	*Candida obtusa, Candida parapsilosis* var. *obtusa*	*Clavispora lusitaniae*
Candida norvegensis		*Pichia norvegensis*
Candida pelliculosa		*Pichia anomala*
Candida pintolopesii (Candida slooffii)		*Arxiozyma telluris*
Candida pulcherrima		*Metschnikowia pulcherrima*
Candida utilis		*Pichia jadinii*

[a] Modified from reference 19.

clinically significant, and isolates from onychomycosis.

C. A variety of relatively inexpensive commercial systems that provide reliable identification of commonly encountered yeasts are available. These include API 20C, IDS RapID Yeast Plus, Vitek Yeast Biochemical Card, Fongiscreen, API Candida, Auxacolor, Microring YT, Fungichrom *I*, Fungifast *I twin*, Uni-Yeast Tek, ID 32C, and the Rapid Yeast Identification Panel (13). Procedures for supplemental tests that may be required and the organisms for which the supplemental tests are most useful are provided in item II below and in procedure 8.5.

With all commercial systems, strict adherence to the manufacturer's directions is required. Errors, such as overfilling cupules (API 20C) and variations from recommended inoculum concentrations, will cause spurious results.

In general, the following tests should be performed as part of the initial routine identification of a yeast along with the commercial system: urease, morphology agar, and glucose assimilation (pellicle formation can also be assessed with this test).

Tables 8.8–4 and 8.8–5 provide some of the characteristics of various pathogenic yeasts.

II. TESTS USEFUL FOR YEAST IDENTIFICATION FROM PRIMARY OR SECONDARY CULTURES

Supplemental tests can sometimes provide the single result that identifies an organism. Colony color is one indication (*see* procedure 8.5), and in combination with microscopic appearance, an identification can nearly be obtained (*see* Fig. 8.5–2 above). It is recommended that morphology agar or cornmeal agar–2% Tween 80 be set up simultaneously with any commercial identification system. Yeast mor-

Table 8.8–2 General considerations of two commercial yeast identification systems

System	Incubation period (h)	Principle	QC organisms	Comments
API 20C	72	Assimilation	*Cryptococcus laurentii* (ATCC 18803) *B. capitatus* (ATCC 10663)	New formulation requires strict adherence to inoculum preparation.
RapID Yeast Plus	4	Preformed-enzyme detection	*C. kefyr* (ATCC 2512) *C. glabrata* (ATCC 2001)	Incubation temperature must be strictly followed.

Table 8.8–3 Examples of useful supplemental tests for yeasts[a]

Procedure	Yeast(s) giving positive reaction (unless otherwise indicated)[b]
Inositol assimilation	*Cryptococcus* spp.; *Trichosporon*
Glycine assimilation/ canavanine resistance	Serotypes (varieties) of *C. neoformans*
Nitrate assimilation	*C. albidus, Cryptococcus terreus, Pichia anomala, Trichosporon pullulans, Rhodotorula glutinis, Sporobolomyces salmonicolor*
Ascospore agar	*Saccharomyces, Schizosaccharomyces, Pichia*
Rhamnose assimilation	*C. lusitaniae*
Melibiose assimilation	*Candida guilliermondii, Trichosporon* spp.
Lactose assimilation	*C. kefyr, Trichosporon* spp., some non-*C. neoformans* species
Morphology agar	Various species (part of subculture routine)
Glucose fermentation[c]	Most *Candida* spp. (+); *Cryptococcus* species (−); ascomycetous yeasts (generally +); basidiomycetous yeasts (generally −)
Cellobiose fermentation[c]	*C. guilliermondii* (+); *P. anomala* (variable)
Maltose fermentation[c]	*C. albicans* (+); *C. tropicalis* (+); other *Candida* spp. (−)
Urease production	Species of *Trichosporon, Cryptococcus, Rhodotorula, Malassezia* (basidiomycetes) (+); *C. krusei* (variable); *C. lipolytica* (+); other yeasts (−)

[a] Note that some tests are components of commercial systems. They are listed because they are key tests.

[b] +, positive; −, negative.

[c] Fermentation tests require that the yeast first be grown on a sugar-free medium, such as malt extract agar or yeast morphology agar.

II. TESTS USEFUL FOR YEAST IDENTIFICATION FROM PRIMARY OR SECONDARY CULTURES *(continued)*

phology on standard primary media and on morphology agar may, at times, be all that is needed to obtain an identification (27). Cycloheximide sensitivity testing and ascospore formation are reserved for problematic identifications (2). Refer to procedure 8.10 for sensitivity results and to the work of Hazen (19) for ascospore and ascus characteristics of ascomycetous yeasts (e.g., *Saccharomyces cerevisiae*). The cycloheximide sensitivity of these yeasts is discussed in reference 2.

The supplemental tests take advantage of characteristics that are restricted to a limited set of organisms (Table 8.8–3). When these characteristics are present, in the absence of other indicative information, you should consider with some caution that the test organism is part of the limited set. Other tests that should be considered are the screening tests described in procedure 8.6.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Media

Recipes for most media mentioned here are available in reference 1.

1. Nitrate assimilation test; yeast carbon agar, Wickerham card, KNO_3-saturated disks (*see* Appendix 8.8–1)
2. Sporulation (ascospore production); ascospore agar deeps, V-8 juice agar deeps, TB Kinyoun stain kit (*see* Appendix 8.8–1)
3. Carbohydrate fermentation test for yeast identification; yeast fermentation with control, dextrose, cellobiose, maltose, sucrose, distilled water, and Vaspar (*see* Appendix 8.8–1)

◩ **NOTE:** The requirement for Vaspar is questionable. It is included here because it does provide a barrier to oxygen penetration into the fermentation broths.

4. Canavanine-glycine-bromthymol blue (CGB) agar; for media and method of preparation, see Appendix 8.8–1

B. General supplies

Petri dishes, microscope slides, coverslips, inoculating loops, Pasteur pipettes, water bath, microscope, and McFarland standards

Table 8.8–4 Culture and biochemical characteristics of yeasts frequently isolated from clinical specimens[a]

Species	Growth at 37°C	Pellicle in broth	Pseudo- or true hyphae	Chlamydospores	Germ tubes	Capsule, India ink	Assimilation of[b]: Glucose	Maltose	Sucrose	Lactose	Galactose	Melibiose	Cellobiose	Inositol	Xylose	Raffinose	Trehalose	Dulcitol	Fermentation of: Glucose	Maltose	Sucrose	Lactose	Galactose	Trehalose	Urease	KNO_3	Phenol oxidation	Ascospores
C. albicans	+	−	+	+[c]	+	−	+	+	+*	−	+	−	−	−	+	−	+	−	F	F	−	−	F	F	−	−	−	−
Candida catenulata	+*	−	+	−	−	−	+	+	−	−	+	−	−	−	+	−	−	−	F*	−	−	−	−	−	−	−	−	−
C. dubliniensis	+	−	+	+[c]	+	−	+	+	+	−	+	−	−	−	+*	−	+	−	F	F	−	−	F	F	−	−	−	−
Candida famata	+	−	−	−	−	−	+	+	+	+*	+	+	+	−	+	+	+	+*	W	−	W	−	−	W	−	−	−	−*
C. glabrata	+	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	+	−	F	−	−	−	−	F	−	−	−	−
Candida guilliermondii	+	−	+	−	−	−	+	+	+	−	+	+	+	−	+	+	+	+	F	−	F	−	F*	F	−	−	−	−*
Candida inconspicua	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
C. kefyr	+	−	+	−	−	−	+	−	+	+	+	−	+*	−	+*	+	−*	−	F	−	F	F*	F	−	−	−	−	−
Candida krusei[d]	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	F	−	−	−	−	−	+*	−	−	−*
Candida lambica	+*	+	+	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	F	−	−	−	−	−	−	−	−	−*
Candida lipolytica[d]	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−*
C. lusitaniae[e]	+	−	+	−	−	−	+	+	+	−	+	−	+	−	+	−	+	−	F	−	F	−	F	F	−	−	−	−*
Candida norvegensis	+	+	−	+	−	−	+	−	−	−	−	−	+	−	−	−	−	−	F*	−	−	−	−	−	−	−	−	+
C. parapsilosis[f]	+	−	+	−	−	−	+	+	+	−	+	−	−	−	+	−	+	−	F	−	−	−	−	−	−	−	−	−
Candida pintolopesii[g]	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	F	−	−	−	−	−	−	−
Candida rugosa	+	−	+	−	−	−	+	−	−	−	+	−	−	−	+*	−	−	−	−	−	−	−	−	−	−	−	−	−
C. tropicalis[e,f]	+	+	+	−[h]	−	−	+	+	+	−	+	−	+	−	+	−	+	−	F	F	F	−	F*	F*	−	−	−	−
Candida viswanathii	+	−	+	+	−	−	+	+	+	−	+	−	+	−	+	−	+	−	F	F	F*	−	+	+	−	−	−	−
Candida zeylanoides	−	−*	+	−	−	−	+	−	−	−	−*	−	−*	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
C. neoformans	+	−	R	−	−	+	+	+	+	−	+	−	+	+	+	+*	+	+	−	−	−	−	−	−	+	−	+	−
C. albidus	−*	−	−	−	−	+	+	+	+	+*	+*	+	+	+	+	+	+	+*	−	−	−	−	−	−	+	+	−	−
Cryptococcus laurentii	+*	−	−	−	−	+	+	+	+	+	+	+*	+	+	+	+*	+	+	−	−	−	−	−	−	+	−	−	−
Cryptococcus luteolus	−	−	−	−	−	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−	−	−
Cryptococcus terreus	−*	−	−	−	−	+	+	+*	−	+*	+*	−		+	+	+	+	−*	−	−	−	−	−	−	+	+	−	−
Cryptococcus uniguttulatus	−	−	−	−	−	+	+	+	+	−	−*	−	−*	+	+	+*	−*	−	−	−	−	−	−	−	+	−	−	−
Rhodotorula glutinus	+	−	−	−	−	−*	+	+	+	−	+*	−	+	−	+	+	+	−	−	−	−	−	−	−	+	+	−	−
Rhodotorula rubra	+	−	−	−	−	−*	+	+	+	−	+	−	+*	−	+	+	+	−	−	−	−	−	−	−	+	−	−	−
S. cerevisiae	+	−	−*	−	−	−	+	+	+	−	+	−	−	−	−	+	+*	−	F	F	F	−	F	F*	−	−	−	+
Pichia anomala	+*	−	−	−	−	−	+	+	+	−	+	−	+	−	+	−	+	−	F	F*	F	−	F	−	−	+	−	+
Geotrichum candidum[i]	−*	+	+	−	−	−	+	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
B. capitatus	+	+	+	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Prototheca wickerhamii[i]	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−

[a] Modified from Warren and Hazen (48) and Pincus et al. (33). +, positive; −, negative; *, some isolates may give the opposite reaction; R, rare; F, the sugar is fermented (i.e., gas was produced); W, weak fermentation.

[b] +, growth greater than that of the negative control.

[c] *C. albicans* typically produces single and no more than two terminal chlamydospores, while some isolates of *C. dubliniensis* will produce terminal chlamydospore in pairs, triplets, and clusters.

[d] *C. lipolytica* assimilates erythritol; *C. krusei* does not. Maximum growth temperatures, 43 to 45°C for *C. krusei* and 33 to 37°C for *C. lipolytica.*

[e] *C. lusitaniae* assimilates rhamnose; *C. tropicalis* usually does not.

[f] *C. parapsilosis* assimilates L-arabinose; *C. tropicalis* usually does not.

[g] *C. pintolopesii* is a thermophilic yeast capable of growth at 40 to 42°C.

[h] Rare strains of *C. tropicalis* produce teardrop-shaped chlamydospores.

[i] Not yeasts but may be confused with several yeast genera.

Table 8.8–5 Characteristics of selected *Trichosporon* spp.[a]

Characteristic[b]	Value[c]							
	T. asahii	*T. asteroides*	*T. cutaneum*	*T. inkin*	*T. mucoides*	*T. ovoides*	*T. louberi*	*T. mycotoxinivorans*
L-Rhamnose assimilation	+	−	+	−	+	+	V	+
Melibiose assimilation	−	−	+	−	+	−	+	+
Raffinose assimilation	−	−	+	−	+	V	+	+
Ribitol assimilation	V	V	+	−	+	−	?	?
Xylitol assimilation	V	+	+	−	+	V	V	W
L-Arabinitol assimilation	+	+	+	−	+	−	V	W
Galactitol assimilation	−	−	−	−	+	−	−	+
Growth at 37°C	+	V	−	+	+	+	+	+
Urease	+	+	+	+	+	+	+	+
0.01% cycloheximide resistance	+	V	−	V	+	+	+	+
0.1% cycloheximide resistance	−	V	−	−	+	−	+	+
Appresoria[d]	−	−	−	+	−	+	−	−

[a] Modified from Hazen and Howell (20) and Guého et al. (16).

[b] The carbohydrates are available from numerous chemical suppliers, including Sigma Chemical Co. (St. Louis, MO).

[c] +, positive; −, negative; V, strain variation.

[d] Also called an infection peg, which penetrates host tissue (hair).

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Media

Use within the expiration date.

B. Organisms

The following organisms should be used for the test indicated at the same time and in the same manner as the test being performed on the clinical isolate.

1. Nitrate assimilation test

Cryptococcus albidus ATCC 10231 is positive for nitrate utilization and positive for peptone growth, and *C. albicans* ATCC 60193 is negative for nitrate utilization but positive for peptone growth.

2. Sporulation (ascospore production)

S. cerevisiae ATCC 24903 produces ascospores on ascospore agar.

3. Carbohydrate fermentation test for yeast identification

Performed at each receipt of medium and when testing a clinical isolate. We test only glucose and cellobiose fermentations, as they are helpful for organisms (e.g., *Candida lusitaniae*) which have equivocal results with commercial assimilation kits. For glucose assimilation, the QC organisms are *C. lusitaniae* ATCC 66032 (positive) and *C. albicans* ATCC 10231 (negative). For cellobiose fermentation, the QC organisms are *C. albicans* ATCC 10231 (positive) and *Blastoschizomyces capitatus* ATCC 10663 (negative). Other species which are useful for QC of other carbohydrate fermentation broths include *Candida glabrata* ATCC 2001 (trehalose positive) and *Candida kefyr* ATCC 8553 (lactose, sucrose, and galactose positive). *C. albicans* ATCC 10231 can be used for maltose fermentation.

4. CGB agar

Cryptococcus laurentii ATCC 66036 can be used as a positive control.

V. PROCEDURES

A. Carbon assimilation test for yeast identification to species level

Carbon assimilation, in practical terms, is the ability to use a sole carbon substrate for growth; thus, an organism that assimilates a substrate will show growth

V. PROCEDURES *(continued)*

Observe standard precautions.

or increased turbidity. There are several commercial kits which provide standardized carbon assimilation tests. Each kit is combined with a typically extensive database by which to identify an isolate based on the organism's assimilation profile. Examples of such assimilation test systems are the API 20C test (bioMérieux), the Vitek Yeast Biochemical Card (bioMérieux), and the YT MicroPlate (Biolog, Hayward, CA). Table 8.8–2 provides the QC organisms for the API 20C test. Assimilation tests can also be performed using nonautomated methods. These methods are described by Larone (27).

B. Nitrate assimilation test for yeast identification to species level

Yeast carbon agar with the addition of various nitrogen sources tests the ability of yeasts to assimilate nitrogen. Potassium nitrate and peptone are used as the nitrogen test sources. The peptone serves as a positive growth control. The nitrate reductase test *does not* substitute for the nitrate assimilation test.

Procedure

1. Melt two tubes of yeast carbon base medium per isolate or control.
2. In a separate tube of sterile distilled water, make a suspension of the yeast isolate to be identified in which the density is not greater than 1+ with a Wickerham card (line seen clearly but with rough edges).
3. When the tubes of yeast carbon base medium have melted and cooled to 50°C, so as not to kill the yeast, add 0.1 ml of the yeast suspension to each tube of medium.
4. Mix thoroughly but gently, taking care to avoid air bubbles, and pour into the sterile petri dish.
5. When agar has hardened, place a KNO_3 disk and a peptone disk on opposite sides of the plate at least 1 cm from the edge.
6. Incubate the plate with the inoculated surface up at 30°C for 48 to 96 h.

C. Sporulation (ascospore production)

One of the characteristics used to identify most fungi is the method of sporulation and the arrangement and morphology of the spores produced. In some fungi, sexual reproduction produces spores in an ascocarp. This contains small sacs, or asci, which contain ascospores. The medium used to demonstrate the production of ascospores in yeasts depends on which species of yeast is suspected; i.e., *Saccharomyces, Schizosaccharomyces,* and *Candida norvegensis* produce ascospores on acetate (ascospore) agar, but all other yeasts produce ascospores more readily on V-8 juice agar.

Procedure

1. For those yeasts that produce ascospores on ascospore agar
   - **a.** Melt the ascospore agar deep in a boiling water bath and cool to 45 to 50°C.
   - **b.** Mix and pour into a sterile 15- by 100-mm petri dish. Allow the agar to solidify.
   - **c.** Inoculate the medium with the yeast to be identified.
   - **d.** Incubate at 25 to 30°C for 3 to 10 days.
   - **e.** After 3 days of incubation, perform a wet prep to check for ascospores.
   - **f.** If ascospores are seen or suspected, prepare a suspension of the yeast in a drop of water on a microscope slide.
   - **g.** Let the smear dry, heat fix it, and stain it by Kinyoun's acid-fast staining method (*see* procedure 7.2).
   - **h.** Examine microscopically for acid-fast (red) ascospores.
   - **i.** If no ascospores are seen, reincubate and reexamine daily for 7 more days. If the control *(S. cerevisiae)* is positive and the test is negative at the end of 7 days, the result is considered negative.
2. For those yeasts that produce ascospores on V-8 juice agar
   - **a.** Melt the V-8 juice agar deep in a boiling water bath and cool to 45 to 50°C.

V. PROCEDURES *(continued)*

b. Mix and pour into a sterile 15- by 100-mm petri dish. Allow the agar to solidify.

c. Follow the procedure above for yeasts that produce ascospores on ascospore agar.

D. Carbohydrate fermentation test for yeast identification

Fermentation reactions are rarely used to identify yeasts because of the incubation period required and because assimilation reactions can identify most species isolated in the clinical laboratory. However, fermentation tests have their value when other tests cannot resolve an identification. A positive fermentation reaction is indicated by the presence of gas (bubbles) in the inverted Durham tube, *not* by a change in the bromthymol blue indicator.

Procedure

1. Prepare a diluted suspension of a pure, 24- to 48-h-old yeast culture in distilled water equal to a no. 1 McFarland standard. Select control organisms that demonstrate a positive and negative test for each carbohydrate tested.
2. Add a single drop of this suspension to each tube of yeast fermentation broth.
3. Add a layer of molten Vaspar directly onto the top of the broth.
4. Incubate at *25 to 30°C* and shake daily. Higher temperatures lead to breakdown of disaccharides.
5. Read the tubes every 2 to 3 days for the first week and then weekly up to 24 days.

It is imperative that these cultures be handled in a biological safety cabinet.

E. CGB agar for differentiation of the three varieties of *Cryptococcus neoformans* (var. *grubyi* [serotype A], var. *neoformans* [serotype D], and var. *gattii* [serotypes B and C])

In some current literature, serotypes A and D are referred to as *C. neoformans*, while serotypes B and C are designated the distinct species epithet *Cryptococcus gattii.* In other literature, serotype A is designated its own species epithet, *Cryptococcus grubii.*

CGB agar contains canavanine, which inhibits the growth of serotypes A and D and provides presumptive differentiation of the varieties of *C. neoformans.* In addition, serotypes B and C are able to assimilate glycine as a carbon source and will turn the medium cobalt blue (25). When growth of serotypes A and D does occur, it is typically weak, and there is no change in the medium color. This medium has relatively high sensitivity and specificity (38). The need to differentiate the varieties for the purpose of medical management is debatable, although *C. neoformans* var. *gattii* can cause central nervous system disease in immunocompetent individuals and may cause more severe disease than *C. neoformans* in immunocompromised patients. Knowledge of the varieties causing cases of cryptococcosis in a geographic area may be useful epidemiologically. If the specific serotype of a *C. neoformans* isolate is critical, then molecular methods are recommended. In the previous edition of this handbook, a serologic method was suggested (Crypto Check Kit [Iatron Laboratories]); however, this product is no longer marketed by the manufacturer.

Procedure

1. Streak yeast inoculum onto medium. The medium should be greenish yellow. Use a heavy inoculum.
2. Incubate at 25 to 30°C.
3. At 5 days, evaluate plate growth. A positive test result is a color change to blue and indicates that the yeast is the variety *gattii.* If a heavy inoculum is used, typically only 48 h is needed to obtain the color change.

F. Other methods for yeast identification

There are a variety of commercial systems for rapid (<8 h) and nonrapid identification of yeasts. Some of these systems are automated. The systems are reviewed by Freydiere et al. (13) and by Wolk and Roberts (53) and are not described here.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING AND INTERPRETATION

A. Nitrate assimilation test for yeast identification

1. Positive growth control (peptone-inoculated portion)
   - **a.** All yeasts utilize peptone as a source of nitrogen—if this portion of the plate has *no* growth after 48 to 72 h, the test is invalid.
   - **b.** If growth is present, proceed to examine the KNO_3 area.
2. Positive test result
   - **a.** Growth control positive
   - **b.** Growth also present on KNO_3 portion of plate
3. Negative test result
   - **a.** Growth control positive
   - **b.** No growth on KNO_3 portion of plate

B. Sporulation (ascospore production)

1. Positive result: acid-fast (red) ascospores are seen among the counterstained yeast cells
2. Negative result: no ascospores are seen

C. Carbohydrate fermentation tests

Positive fermentation is indicated by turbidity and accumulation of gas (CO_2) in the Durham tube or underneath the Vaspar seal. The bromthymol blue indicator will turn yellow due to carbohydrate assimilation. (All fermented carbohydrates will be assimilated, but not all assimilated carbohydrates will be fermented.) Fermentation reactions for some *Candida* spp. are given in Table 8.8–6.

VII. LIMITATIONS

For carbohydrate fermentation tests, a false-positive reaction may occur due to endogenous carbohydrates present in the yeast cells if a heavy inoculum is used.

VIII. MOLECULAR METHODS FOR THE IDENTIFICATION OF YEASTS

At present, yeasts are not routinely identified using nucleic acid technology; however, a number of different methods have been developed for use in research, in the development of diagnostic methods, and for epidemiological investigations. The literature contains frequent references to sequencing technology and its utility for species identification. Indeed, sequencing rRNA genes has differentiated closely

Table 8.8–6 Fermentation reactions for some Candida spp.[a]

Species	Fermentation reaction[b]			
	Glucose	Maltose	Cellobiose	Sucrose
C. albicans	+	+	−	−
C. dubliniensis	+	+	?	−
C. guilliermondii	+	−	−	+(w)
C. krusei	+	−	o	−
C. lusitaniae	+	−	+	V
C. parapsilosis	+	−	−	−
C. tropicalis	+	+	−	+(v)

[a] Note that *C. guilliermondii* and *C. parapsilosis* produce distinctively different morphologies on morphology media. *C. albicans* and *C. dubliniensis* are physiologically very similar. The difficulty in distinguishing these organisms is that no single differential test (e.g., growth at 45°C, color formation on CHROMagar, or assimilation profile) is definitive. However, several (at least three) differential tests in combination provide sufficient information to identify an organism as one or the other species. Otherwise, molecular techniques should be employed to distinguish the species.

[b] −, negative; +, positive; o, not tested routinely; v, rare negatives; V, strain variation; w, weak.

VIII. MOLECULAR METHODS FOR THE IDENTIFICATION OF YEASTS *(continued)*

related organisms, such as *Candida dubliniensis, C. albicans,* and *Candida stellatoidea* (44) and the *Malassezia* spp. (17, 21, 41–43). However, the equipment required for sequencing is not widely available to routine microbiology laboratories, so a variety of genetic recognition methods have been developed that utilize generally available equipment. These methods include restriction enzyme analysis (REA), pulsed-field gel electrophoresis (PFGE), probes, and applications of PCR. As there are no routine procedures to follow, descriptions of some of the available techniques and their applications are provided below.

REA or restriction fragment length polymorphism (RFLP) has been used for species and strain recognition, with the results dependent on the restriction enzyme used. The method requires extraction of DNA, followed by digestion with a suitable restriction enzyme and electrophoresis to separate the DNA fragments. The fragment band pattern is then compared for similarity to reference strains or other isolates of the same species. The enzymes EcoRI (31) and HinfI (14) have been used for a variety of *Candida* spp., and species-specific patterns have been described. These enzymes have been used for strain typing, but the discrimination of strains differs for each species; isolates of *C. albicans* are easily discriminated, but isolates of *Candida parapsilosis* show few differences.

The DNA pattern from restriction digests can be transferred to membranes by Southern blotting and hybridized to DNA probes. Probes have been designed that are species specific and do not hybridize to DNA from unrelated species: for example, 27A (36) and Ca3 (37) for *C. albicans,* Ct3 for *C. tropicalis* (24), Cg6 and Cg12 for *C. glabrata* (30), and Cp3-13 for *C. parapsilosis* (12). Variations within the hybridization patterns of such species-specific probes have been used to differentiate strains and in some cases have been used to demonstrate minor genetic changes within a single strain over a period of time, a process referred to as microevolution.

PFGE is a technique used to separate large fragments of DNA and whole yeast chromosomes which do not resolve using conventional gel electrophoresis. The method of extraction is unlike other DNA methods, as yeast cells are exposed to cell wall-digestive enzymes and lysed while immobilized inside agarose blocks. This ensures that chromosomes remain intact and are not fragmented by mechanical processes, such as pipetting or vortexing. The electrophoretic chromosome profiles, or karyotypes, can be used for identification, especially for species that exhibit little or no variation in the karyotype pattern between isolates. *Malassezia* species are not easily differentiated phenotypically (Table 8.8–7) but have unique karyotypes and demonstrate virtually no strain variation (3, 22), whereas there is sufficient variation to distinguish between isolates of other species, such as *C. albicans* and *C. glabrata* (8, 10).

PCR is becoming a powerful tool for species identification, as it requires less time than the other methods and can be performed on samples containing very small amounts of DNA. PCR amplifies specific sections of DNA, which are determined by the choice of primers, and the amplified products are separated by electrophoresis. Primers have been designed that recognize sequences common to all fungi, such as the internal transcribed spacer (ITS) and nuclear small sequences in the ribosomal DNA regions (49). Amplification using these primers produces DNA that can be used for identification by comparing the size of the amplicon and by its restriction digestion patterns (15, 51). Another approach has used the M13 minisatellite sequence as a primer, which produced characteristic band patterns for each species and has even been used to identify anamorph and teleomorph pairs (28). Using a repetitive extragenic palindromic sequence has led to the development of an automated tool for identification of *Candida* species (52). Random amplification of polymorphic DNA using single oligomer primers and low annealing temperatures has generated fingerprints characteristic of species (46) and has facilitated strain

Table 8.8–7 Key characteristics to differentiate *Malassezia* species

Species	Growth on Sabouraud dextrose agar[a]	Catalase	Assimilation of Tween[b]:		
			20	40	80
M. pachydermatis	+	−/w[c]	−	+	+
M. restricta	−	−	−	−	−
M. sympodialis	−	+	−[d]	+	+
M. furfur	−	+	+	+	+
M. slooffiae	−	+	+	+	−/w
M. globosa[e]	−	+	−	−	−
M. obtusa[e]	−	+	−	−	−
M. dermatis	−	+	+	+	+
M. japonica	−	+	−	+/−	−
M. nana	−	+	+/−	+	+/−
M. yamatoensis	−	+	+	+	+

[a] All species except *M. pachydermatis* require lipid supplementation for growth. +, positive; −, negative.

[b] This test is performed as described in references 16 and 17.

[c] w, weak. This test is performed by placing a drop of H_2O_2 (10%) either directly on the plate culture or onto a smear of the culture on a glass slide (17, 18).

[d] Growth of *M. sympodialis* is inhibited by high concentrations of Tween 20. At lower concentrations, the organism exhibits Tween 20 assimilation (17, 18).

[e] *M. globosa* and *M. obtusa* can be distinguished by their morphologies. *M. globosa* is globose, whereas *M. obtusa* produces long, cylindrical cells.

VIII. MOLECULAR METHODS FOR THE IDENTIFICATION OF YEASTS *(continued)*

typing of many organisms. However, reproducibility problems, particularly for strain typing, make interlaboratory comparisons of fingerprint patterns difficult (40). Species-specific primers have been developed for yeast identification and typing (6) and have been used in combination with universal primers for the detection of yeast DNA in mixed cultures (32) and from clinical specimens (4). DNA amplified using universal primers has been hybridized with species-specific probes to detect *Candida* spp. in blood samples (11, 39, 47) and has detected *C. albicans* in ocular samples (23). Yeasts have been detected in infected blood and CSF samples using primers for the ERG11 gene and hybridization to species-specific probes (34).

Amplification of the ITS2 region, combined with precise determination of amplicon length and sequence polymorphism, distinguished 34 species of yeasts, some of which differed by <2 bp in length (7). However, other studies have found that some unrelated organisms had very similar fragment lengths (9, 26). Amplification and sequencing of the D1/D2 region of the large ribosomal subunit identified over 45 yeast species (29). Sequence analysis of the D1-D2 and ITS1 genes and several other genes confirmed the three major genotypes detected in *C. parapsilosis* by RFLP, karyotyping, and DNA reassociation studies (35) and led to the naming of groups II and III as *Candida orthopsilosis* and *Candida metapsilosis*, respectively (45). The results of these studies also suggested that *C. parapsilosis* might have evolved from *C. orthopsilosis*, as the former species demonstrated virtually no sequence variation, in contrast to the high levels of variation seen in *C. orthopsilosis* sequences. A new technique using microarrays with probes complementary to the ITS1 and ITS2 regions and an APEX detection system also distinguished *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* among 24 other fungal pathogens, including 10 other *Candida* species (5).

DNA sequence databases are well established, and the application of automated technologies is becoming more commonplace. Identification of fungal pathogens directly from clinical material is still mainly a research-based process and requires further development before it will be part of the routine diagnostic laboratory.

REFERENCES

1. **Atlas, R. M.** 1993. *Handbook of Microbiological Media*. CRC Press, Boca Raton, FL.
2. **Barnett, J. A., R. W. Payne, and D. Yarrow.** 2000. *Yeasts: Characteristics and Identification*, 3rd ed. Cambridge University Press, Cambridge, United Kingdom.
3. **Boekhout, T., and R. W. Bosboom.** 1994. Karyotyping of *Malassezia* yeasts: taxonomic and epidemiological implications. *Syst. Appl. Microbiol.* **17:**146–153.
4. **Burgener-Kairuz, P., J.-P. Zuber, P. Jaunin, T. G. Buchman, J. Bille, and M. Rossier.** 1994. Rapid detection and identification of *Candida albicans* and *Torulopsis (Candida) glabrata* in clinical specimens by species-specific nested PCR amplification of a cytochrome P-450 lanosterol-α-demethylase (L1A1) gene fragment. *J. Clin. Microbiol.* **32:**1902–1907.
5. **Campa, D., A. Tavanti, F. Gemignani, C. S. Mogavero, I. Bellini, F. Bottari, R. Barale, S. Landi, and S. Senesi.** 2008. DNA microarray based on arrayed-primer extension technique for identification of pathogenic fungi responsible for invasive and superficial mycoses. *J. Clin. Microbiol.* **46:**909–915.
6. **Carlotti, A., F. Chaib, A. Couble, N. Bourgeois, V. Blanchard, and J. Villard.** 1997. Rapid identification and fingerprinting of *Candida krusei* by PCR-based amplification of the species-specific repetitive polymorphic sequence CKRS-1. *J. Clin. Microbiol.* **35:**1337–1343.
7. **Chen, Y. C., J. D. Eisner, M. M. Kattar, S. L. Rassoulian-Barrett, K. LaFe, S. L. Yarfitz, A. P. Limaye, and B. T. Cookson.** 2000. Identification of medically important yeasts using PCR-based detection of DNA sequence polymorphisms in the internal transcribed spacer 2 region of the rRNA genes. *J. Clin. Microbiol.* **38:**2302–2310.
8. **Clemons, K. V., F. Feroze, K. Holmberg, and D. A. Stevens.** 1997. Comparative analysis of genetic variability among *Candida albicans* isolates from different geographic locales by three genotypic methods. *J. Clin. Microbiol.* **35:**1332–1336.
9. **De Baere, T., G. Claeys, D. Swinne, G. Verschraegen, A. Muylaert, C. Massonet, and M. Vaneechoutte.** 2002. Identification of cultured isolates of clinically important yeast species using fluorescent fragment length analysis of the amplified internally transcribed rRNA spacer 2 region (ITS2). *BMC Microbiol.* **2:**21.
10. **Defontaine, A., M. Coarer, and J. P. Bouchara.** 1996. Contribution of various techniques of molecular analysis to strain identification of *Candida glabrata*. *Microbiol. Ecol. Health Dis.* **9:**27–33.
11. **Elie, C. M., T. J. Lott, E. Reiss, and C. J. Morrison.** 1998. Rapid identification of *Candida* species with species-specific DNA probes. *J. Clin. Microbiol.* **36:**3260–3265.
12. **Enger, L., S. Joly, C. Pujol, P. Simonson, M. A. Pfaller, and D. A. Soll.** 2001. Cloning and characterization of a complex DNA fingerprinting probe for *Candida parapsilosis*. *J. Clin. Microbiol.* **39:**658–669.
13. **Freydiere, A.-M., R. Guinet, and P. Boiron.** 2001. Yeast identification in the clniical microbiology laboratory: phenotypical methods. *Med. Mycol.* **39:**9–33.
14. **Fujita, S.-I., and T. Hashimoto.** 2000. DNA fingerprinting patterns of *Candida* species using *Hin*fI endonuclease. *Int. J. Syst. Evol. Microbiol.* **50:**1381–1389.
15. **Gaitanis, G., A. Velegraki, E. Frangoulis, A. Mitroussia, A. Tsigonia, A. Tzimogianni, A. Katsambas, and N. J. Legakis.** 2002. Identification of *Malassezia* species from patient skin scales by PCR-RFLP. *Clin. Microbiol. Infect.* **8:**162–173.
16. **Guého, E., L. Improvisi, G. S. de Hoog, and B. Dupont.** 1994. Trichosporon on humans: a practical account. *Mycoses* **37:**3–10.
17. **Gueho, E., G. Midgley, and J. Guillot.** 1996. The genus *Malassezia* with description of four new species. *Antonie van Leeuwenhoek* **69:**337–355.
18. **Guillot, J., E. Guého, M. Lesourd, G. Midgley, G. Chévrier, and B. Dupont.** 1996. Identification of *Malassezia* species. *J. Mycol. Med.* **6:**103–110.
19. **Hazen, K. C.** 1995. New and emerging yeast pathogens. *Clin. Microbiol. Rev.* **8:**462–478.
20. **Hazen, K. C., and S. A. Howell.** 2003. *Candida, Cryptococcus*, and other yeasts of medical importance, p. 1693–1711. *In* P. R. Murray, E. J. Baron, K. Chapin, M. A. Pfaller, and F. C. Tenover (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, DC.
21. **Hirai, A., R. Kano, K. Makimura, E. R. Duarte, J. S. Hamdan, M. A. Lachance, H. Yamaguchi, and A. Hasegawa.** 2004. *Malassezia nana* sp. nov., a novel lipid-dependent yeast species isolated from animals. *Int. J. Syst. Evol. Microbiol.* **54:**623–627.
22. **Howell, S. A., C. Quin, and G. Midgley.** 1993. Karyotypes of oval cell forms of *Malassezia furfur*. *Mycoses* **36:**263–266.
23. **Jaeger, E. M. E., N. M. Carrol, S. Choudhury, A. A. S. Dunlop, H. M. A. Towler, M. M. Matheson, P. Adamson, N. Okhravi, and S. Lightman.** 2000. Rapid detection and identification of *Candida, Aspergillus*, and *Fusarium* species in ocular samples using nested PCR. *J. Clin. Microbiol.* **38:**2902–2908.
24. **Joly, S., C. Pujol, K. Schröppel, and D. R. Soll.** 1996. Development of two species-specific fingerprinting probes for broad computer-assisted epidemiological studies of *Candida tropicalis J. Clin. Microbiol.* **34:**3063–3071.
25. **Kwon-Chung, K. J., I. Polacheck, and J. E. Bennett.** 1982. Improved diagnostic medium for separation of *Cryptococcus neoformans* var. *neoformans* (serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (serotypes B and C). *J. Clin. Microbiol.* **15:**535–537.
26. **Landlinger, C., L. Baskova, S. Preuner, B. Willinger, V. Buchta, and T. Lion.** 23 December 2008. Identification of fungal species by fragment length analysis of the internally transcribed spacer 2 region. *Eur. J. Clin. Microbiol. Infect. Dis.* doi: 10.1007/s10096-008-0683-3.

REFERENCES *(continued)*

27. **Larone, D. H.** 2002. *Medically Important Fungi: a Guide to Identification*, 4th ed. ASM Press, Washington, DC.
28. **Latouche, G. N., H.-M. Daniel, O. C. Lee, T. G. Mitchell, T. C. Sorrell, and W. Meyer.** 1997. Comparison of use of phenotypic and genotypic characteristics for identification of species of the anamorph genus *Candida* and related teleomorph yeast species. *J. Clin. Microbiol.* **35:**3171–3180.
29. **Linton, C. J., A. M. Borman, G. Cheung, A. D. Holmes, A. Szekely, M. D. Palmer, P. D. Bridge, C. K. Campbell, and E. M. Johnson.** 2007. Molecular identification of unusual pathogenic yeast isolates by large ribosomal subunit gene sequencing: 2 years of experience at the United Kingdom Mycology Reference Laboratory. *J. Clin. Microbiol.* **45:**1152–1158.
30. **Lockhart, S. R., S. Joly, C. Pujol, J. D. Sobel, M. A. Pfaller, and D. R. Soll.** 1997. Development and verification of fingerprinting probes for *Candida glabrata. Microbiology* **143:**3733–3746.
31. **Magee, B. B., T. M. D'Souza, and P. T. Magee.** 1987. Strain and species identification by restriction fragment length polymorphisms in the ribosomal DNA repeat of *Candida* species. *J. Bacteriol.* **169:**1639–1643.
32. **Mannarelli, B. M., and C. P. Kurtzman.** 1998. Rapid identification of *Candida albicans* and other human pathogenic yeasts by using short oligonucleotides in a PCR. *J. Clin. Microbiol.* **36:**1634–1641.
33. **Pincus, D. H., D. C. Coleman, W. R. Pruitt, A. A. Padhye, I. F. Salkin, M. Geimer, A. Bassel, D. J. Sullivan, M. Clarke, and V. Hearn.** 1999. Rapid identification of *Candida dubliniensis* with commercial yeast identification systems. *J. Clin. Microbiol.* **37:**3533–3539.
34. **Posteraro, B., M. Sanguinetti, L. Masucci, L. Romano, G. Morace, and G. Fadda.** 2000. Reverse cross hybridization assay for rapid detection of PCR-amplified DNA from *Candida* species, *Cryptococcus neoformans*, and *Saccharomyces cerevisiae* in clinical samples. *J. Clin. Microbiol.* **38:**1609–1614.
35. **Roy, B., and S. A. Meyer.** 1998. Confirmation of the distinct genotype groups within the form species *Candida parapsilosis. J. Clin. Microbiol.* **36:**216–218.
36. **Scherer, S., and D. A. Stevens.** 1988. A Candida albicans dispersed, repeated gene family and its epidemiologic applications. *Proc. Natl. Acad. Sci. USA* **85:**1452–1456.
37. **Schmid, J., E. Voss, and D. R. Soll.** 1990. Computer-assisted methods for assessing strain relatedness in *Candida albicans* by fingerprinting with the moderately repetitive sequence Ca3. *J. Clin. Microbiol.* **28:**1236–1243.
38. **Shadomy, H. J., S. Wood-Helie, S. Shadomy, W. E. Dismukes, R. Y. Chau, and the NIAID Mycoses Study Group.** 1987. Biochemical serogrouping of clinical isolates of *Cryptococcus neoformans*. *Diagn. Microbiol. Infect. Dis.* **6:**131–138.
39. **Shin, J. H., F. S. Nolte, and C. J. Morrison.** 1997. Rapid identification of *Candida* species in blood cultures by a clinically useful PCR method. *J. Clin. Microbiol.* **35:**1454–1459.
40. **Soll, D. A.** 2000. The ins and outs of DNA fingerprinting the infectious fungi. *Clin. Microbiol. Rev.* **13:**332–370.
41. **Sugita, T., M. Tajima, M. Takashima, M. Amaya, M. Saito, R. Tsuboi, and A. Nishikawa.** 2004. A new yeast, *Malassezia yamatoensis*, isolated from a patient with seborrheic dermatitis, and its distribution in patients and healthy subjects. *Microbiol. Immunol.* **48:**579–583.
42. **Sugita, T., M. Takashima, M. Kodama, R. Tsuboi, and A. Nishikawa.** 2003. Description of a new yeast species, *Malassezia japonica*, and its detection in patients with atopic dermatitis and healthy subjects. *J. Clin. Microbiol.* **41:**4695–4699.
43. **Sugita, T., M. Takashima, T. Shinoda, H. Suto, T. Unno, R. Tsuboi, H. Ogawa, and A. Nishikawa.** 2002. New yeast species, *Malassezia dermatis*, isolated from patients with atopic dermatitis. *J. Clin. Microbiol.* **40:**1363–1367.
44. **Sullivan, D. J., T. J. Westerneng, K. A. Haynes, D. E. Bennett, and D. C. Coleman.** 1995. *Candida dubliniensis* sp. nov.: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. *Microbiology* **141:**1507–1521.
45. **Tavanti, A., A. D. Davidson, N. A. Gow, M. C. J. Maiden, and F. C. Odds.** 2005. *Candida orthopsilosis* and *Candida metapsilosis* spp. nov. to replace *Candida parapsilosis* groups II and III. *J. Clin. Microbiol.* **43:**284–292.
46. **Thanos, M., G. Schönian, W. Meyer, C. Schweynoch, Y. Gräser, T. G. Mitchell, W. Presber, and H.-J. Tietz.** 1996. Rapid identification of *Candida* species by DNA fingerprinting with PCR. *J. Clin. Microbiol.* **34:**615–621.
47. **Wahyuningshi, R., H.-J. Freisleben, H.-G. Sonntag, and P. Schnitzler.** 2000. Simple and rapid detection of *Candida albicans* DNA in serum by PCR for diagnosis of invasive candidiasis. *J. Clin. Microbiol.* **38:**3016–3021.
48. **Warren, N. G., and K. C. Hazen.** 1999. *Candida, Cryptococcus*, and other yeasts of medical importance, p. 1184–1199. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 7th ed. ASM Press, Washington, DC.
49. **White, T. C., T. Bruns, S. Lee, and J. Taylor.** 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315–322. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. C. White (ed.), *PCR Protocols. A Guide to Methods and Applications.* Academic Press, San Diego, CA.
50. **Wickerham, L. J., and K. A. Burton.** 1948. Carbon assimilation tests for the classification of yeasts. *J. Bacteriol.* **56:**363–371.
51. **Williams, D. W., M. J. Wilson, M. A. O. Lewis, and A. J. C. Potts.** 1995. Identification of *Candida* species by PCR and restriction fragment length polymorphism analysis of intergenic spacer regions of ribosomal DNA. *J. Clin. Microbiol.* **33:**2476–2479.

REFERENCES *(continued)*

52. **Wise, M. G., M. Healy, K. Reece, R. Smith, D. Walton, W. Dutch, A. Renwick, J. Huong, S. Young, J. Tarrand, and D. P. Kontoyiannis.** 2007. Species identification and strain differentiation of clinical *Candida* isolates using the DiversiLab system of automated repetitive sequence-based PCR. *J. Med. Microbiol.* **56:**778–787.

53. **Wolk, D. M., and G. D. Roberts.** 2002. Commercial methods for identification and susceptibility testing of fungi, p. 225–255. *In* A. L. Truant (ed.), *Manual of Commercial Methods in Clinical Microbiology*. ASM Press, Washington, DC.

SUPPLEMENTAL READING

Remel. 1993. Technical information, ascospore agar. TI no. 9046-A. Remel, Lenexa, KS.

APPENDIX 8.8–1

Medium Preparation and Sources

A. Morphology agar

1. Available commercially as a dehydrated powder from Remel (catalog no. 459962)
2. An alternative to morphology agar is to use cornmeal-Tween 80 with or without trypan blue (available as prepared plates from Remel [catalog no. 01328 and 01330, respectively]).
3. If not available commercially, cornmeal-Tween 80 can be prepared by the following recipe.

cornmeal	40.0 g
agar	20.0 g
Tween 80 (polysorbate 80)	10 ml
distilled water	1 liter

Mix cornmeal with 500 ml of water and heat for 1 h at 65°C. Filter through cheesecloth to clarify solution and add sufficient water to bring volume back to original. Adjust to pH 6.6 to 6.8. Dissolve agar in 500 ml of water by heating and add to cornmeal solution. Add Tween 80 and autoclave (121°C; 15 min). Dispense into petri dishes.

B. Nitrate assimilation test for yeast identification to species level

1. Yeast carbon agar
   a. Purchased commercially from Remel (catalog no. 09-984)
   b. Stored at 2 to 8°C
2. Disks impregnated with saturated KNO_3 or peptone solutions
3. Wickerham card—this can be made by drawing three parallel black lines 0.75 mm thick and 5 mm apart
4. If yeast carbon agar is not available commercially, use the recipe provided by Atlas (1).

C. Sporulation (ascospore production)

1. Ascospore (also called acetate ascospore) agar deeps
   a. Purchased from Remel (catalog no. 09-046)
   b. Stored at 2 to 8°C
   c. If not available commercially, use the following recipe.

potassium acetate	5 g
yeast extract	1.25 g
glucose	0.5 g
agar	15.0 g
distilled water	500 ml

Dissolve by boiling for 1 min and dispense into tubes. Autoclave (121°C; 15 min), and allow tubes to cool in slanted position.

2. V-8 juice agar deeps
   a. Purchased from Smith River Biologicals (catalog no. 24V8)
   b. Stored at 2 to 8°C

APPENDIX 8.8–1 *(continued)*

c. If not available commercially, use the following recipe.

V-8 juice	500 ml
dry baker's yeast	10.0 g
agar	20.0 g
distilled water	500 ml

Dissolve agar by boiling it in water. Mix juice and dry yeast; adjust to pH 6.8 with 20% KOH and then add to dissolved agar solution and mix well. Dispense into tubes. Autoclave (121°C; 15 min), and allow tubes to cool in slanted position.

3. TB Kinyoun stain kit
   a. Purchased from BBL (catalog no. 12318)
   b. Contents of kit
      (1) TB Kinyoun stain carbolfuchsin
      (2) TB Kinyoun stain decolorizer
      (3) TB Kinyoun stain brilliant green
   c. Stored at room temperature

D. Carbohydrate fermentation test for yeast identification

1. Yeast fermentation broth
   a. Purchased commercially from Remel (with control, 06-5402; dextrose, 06-5314; cellobiose, 06-5310; maltose, 06-5330; sucrose, 06-5462). Alternative source is PML Microbiologicals (control, T-79-00; glucose, T-79-02; maltose, T-79-08; cellobiose, not available; sucrose, T-79-10).
   b. Stored at 2 to 8°C
2. Sterile distilled water
   a. Stored at 2 to 8°C
   b. Expiration date is 3 months from preparation.
3. No. 1 McFarland standard
   a. Purchased from Remel (catalog no. 20-351)
   b. Stored at room temperature
4. Vaspar (1 part white petrolatum and 2 parts paraffin tissue embedding medium)
   a. Expiration date is 1 year after preparation.
   b. Stored at room temperature

E. CGB agar for differentiation of the three varieties of *Cryptococcus neoformans* (var. *grubii* [serotype A], var. *neoformans* [serotype D], and var. *gattii* [serotypes B and C])

1. Agar

agar	20 g

2. Solution A

glycine	10 g
KH_2PO_4	1 g
$MgSO_4$	1 g
thiamine-HCl	1 mg
L-canavanine sulfate	30 mg
distilled water	100 ml

Adjust to pH 5.6 and filter sterilize.

3. Solution B

sodium bromthymol blue	0.4 g
distilled water	100 ml

Mix 880 ml of distilled water, 20 ml of solution B, and 20 g of agar. Autoclave (121°C; 15 min), and cool to 50°C. Add 100 ml of solution A. Mix thoroughly and dispense into petri dishes (15 to 20 ml/plate).

Reference

1. **Snyder, J. W., and R. H. Atlas.** 2006. *Handbook of Media for Clinical Microbiology*, 2nd ed. CRC Press, Boca Raton, FL.

8.9 Mould Identification

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

"Mould" is an informal term signifying a fungus growing mostly or entirely in the form of diffuse filaments and usually producing an asexual reproductive state or a sexual state that is not a large, complex fruiting body. Most such fungi are recognized as asexual states or asexual species (the former have known sexual states, while the latter do not) related to various fungi in the phyla Ascomycota and Basidiomycota. In addition, members of the phylum Zygomycota generally grow as moulds.

Current conventions for naming moulds depend on which group they come from. Members of the Zygomycota, in which the asexual state is dominant, possess only a single name per biological species. Members of the Ascomycota and Basidiomycota, however, may or may not produce complex sexual states that, in premolecular times, were the best sources of information for systematic classification. Therefore, many species had one nonsystematic "form" or "anamorph" name for the asexual state as it was commonly seen in the laboratory and another, fully systematic "teleomorph" or "holomorph" name for the sexual state, if it could be produced in the laboratory or found in the field. Sometimes these names were unknowingly coined independently and were not connected until years later. In addition, a few species had more than one anamorph name for radically different growth forms that were difficult to connect as being part of the same species—this convention is known as the "synanamorph name." As molecular biology proceeds, the anamorph and synanamorph names could in theory be eliminated. They are, however, almost universally the best-known and most widely used names and also connect to common disease names, such as coccidioidomycosis, histoplasmosis, and aspergillosis. Nomenclatural stability is preferred whenever possible by everyone working with fungi, and therefore the anamorph names have been retained. Higher-level taxonomic nomenclature is likely to change as a result of phylogenetic studies. For example, it has been proposed that the medically important species of zygomycetes be classified into the subphylum Mucormycotina (9). Current phylogenetic classifications can be found on GenBank at http://www.ncbi.nlm.nih.gov/taxonomy.

Although molecular and biochemical characters more and more facilitate identification of some moulds, it remains difficult in the "real world" to compete with morphological identification. It may require knowledge, but it is generally inexpensive and rapid. The more the laboratorian knows, the more these advantages can be maximized. Characteristics learned from cultures can sometimes even be recognized in direct specimen microscopy or histopathology (e.g., *Scedosporium* conidia in sinus wall scrapings), making possible the medical laboratory ideal of a 2-min preliminary identification directing optimal therapy, with more time-consuming techniques directed toward confirmation.

Morphological classification schemes for moulds of ascomycetous and basidiomycetous affinities emphasize conidial ontogeny, that is, the process by which asexual propagules form. Conidial ontogeny provides a grounding in very stable characters that can then allow consideration of more variable characters, such as colony color and conidial septation. In some fungi in which conidia are similar among different species or are lacking or slow to form, supplementary characteristics, such as temperature tolerance, cycloheximide resistance, conversion in vitro to a host-adapted particulate state (e.g., yeast, spherule, or fission yeast), nutritional requirements, and enzymatic activities, may be used. In a few cases, immunological or nucleic acid-based confirmatory techniques are needed.

This procedure first surveys the types of characteristics that are examined in the different fungal groups. Then, using the terminology from this survey where necessary, procedures are outlined. Some extra terminology usually applied only to dermatophytes is mentioned in a separate item.

II. MOULD MICROMORPHOLOGY

Techniques for making microscopic mounts and observing mould structures are dealt with in procedure 8.7. The information below applies to the structures seen.

A. General mould micromorphological characteristics

A few general structural names are applied to morphologies seen across a wide range of moulds (Table 8.9–1); a few terms are seen mainly in mycological specialist literature and need not be learned unless such literature is approached.

B. Mould micromorphologies found in specific groups

1. Zygomycota

Most Zygomycota (Mucormycotina [9]) seen in the clinical laboratory are members of the order Mucorales. In culture, these fungi are rapidly growing, with woolly, brownish-gray colonies often over 1 cm deep. Less common Zygomycota that can be isolated from serious infections include members of the order Entomophthorales. These produce filaments that, while initially coenocytic, become septate much more rapidly than those of the Mucorales. Importantly, therapy of entomophthoromycoses may be very different from that of mucormycoses and other fungal diseases, making rapid identification valuable.

The morphological characteristics of these two orders are described and examples are given in Table 8.9–2 and Fig. 8.9–A1 and 8.9–A2 in Appendix 8.9–1.

2. Ascomycota

Ascomycota make up most of the mould fungi of the medical laboratory, including all the dimorphic fungi, the dermatophytes, and the common opportunistic moulds producing normally septate filaments in tissue. Their colonies include all colors, growth rates, and textures. One distinctive charac-

Table 8.9–1 Definitions of terms used to describe morphological structures

Morphological characteristic	Definition
Ameroconidia	Single-celled conidia
Chlamydospores (resting cells)	Differentiated, swollen cells that remain attached to the mycelium on which they were formed; not disseminated (Note: occurrence of "spore" in "chlamydospore" is an anachronistic holdover that cannot be rationalized with modern structural terminology, but "chlamydospore" is still in use.)
Conidia (singular, conidium)	Propagules formed asexually by breaking off of cells or morphologically differentiated branches
Dictyoconidia (or muriform conidia)	Conidia divided in more than one plane by septa and thus appearing to be composed of squarish or polygonal cells
Didymoconidia	Conidia with a single cross wall dividing them into two cells
Hyphae (singular, hypha)	Fungal filaments
Mycelium	An aggregation or mass of hyphae (Note: singular word)
Phragmoconidia	Conidia with two or more parallel septa in a linear series
Sclerotia (singular, sclerotium)	Large multicelled resting structures formed on colony surface as round, rarely tubular, masses of compacted cells
Septa (singular, septum)	Cross walls dividing hyphae
Spores	Sexually formed propagules or propagules formed by free cell formation

Table 8.9–2 Phenotypic characteristics useful for identification of Zygomycota[a]

Characteristic	Definition	Identification aid
Aseptate coenocytic filaments; >4 μm wide; often undulate	No cross walls; one extended, multinucleate, tubular cell; large diameter; often wavy outline	Mucoralean zygomycetes visible by direct microscopy of specimen and culture. Note that adventitious septa may be present.
Adventitious septa	With age, parts of coenocytic filaments die and are cut off from living portions by cross walls, the adventitious septa.	These septa are highly irregular in spacing and may be found in older hyphae connected to tracts of younger, aseptate hyphae.
Sporangiophores (Fig. 8.9–A1)	Tall upright branches that end in a sporangium	Useful to distinguish fungi such as *Rhizopus* and *Absidia*[b] as members of the Mucorales in culture.
Sporangium	Sac-like inflated apex of a sporangiophore in which a mass of sporangiospores is produced	Seen in *Rhizopus, Mucor,* and *Absidia*[b]
Sporangiospores	One-celled spores found inside a sporangium	
Columella	Apex of sporangiophore forms an empty, swollen structure that supports the sporangium	
Collarette	The collarette is the remnant of the sporangial wall attached to the columella; it is seen when the spore mass is removed.	
Apophysis (Fig. 8.9–A1)	The sporangiophore has a funnel-shaped apex supporting the columella, beneath the collarette.	*Absidia*[b] has typical funnel- or trumpet-bell-shaped apophysis. The apophysis of *Apophysomyces elegans* is vase shaped.
Rhizoids (Fig. 8.9–A2)	Tuft of root-like structures that act as counterbalance to clusters of sporangiophores	*Rhizopus*—sporangiophores unbranched in tufts joined at base with rigid, thick-walled, often brown rhizoids located opposite (i.e., nodal) sporangial tufts *Rhizomucor*—multiply branched sporangiophores; rudimentary rhizoids *Absidia*[b]—some rhizoids produced when long aerial hyphae touch agar surface; not formed near sporangiophores; often not seen in medically important *A. corymbifera*[b]
Vesicle	Empty bulbous structure at apex of sporangiophore gives rise to one-celled sporangiospores on individual short pegs	*Cunninghamella*—swollen vesicle If spores are knocked off, vesicle appears spiny due to stubs of spore pegs.
Merosporangia	Vesicles that produce cylindrical finger-like sacs containing spores in a linear arrangement	*Syncephalastrum racemosum* merosporangia have 3 to 18 spores.
Primary spores	Large (~40-μm diameter) spores formed on short stalks and mechanically discharged by the sudden popping out of a papilla	Seen in *Conidiobolus* (Zygomycota,[a] order Entomophthorales) (Note: other Entomophthorales may not have these structures.)
Papilla	Small mammary shape at base of the primary *Conidiobolus* spore	
Secondary microspore	Discharged primary *Conidiobolus* spores may produce a crown-like apical zone of short pegs bearing secondary microspores at their tips.	
Zygospores	As seen in *Basidiobolus,* thick-walled spores found in subsurface mycelium. These are sexual spores with beak-like structure composed of two cells fused side by side.	Different types of zygospores are formed by other zygomycetes, but except in *Basidiobolus,* these structures are seldom seen in the clinical laboratory.

[a] In 2007, Hibbett et al. (8) revised the Zygomycota, and the medically important species were placed in the Mucormycotina, order Mucorales, and *Conidiobolus ranaram* was placed in the Entomophthoromycotina, order Entomophthorales.

[b] In 2007, Hoffman et al. (9) revised the genus *Absidia,* and the medically important species, including *A. corymbifera,* have been grouped into a new genus, *Mycocladus.*

II. MOULD MICROMORPHOLOGY *(continued)*

teristic, melanization, resulting in strongly olive-gray to black colonies, is found in at least some members of three major groups, the Pyrenomycetes, Discomycetes, and Loculoascomycetes. Simply structured asexual stages of such melanized fungi were previously referred to as the form family Dematiaceae (hence the description of pigmented moulds as dematiaceous).

II. MOULD MICROMORPHOLOGY *(continued)*

Simply structured asexual states lacking such dark pigmentation were placed in the form family Moniliaceae. Asexual states with complex, flask- or blister-shaped fruiting structures were referred to as the class Coelomycetes. Finally, asexual states with no reproduction in culture were called Mycelia sterilia. The well-known names Dematiaceae, Moniliaceae, Coelomycetes, and Mycelia sterilia have been dropped from formal use but remain in informal use. At the family level and above, asexual states are now said to belong to the holomorphic groups to which they have shown genetic affinity, e.g., *Aspergillus* is considered to be a eurotiaceous fungus (related to the family Eurotiaceae), not a member of the Moniliaceae.

Both asexual and sexual states may be seen in ascomycetous fungi, but asexual states are more common and are usually quicker to form. The basis of conidial ontogeny (i.e., mode of production), rather than other characteristics, such as color and shape, is used for identification (Table 8.9–3 and Fig. 8.9–A3 to 8.9–A12 in Appendix 8.9–1).

3. Basidiomycota

Most clinically significant Basidiomycota are yeasts, not moulds. Members of the basidiomycetous yeast genus *Trichosporon* may be wrinkled-velvety in texture and look like arthroconidium-producing moulds, but they can ordinarily be found to also produce at least a few hyaline budding cells, identifying them (somewhat arbitrarily) as yeasts. Most other basidiomycetes of the clinical laboratory are seen as fast-growing, cottony moulds that are among the most common contaminants of certain types of specimens, especially respiratory secretions. They are very susceptible to cycloheximide but will grow on media like plain blood agar intended to isolate cycloheximide-sensitive systemic pathogens, such as the basidiomycetous yeast *Cryptococcus neoformans.* About half the isolates seen produce copious arthroconidia (usually simple, but sometimes alternate), while the other half remain sterile (nonreproductive). In nature, these isolates would ordinarily mate with others of the same species and produce a conspicuous sexual fruiting body (basidiome), such as a mushroom or wood decay conk (defined as the visible fruiting body of a bracket fungus). Those with arthroconidia are morphologically recognizable, especially when their cycloheximide intolerance has been ascertained. The others may be confirmed as probable basidiomycetes by their good growth on agar amended with 10 μg of benomyl/ml (22).

On rare occasions, basidiomycetous moulds cause infections. The most commonly significant organism is the split-gill mushroom *Schizophyllum commune,* which regularly causes chronic mycotic sinusitis and rarely other infections. It may occur as an unmated, haploid form with ordinary septate filaments and sterile, cottony cultures, but more commonly it occurs as a mated, dikaryotic form (that is, containing pairs of nuclei with one member of each pair derived from each of the two mating parents) whose hyphae, at each septum, bear a characteristic side loop called a clamp connection (Fig. 8.9–A13 in Appendix 8.9–1) looping laterally from the end of one adjacent cell to the beginning of the next. These connecting loops, derived from the complex cell division typical of dikaryotic hyphae, can be seen in histopathology and in sterile cultures, allowing instant identification of the organism as a basidiomycete. After 14 or more days in culture, most dikaryotic *S. commune* isolates form mushrooms in vitro, and in some cases the continuation of the sexual cycle may be followed. The mushroom gills give rise to a layer of club-shaped sexual cells called basidia (Fig. 8.9–A14 in Appendix 8.9–1), and nuclear fusion and meiosis take place in each one. Then, the four nuclear products of this reduction-division are incorporated into basidiospores attached by small outgrowths at the apex of each basidium. Each basidiospore is then forcibly shot into the air.

Table 8.9–3 Description of terms for conidial ontogeny and reproductive structures of the Ascomycota

Characteristic	Definition	Example(s) of organism(s) identified
Thallic	Simplest mode of conidiation. Filament differentiates so that it separates into individual cells or produces specialized short side branches that break away.	
Blastic	Conidiation begins at a focal site. An initially thin-walled, rounded, or tubular daughter cell begins to extend by a budding-like process out of a mother cell. It then differentiates into its characteristic form as it matures and is cut off by a septum so that it can break away.	
Arthroconidia	Example of thallic conidiation (*see* next two entries)	
Simple arthroconidia (Fig. 8.9–A3)	Produced when every cell of a fragmenting filament becomes a separate conidium	*Geotrichum, Scytalidium*
Alternate arthroconidia (Fig. 8.9–A4)	Nutrient-filled, healthy conidia are linked on either side by dead, empty disjunctor cells that autodigest and break up.	*Coccidioides immitis, Malbranchea*
Aleurioconidia	Second form of thallic conidiation; specialized side branches break off	
Micro(aleurio)conidia (Fig. 8.9–A5, right)	Small, single-celled side branches with bulbous tips break off.	Dermatophytes, mould phase of *Histoplasma capsulatum*
Macro(aleurio)conidia (Fig. 8.9–A5, left)	Large, sometimes multicelled, more strongly inflated and differentiated side branches that break off	Dermatophytes, mould phase of *H. capsulatum*
Spherule	Unique form of thallic conidiation; swollen, large spherical structure that divides internally into endoconidia, which fragment apart at maturity	Found in particulate host phase of *C. immitis*
Sclerotic fission cells	Rounded single cells split by arthroconidium-like fission into two daughter cells	*Fonsecaea pedrosoi* and related agents of chromoblastomycosis
Blastoconidia; yeast budding (Fig. 8.9–A6)	Simplest form of blastic conidiation; yeast buds usually single celled; blastoconidia from filaments may be larger and more specialized	Black yeasts, e.g., *Exophiala;* yeast states of dimorphic fungi, e.g., *H. capsulatum* *Cladosporium* and *Cladophialophora* produce bud-like conidia that initially fail to separate from each other, giving rise to upright conidial chains.
Conidiophore	Specialized fertile filament	
Sympodial proliferation	Conidiophore produces a budded conidium, elongates, and produces another conidium, etc. Each elongation is called a proliferation. In sympodial structures, proliferation often occurs at a Y-shaped angle to the conidium just produced.	
Rosette (Fig. 8.9–A7)	The proliferations of sympodial conidiophores are short, and when conidia remain attached, the whole structure resembles a flower.	*Sporothrix schenckii*
Rhachis	Longer extensions between conidial proliferations produce a zigzag structure.	*Beauveria bassiana*
Poroconidia	Conidia formed from a conidiophore, usually a rhachis, that shows a conspicuous, small, dark, ring-shaped scar at each place where a conidium formed and broke away	*Curvularia, Alternaria*
Collarette	Collar-like remnant of cell wall around the tip of a conidiophore producing phialo- or annelloconidia	
Enteroblastic	Successive conidia bud out from within the collarette as extensions of the inner cell wall.	
Phialide (Fig. 8.9–A8)	Simplest type of conidiogenous cell that produces enteroblastic conidia	Bottle-shaped phialides—*Phialophora, Aspergillus.* Needle-shaped phialides—*Acremonium*
Uniseriate (Fig. 8.9–A9)	In *Aspergillus,* phialides attached to a swollen vesicle located at apex of a conidiophore	*A. fumigatus*
Biseriate (Fig. 8.9–A9)	*Aspergillus* vesicle forms short branches (metulae), which bear tufts of phialides.	*A. terreus*

Table 8.9–3 *(continued)*

Characteristic	Definition	Example(s) of organism(s) identified
Annellide (Fig. 8.9–A10)	Each time a conidium is produced, an additional collarette or swelling is formed on the apex of the phialide.	*Scopulariopsis, Scedosporium, Exophiala*
Synnema	Formed where several hyphae bundle together and produce a tall, heavily stalked composite conidiogenous structure	*Graphium* synanamorph of *Pseudallescheria boydii*
Sporodochium	Hyphal bundles resemble cushion-like structures bearing masses of conidia.	*Fusarium* spp.
Pycnidium (Fig. 8.9–A11)	Flask-like structure that has differentiated opening (ostiole) and that contains conidia produced on conidiogenous cells	
Ascomata (Fig. 8.9–A12)	Sexual structure of an ascomycetous fungus; in the type most common in medically important fungi, the cleistothecium, a hollow ball or flask-like structure, produces single-celled sacs (asci) in the interior. Each ascus produces a set number of ascospores, 4 or 8, or, rarely, 16 or 32. The outer wall of the cleistothecium is called the peridium.	Ascal structure helps to differentiate ascomata from asexual pycnidia.
Homothallism	Condition in which a single isolate goes through a sexual cycle without mating; this is seen as formation of teleomorphs (sexual states) in single strains.	Some *Aspergillus* spp., *P. boydii*
Heterothallism	Sexual cycle that results from mating of two strains of opposite mating types	Some dermatophytes, *H. capsulatum*
Cleistothecium	Asci formed within a closed ball-shaped ascoma that must crack to release ascospores	*Emericella nidulans,* teleomorph of *Aspergillus nidulans*
Evanescent	Walls of asci dissolve shortly after ascospore formation.	*Pseudallescheria*
Perithecium	An ostiole develops at the apex of a short protuberant neck of the ascoma.	*Chaetomium*
Hülle cells	Ascomata surrounded by a layer of round cells with heavily thickened walls	*Emericella*
Setae	Stiff, sharp, elongated spines on the ascomata	*Arthroderma* has helical setae, or "spirals," also found in conidium-producing tufts of *Trichophyton mentagrophytes*

III. DIMORPHIC FUNGI

Dimorphic fungi are thermal or thermal-nutritional dimorphic systemic pathogens that convert from a mould phase in ordinary culture or the environment to a budding yeast phase in the host or under special growth conditions at 37°C. Examples include *Histoplasma capsulatum, Blastomyces dermatitidis, Paracoccidioides brasiliensis,* and *Sporothrix schenckii.* Certain dimorphic fungi produce thallic rather than yeast-like particulate phases, e.g., *Coccidioides immitis,* which produces spherules, and *Penicillium marneffei,* which produces elongated arthroconidia that fragment into daughter arthroconidia.

Once an isolate is suspected of being a dimorphic pathogen, based on colony and microscopic morphologies, it is essential to confirm its identity. Conversion to the particulate phase in vitro can be demonstrated for *B. dermatitidis* on specialized media (*see* item VII.C.3 below) within 48 to 72 h, although a type from sub-Saharan Africa may take several weeks to convert. *H. capsulatum* and *P. brasiliensis* also require specialized media for conversion, although the latter can also convert on BHI-blood agar. The medium of choice for *P. marneffei* is Sabouraud dextrose agar, and for *S. schenckii* it is BHI agar with overlaid liquid 10% yeast extract. Conversion of *C. immitis* is not reliable as a diagnostic test, even on specialized media; therefore, alternative confirmatory tests are required.

III. DIMORPHIC FUNGI *(continued)*

Nucleic acid probes and exoantigen tests are available for the identification of *B. dermatitidis, H. capsulatum,* and *C. immitis,* but not for *P. marneffei* or *S. schenckii.* Exoantigen testing is an alternative for identification but always requires time for subcultures to grow. These molecular tests are subject to a proportion of false results; therefore, the strict use of controls is required to minimize erroneous reporting.

IV. MATERIALS

Include QC information on reagent container and in QC records.

In order to segregate preanalytical, analytical, and postanalytical considerations, the present procedure is structured to divide individual processes into up to five separate items. These are materials, given in item IV; QC, given in item V; identification techniques, given in item VII (for most moulds) or VIII (for dermatophytes); reporting and interpretation, given in item IX; and media and reagents, given in Appendix 8.9–2. Generally, to obtain complete information on a process, the notes for the process must be followed through these items. Connecting links are written into the text to facilitate this.

A. Fungal sporulation media for best visualization of reproductive structures in moulds
Several types of media are available for sporulation or conidiation of a wide range of fungi (Table 8.9–4). Some commonly used types in clinical mycology are listed below. See Appendix 8.9–2 for sources and recipes.

B. General supplies
1. Incubators at 35, 37, 40, 42, 45, 50, and 55°C (for temperature testing as outlined in item VII.B for species listed in Table 8.9–5)
2. Pipettes (variable volume)
3. Needles and loops
4. Slides and coverslips
5. Petri dishes
6. Glass tubes for media

C. Nucleic acid probe
1. AccuProbe kit
2. Luminometer
3. Sonicator
4. Heating block
5. Water bath
6. Vortex
7. Glass rods

See items V.B and VII.C.2 for QC and procedure and supplier information, respectively.

D. Conversion of dimorphic fungi
For specialized growth media needed, see sources and recipes in Appendix 8.9–2, as well as the interconnected items on QC (V.D) and procedure (VII.C.3).

E. Exoantigen test
Antiserum, with test antibody and control antigens, is available as kits, and the immunodiffusion agar plates are available commercially (see Appendix 8.9–2 for sources and recipes). Antigens are extracted using Merthiolate and are concentrated with an Amicon Minicon macrosolute B-15 concentrator. More details, including supplier information, are given in the procedure for this method outlined in item VII.D below. A moist chamber or plastic bag is needed for the immunodiffusion plates.

F. Dermatophyte identification
Media include Sabouraud dermatophyte decontamination broth, *Trichophyton* agars, Christensen's urea agar and broth, bromcresol purple (BCP) milk glucose agar, 10% yeast extract broth, and cornmeal glucose agar. See Appendix 8.9–2 for sources and recipes and item VIII below for procedures.

Table 8.9–4 Common media used for moulds

Medium	Fungus
Potato glucose agar	Most fungi
Leonian's agar	Most fungi, especially ascomycetous moulds, basidiomycetes, and *Microsporum canis*
Potato flake agar	Most fungi; especially useful for dermatophytes
Cereal agar	Dermatophytes, to reduce pleomorphic degeneration
Tap water agar	*Alternaria, Bipolaris, Exserohilum* (order Pleosporales), or similar isolates with fast-growing, nonsporulating, dark mycelia

Table 8.9–5 Temperature tests commonly used in identification of clinically important moulds

Mould	Temp (°C) (characteristic)[a]	Status
Zygomycota (Mucormycotina)		
Absidia corymbifera	37 (+)	Opportunistic
Other *Absidia* spp.	37 (−)	Nonpathogenic
Cunninghamella bertholletiae	45 (+)	Opportunistic
Cunninghamella elegans	37 (−)	Nonpathogenic
Mortierella wolfii	45 (+)	Opportunistic
Other *Mortierella* spp.	45 (−)	*M. polycephala* rarely opportunistic; others nonpathogenic
Mucor circinelloides	37 (+/w/−)	Opportunistic when thermotolerant
Mucor ramosissimus	37 (−)	Reported as opportunistic (maximum growth temp, 36°C)
Most other *Mucor* spp.	37 (−)	Nonpathogenic
Rhizomucor miehei	55 (+)	Rarely opportunistic
Rhizomucor pusillus	50 (+); 55 (+/w/−)	Opportunistic
Rhizomucor microsporus var. *microsporus*	45 (+); 50 (−)	Opportunistic
Rhizomucor microsporus var. *rhizopodiformis*	45 (+); 50 (+/w); 55 (−)	Opportunistic
Rhizopus oryzae	37 (+); 40 (+); 45 (w/−); 50 (−)	Opportunistic
Rhizopus stolonifer	37 (−)	Nonpathogenic
Ascomycota		
Eurotiales		
Aspergillus fumigatus	42 (+); 45 (+ [rarely −])	Opportunistic
A. flavus	45 (+)	Opportunistic
A. terreus	45 (+)	Opportunistic
A. nidulans	45 (+)	Opportunistic
A. versicolor	37 (+); 45 (−)	Normally nonpathogenic; opportunistic in nails
A. sydowii	37 (+); 45 (−)	Normally nonpathogenic; opportunistic in nails
Paecilomyces variotii	40 (+); 50 (+ or −)	Opportunistic
Penicillium chrysogenum	37 (+); 40 (−)	Rarely opportunistic
Penicillium citrinum	37 (+); 40 (−)	Rarely opportunistic
Penicillium decumbens	37 (+); 40 (−)	Rarely opportunistic
Most other *Penicillium* spp. in subgenera *Penicillium, Furcatum, Aspergilloides*	37 (−)	Nonpathogenic
P. marneffei	37 (+) (converts to fission cells on SAB)	Pathogenic
P. purpurogenum	37 (+) (mycelium only); 45 (−)	Rarely opportunistic
Other *Penicillium* spp. in subgenus *Biverticillium* (including red-pigmented species and *Talaromyces* anamorphs)	37 (+ or −); 45 (+ or −); 55 (+ or −)	Rarely opportunistic when thermotolerant (e.g., *P. piceum, P. verruculosum*)
Onygenales		
Trichophyton gloriae	37 (+)	Nonpathogenic; rare
T. mentagrophytes	37 (+)	Pathogenic
T. rubrum	37 (+)	Pathogenic
T. terrestre	37 (−)	Nonpathogenic; common
T. tonsurans	37 (+)	Pathogenic
Chrysosporium georgiae	37 (+ or −)	Nonpathogenic; uncommon
Chrysosporium state of *Arthroderma cuniculi*	37 (−)	Nonpathogenic; uncommon
Mycosphaerellaceae		
Cladosporium cladosporioides	35 (−)	Rarely reported as opportunistic; even case isolates do not grow at 37°C
Cladosporium herbarum	35 (−)	Nonpathogenic; occasional case reports dubious

(continued)

Table 8.9–5 Temperature tests commonly used in identification of clinically important moulds *(continued)*

Mould	Temp (°C) (characteristic)[a]	Status
Herpotrichiellaceae		
Cladophialophora arxii	40 (+)	Opportunistic (rare)
Cladophialophora bantiana	40 (+)	Opportunistic
Cladophialophora boppii	37 (+); 40 (−)	Opportunistic (rare)
Cladophialophora carrionii	37 (+); 40 (−)	Opportunistic
Cladophialophora devriesii	37 (+); 40 (−)	Opportunistic (rare)
Cladophialophora emmonsii	37 (+); 40 (+ or −)	Opportunistic
Exophiala (Wangiella) dermatitidis	40 (+)	Opportunistic
Exophiala jeanselmei	37 (+); 40 (−)	Opportunistic
Exophiala mesophila	35 (−)	Nonpathogenic
Exophiala moniliae	30 (−) (former type strain)	Reported as opportunistic, but probably misidentified
Exophiala spinifera	37 (+); 40 (−)	Opportunistic

[a] +, positive; −, negative; w, weak; SAB, Sabouraud agar.

ANALYTICAL CONSIDERATIONS

V. QUALITY CONTROL

◩ **NOTE:** QC strains are listed for the tests below. Strains recommended by manufacturers of commercial products have been tested specifically and verified as reliable. On the other hand, "possible QC strains" listed for the remaining tests are known to be typical representatives of the species in question, but in most cases their performance in the specific test mentioned has not to my knowledge been validated in the published literature. Exceptions for some very limited applications may be found in NCCLS document M22-A2 (16). Expected results for a number of additional strains are listed by Sewell (19), who also states that "any strain that yields identical results is acceptable" for QC. In general, however, the strains listed here should be used with caution until validation studies have been done to ascertain the reliability of their QC application and the durability of this status under various conditions. It must be understood that dermatophyte and dimorphic fungal colonies stored as living cultures, whether refrigerated or kept at room temperature, may degenerate slowly or rapidly over time. It is best to make subcultures of QC strains immediately upon receipt and to store these subcultures in a −20 to −80°C freezer or, where possible, to make lyophils or liquid nitrogen storage preparations. New subcultures can then be made from the frozen or lyophilized material on a regular basis or at least when the living strain appears to have degenerated, that is, to have lost its original morphological or pigment features. The actual functional shelf lives of metabolically active cultures are not known for any of the strains cited below. There are as yet many unsolved problems in applying QC techniques to mycological testing. At present, pending formal studies of individual QC strains, a fresh isolate shown in local preliminary tests to give good performance may be substituted for the strains listed below, except for strains recommended by manufacturers. This is because undegenerated fresh isolates may be more reliably typical in such tests than any stored isolate.

Include QC information on reagent container and in QC records.

A. Media

1. Always use commercial media within their expiration dates.
2. Ensure that the media are performing as expected by testing each batch with an isolate of known morphology.
   - **a.** Potato glucose agar (PGA)—St. Germain and Summerbell (21) suggest verifying yellow pigment production in a typical *Microsporum canis* isolate. Heavy production of macroconidia should also be seen. Possible QC

V. QUALITY CONTROL
(continued)

strains: CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) 496.86, ATCC (American Type Culture Collection, Manassas, VA) 36299, and UAMH (University of Alberta Microfungus Collection and Herbarium, Edmonton, Canada) 4174. For specific storage considerations involving the QC strain, see Appendix 8.9–2. There is no negative control for this medium.

b. Leonian's agar—use a typical *M. canis* isolate, as for PGA.

◩ **NOTE:** Dermatophyte pigments are not expected to develop well on this medium.

c. Potato flake agar (for the commercial formulation, see Appendix 8.9–2; for the procedure, see item VIII.C.4 below)

(1) Ensure that the indicator color is yellow.

(2) A typical red-pigmented *Trichophyton rubrum* isolate acts as a positive control, turning the indicator to blue-green in 7 days at 25 to 32°C. Visible red colony on reverse side after 7 to 10 days. Possible *T. rubrum* QC strains: ATCC 28188 and CBS 392.58.

B. Biochemical test

Cycloheximide resistance (see item VII.A below for procedure)—for a negative control, use *Candida glabrata* (CBS 138 = ATCC 2001 = IFO [Institute for Fermentation, Osaka, Japan] 0622 [same strain in all collections]), *Candida krusei* ATCC 14243, or *Aspergillus niger* ATCC 16404, which will not grow in the presence of 0.5 g of cycloheximide/liter, and use *Candida albicans* (ATCC 10231 = CBS 6431 = IFO 1594 [same strain in all collections]) as the resistant positive control.

C. Nucleic acid probe (see item VII.C.2 below for procedure)

1. Positive control organism—known strain of the test species. Recommended cultures: ATCC 60916 or UAMH 3604 *(B. dermatitidis)*, ATCC 38904 or UAMH 3536 *(H. capsulatum)*, and ATCC 28868 *(C. immitis)*. As of this writing, ATCC 38904 and ATCC 28868 are no longer available from the ATCC; indeed, all distribution of *C. immitis* has been discontinued in connection with bioterrorism concerns. Several *H. capsulatum* strains remain available. In the absence of a recommended QC strain, a typical, well-confirmed clinical isolate giving an equally strong positive signal may of necessity be substituted. Typical *C. immitis* isolates may still be obtained from other culture collections, such as CBS, but there may be restrictions on shipment to some jurisdictions. See the comments in item VII.C.2 below on ongoing maintenance of QC strains in good metabolic condition to permit rapid, controlled testing.

2. Negative control—dimorphic fungus closely related to the test strain. Use *H. capsulatum* when *C. immitis* or *B. dermatitidis* is tested, and use *B. dermatitidis* when *H. capsulatum* is tested. Recommended cultures are as given above.

D. Conversion of dimorphic fungi (see item VII.C.3 below for the procedure and Appendix 8.9–2 for medium recipes and sources)

1. *B. dermatitidis*—use a readily converting North American or North African isolate; look for detached yeast cells budding with a broad base within 72 h at 37°C. Possible QC strains: ATCC 60916, CBS 673.78, and UAMH 3604.

2. *H. capsulatum*

a. Medium (Kurung-Yegian)—should be opaque, smooth, and yellow, with final pH of ~6.6

b. Use a strain that converts to yeast phase within 5 to 15 days at 37°C. Possible QC strains: CBS 214.53, ATCC 8136, and UAMH 3536.

3. *P. marneffei*—reference strain should convert to a heaped, pasty, cream-colored colony composed of short branches rapidly fragmenting into arthro-

V. QUALITY CONTROL
(continued)

conidia, with the colony composed mostly of free arthroconidia in early growth and entirely at maturity (7 days postconversion). Possible QC strains: ATCC 18224 = CBS 334.59 (same strain) or ATCC 24100 = UAMH 4181. If a negative control is required, *Penicillium purpurogenum* (possible QC strain: CBS 364.48 = ATCC 9777 [same strain]) will produce flattened, restricted colonies of hyphae, often with swellings and other irregularities and generally with relatively dark colony color and reverse colony pigments.

E. Identification of dermatophytes (see item VIII below for procedures and Appendix 8.9–2 for media and sources)

1. Christensen's urea agar—*T. rubrum*, negative result on days 7 and 10 at 25°C. Possible QC strain: ATCC 28188. *Trichophyton mentagrophytes*, discernible positive result by day 7 and full positive reaction by day 10. Possible QC strains: ATCC 9533, CBS 318.56, and UAMH 5888.

2. BCP agar—positive control for pH change: *T. mentagrophytes* (alkalinity, purple color at 7 to 10 days at 25°C); negative control, *T. rubrum* (no indicator color change until after 14 days). Possible QC strains: ATCC 9533, CBS 318.56, and UAMH 5888 *(T. mentagrophytes);* ATCC 28188, CBS 392.58, and UAMH 8547 *(T. rubrum).*

3. Cornmeal glucose agar—typical *T. rubrum* strains should develop wine red reverse pigmentation; *T. mentagrophytes* will be uncolored to reddish brown. QC strains as in item 2 above.

4. Polished-rice medium—*Microsporum audouinii* reference isolate shows feeble growth and may or may not produce some brown pigment (possible QC strains: CBS 108934 [African variant usually seen today] and ATCC 10216 [classic strain type]); *M. canis* reference isolate (possible QC strains: CBS 496.86, ATCC 36299, and UAMH 4174) shows vigorous growth and abundant yellow pigmentation.

5. Hair perforation test—positive control: *T. mentagrophytes* (possible QC strains: ATCC 9533, CBS 318.56, and UAMH 5888) or *M. canis.* Negative control: *T. rubrum* (possible QC strains: ATCC 28188, CBS CBS 392.58, and UAMH 8547).

VI. PRELIMINARY IDENTIFICATION AND SUBSEQUENT CONFIRMATORY TESTS

Preliminary identification of isolates from primary culture is outlined in procedure 8.7. The colony appearance, microscopic morphology, temperature tolerance, cycloheximide sensitivity, and growth rate will provide clues to the genus of the isolate. This information should allow a suitable choice of culture medium for a slide culture to induce better production of characteristic microscopic morphology. In some instances, all of this information and the experience of the laboratorian will be sufficient for species identification. Where identification is uncertain or not possible, culture on the most suitable agar to induce conidiation and growth and a series of biochemical tests most appropriate for the suspected fungus (*see* below) should be performed.

VII. BIOCHEMICAL AND PHYSIOLOGICAL TECHNIQUES USED IN GENERAL MOULD IDENTIFICATION

It is imperative that these cultures be handled in a biological safety cabinet.

Mould identification techniques differ to some extent among different types of laboratory mycology, and in some areas, using micromorphology alone may be inappropriate or too slow. In particular, many dermatophyte types have undergone evolutionary processes that tend to diminish both sexual and normal conidial reproduction. Some species essentially never produce conidia, apart from substrate arthroconidia formed in host tissue. Therefore, physiological or other characteristics are commonly used in the laboratory. Dimorphic fungi in their mould states all strongly resemble at least one nonpathogenic fungal species and thus need to be confirmed by specialized techniques. Confirmatory tests for dimorphic fungi, as

VII. BIOCHEMICAL AND PHYSIOLOGICAL TECHNIQUES USED IN GENERAL MOULD IDENTIFICATION *(continued)*

well as physiological tests for dermatophyte identification, are dealt with in specific items below.

Certain biochemical and physiological characteristics are used across a wide range of fungi. In particular, cycloheximide responses, widely applied in strategies of selective isolation (*see* procedures 8.6 and 8.7), are also useful to assist in identifying mould isolates. Temperature tolerance tests have also become applicable in many specific mould groups. These tests are outlined below.

QC procedures and isolates for many of the tests in this section are dealt with in items V.A to D above.

A. Cycloheximide responses

1. Procedure

Sensitivity may be inferred by examining selective cycloheximide-containing primary isolation media in contrast to cycloheximide-free isolation media used for the same specimen. It can better be determined, however, by subculturing colonies onto a cycloheximide medium (e.g., cycloheximide-supplemented Sabouraud's peptone glucose agar) and its cycloheximide-free equivalent control medium (e.g., plain Sabouraud's peptone glucose agar) and incubating them at a suitable temperature and for a duration appropriate to the control growth rate of the organism.

2. Results

Table 8.9–6 lists common or significant moulds showing a characteristic cycloheximide response. In general, many of the more virulent mould pathogens and opportunists are cycloheximide tolerant or semitolerant (i.e., with a restricted colony growth rate but not killed at 100 mg of cycloheximide liter^{-1} and usually killed or strongly restricted at 500 mg liter^{-1}).

B. Temperature tolerance tests

1. Procedure

a. Temperature tolerance tests can generally be done on any cycloheximide-free medium commonly used for medically important fungi, e.g., plain Sabouraud agar or PGA.

b. Inoculated plates should be placed in incubators at 35, 37, 40, 42, 45, 50, and 55°C and left until growth is visible on the plate at the lowest temperature setting. Alternatively, a lower number of test temperatures may be selected based on relevance to the fungal group being tested (Table 8.9–5).

◩ **NOTE:** As they require a range of incubators, these tests may be more convenient in larger laboratory operations, especially where various general and mycobacteriology incubators can be shared for occasional fungal incubations. Smaller laboratories wishing to do a temperature test but lacking the appropriate incubator may inoculate the fungus in question into a relatively narrow tube and immerse it to near the cap in an adjustable water bath stabilized at the temperature in question.

2. Results

Table 8.9–5 shows the common temperature responses examined.

C. Species confirmation of dimorphic moulds

1. Presumptive recognition of mould phases

Examine the isolate, noting the colony appearance, microscopic morphology, and conidial ontogeny. The salient characteristics of mould phases of dimorphic fungi are given in Table 8.9–7.

2. Confirmation of dimorphic moulds using nucleic acid probes

DNA probes for confirmation of *C. immitis, H. capsulatum,* and *B. dermatitidis* are marketed as kits under the name AccuProbe (Gen-Probe Inc., San Diego, CA). The method uses a fluorescent label and requires a luminometer

Table 8.9–6 Cycloheximide responses of common or critical clinical laboratory moulds (including abundant contaminants)

Mould group	Example(s)	Cycloheximide response	Status
Zygomycota (Mucormycotina)			
Mucorales	*Rhizopus, Absidia, Mucor*	Semitolerant[a]	Opportunistic or nonpathogenic
Ascomycota			
Saccharomycetes	*Geotrichum*	Tolerant	Normally nonpathogenic[b]
Eurotiales	*Aspergillus, Penicillium, P. variotii*	Semitolerant	Opportunistic or nonpathogenic (*P. marneffei* is pathogenic)
Onygenales	Dermatophytes, e.g., *Trichophyton, Microsporum*	Tolerant	Pathogenic
	Coccidioides, Blastomyces, Histoplasma, Paracoccidioides, Emmonsia	Tolerant	Pathogenic
	Chrysosporium, Malbranchea	Tolerant	Normally nonpathogenic
	Onychocola	Tolerant	Pathogenic
Ophiostomatales	*S. schenckii*	Tolerant	Pathogenic
	Other *Sporothrix* spp.	Tolerant	Normally nonpathogenic
Microascales	*Scopulariopsis, Pseudallescheria*	Semitolerant	Opportunistic
	Scedosporium prolificans	Susceptible	Opportunistic
Hypocreales	*Acremonium, P. lilacinus*	Semitolerant	Opportunistic or nonpathogenic
	Fusarium	Semitolerant or susceptible	Opportunistic or nonpathogenic
Sordariales	*Chaetomium*	Semitolerant	Normally nonpathogenic
	Phaeoacremonium, Phialemonium	Semitolerant	Opportunistic or normally nonpathogenic
	Arthrinium, Nigrospora, Neurospora	Susceptible	Nonpathogenic
Mitosporic Ascomyota	*Ochroconis gallopava, Nattrassia (Scytalidium dimidiatum)*	Susceptible	Opportunistic
	Ochroconis constricta	Tolerant	Nonpathogenic
	Fonsecaea	Tolerant	Opportunistic
Dothidiomycetes	*Aureobasidium*	Semitolerant or susceptible	Normally nonpathogenic
	Hortaea	Susceptible	Pathogenic
	Cladosporium	Susceptible or semitolerant	Normally nonpathogenic
Herpotrichiellaceae	*Exophiala, Cladophialophora*	Tolerant	Opportunistic
Sordariomycetes	*Phialophora verrucosa*	Tolerant	Opportunistic
Pleosporales	*Alternaria, Curvularia, Bipolaris, Exserohilum*	Usually semitolerant	Opportunistic or normally nonpathogenic
	Epicoccum	Susceptible	Normally nonpathogenic
Basidiomycota	*Schizophyllum, Hormographiella*	Susceptible	*Schizophyllum* opportunistic; others normally or always nonpathogenic

[a] Semitolerant, restricted in colony growth rate but not killed at 100 mg of cycloheximide liter^{-1}; usually killed or strongly restricted at 500 mg liter^{-1}.

[b] Normally nonpathogenic, usually insignificant when isolated but rare opportunistic infection is possible.

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to assess the results. It is thus most commonly used by reference laboratories, laboratories in areas of endemicity, or laboratories where similar kits are also used in the mycobacteriology laboratory.

The probe method has the advantage that it can be used on all suspected cultures of the organism, even when they are small (e.g., in early outgrowth on primary isolation plates) or contaminated with bacteria or other fungi. The alternative methods require a culture at least 48 h old, often 7 days old or more, and contamination may cause delay.

The probe test is relatively costly, and in the cases of *B. dermatitidis* and *C. immitis*, consideration may be given to the use of alternative methods in cases where appropriate treatment has already begun on the basis of well-

Table 8.9–7 Distinguishing features for preliminary identification of mould phases of dimorphic systemic pathogens

Species	Mould phase characteristics	Mimics
Blastomyces dermatitidis	Subglobose aleurioconidia, smooth or rarely finely roughened, mostly 2–7 μm in diam, sometimes on swollen stalks, commonly formed on dusty, pale to sandy colonies; however, waxy, whitish, poorly conidial colonies are also common.	Closest, *Emmonsia* spp.; also, various *Chrysosporium* spp., *T. mentagrophytes,* microconidial *Histoplasma*[a]
Coccidioides immitis	Chains of alternate arthroconidia, which are barrel shaped or cylindrical and separated by empty disjunctor cells, are formed on dusty, often thin colonies with a raised margin, tending to creep far up glass tube walls and sides of plastic petri plates.	Closest, *Malbranchea* state of *Uncinocarpus reesei;* also, other *Malbranchea* spp.
Histoplasma capsulatum	Globose macroaleurioconidia with tuberculate (finger-like) roughening, 8–16 μm in diam; subglobose, rough-walled microaleurioconidia 2–5 μm in diam often also formed; colonies are sandy brown or paler and powdery.	Closest, *Chrysosporium* states of *Renispora flavissima* and *Arthroderma tuberculatum; Sepedonium* spp., *R. flavissima,* and *Sepedonium chrysospermum* have lemon yellow to golden colonies; *Sepedonium* usually has a phialidic synanamorph. *A. tuberculatum* has mainly ovoid, not globose, conidia, but similar conidia may also be found in some *H. capsulatum* isolates.[a]
Paracoccidioides brasiliensis	Slow-growing colonies with uncommon aleurioconidia and/or arthroconidia	Closest, an uncharacterized soil organism with similar conversion in vitro but avirulent and lacking gp43 antigen (5); also, *Chrysosporium* spp.
Penicillium marneffei	Colony typically with bright red reverse and soluble pigment around the colony; surface dull brown or reddish and velvety, with sparse, dull green conidia formed on biverticillate penicilli (penicilli with two successive ranks of whorled branches, the upper rank consisting of long, sharply pointed phialides); conidia smooth, ellipsoidal, 2.5–4.0 by 2.0–3.0 μm	Closest, several other *Penicillium* subgenus *Biverticillium* species with red reverse, e.g., *P. purpurogenum, P. minioluteum, P. funiculosum*
Sporothrix schenckii	Flat, slow-growing, whitish then partly deep brown to black; sometimes near black from the start; primary conidia formed in sympodial rosettes on spiny tooth-like denticles, hyaline, obovoidal (egg shaped, attached at the thin end), 3–6 by 2–3 μm; secondary conidia deep brown, often subglobose, sometimes triangular, attached individually on denticles on sides of hyphae, especially substrate (in the agar) hyphae, often densely packed and surrounding the hyphae in sleeve-like formation, at the same time turning colony dark	Closest, uncharacterized *Sporothrix* spp. sharing peat moss and decaying vegetation habitat; also, *Sporothrix* state of *Ophiostoma stenoceras;* most mimics lack secondary conidia. According to Dixon et al. (4), mimics possessing these structures can be distinguished by lack of 37°C growth in vitro; however, I have had isolates from confirmed sporotrichosis growing poorly or not at all at 37°C. *O. stenoceras* forms black, long-necked perithecia after 3–4 wk in culture on PGA or Leonian's agar.

[a] See reference 20 for detailed descriptions of important mimic fungi.

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demonstrated, characteristic direct microscopy (histopathology or direct hydroxide mount) plus isolation of a micromorphologically typical culture. The probability of a nonpathogenic mimic species (e.g., various *Malbranchea* spp. in the case of *C. immitis;* various *Chrysosporium, Emmonsia,* and *Trichophyton* spp. in the case of *B. dermatitidis;* and various cycloheximide-susceptible basidiomycetes in both cases) being isolated from such a microscopically diagnostic lesion is minimal, and such a coincidental isolation, if it occurred, would not by any means indicate that the interpretation of structures seen in vivo was incorrect. In the case of *H. capsulatum,* which is much less distinctive in tissue and has known in vivo mimics (e.g., *C. glabrata* cells in macrophages), the probe is best utilized in each case if available. For all three organisms, the probe may be advantageously used immediately whenever a suspected culture grows from a lesion for which direct microscopy was negative or whenever direct microscopy was done elsewhere (i.e., in another institution) with an unknown or questionable result.

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The probe test is based on the detection of rRNA, which is present in high quantity in cells. A specific DNA probe labeled with an acridinium ester fluorophore by the manufacturer hybridizes with the species-specific cellular RNA of test isolates; a subsequent luminometer reading then detects the double-stranded hybrids between the test isolate RNA and the probe DNA. Detailed procedures are found in the package inserts that accompany the kits. The information in this procedure should not be substituted for the package insert, as specifications may change if technical improvements are introduced.

Running the AccuProbe tests reliably depends on comparing the test results with results from a simultaneously tested known positive control strain of the test species (potential control strains are discussed above in item V.C), as well as a negative control strain of another species (protocols with less frequently used controls can be designed for laboratories with a high volume of these tests). Generally, a closely related dimorphic fungus is run as a negative control, e.g., *H. capsulatum* when *C. immitis* or *B. dermatitidis* is tested and *B. dermatitidis* when *H. capsulatum* is tested. If a probe is to be run the same day a new isolate is recognized as a potential dimorphic species, such a stored stock subculture of the same species will suffice as a positive control provided its signal level when the test is completed proves to be above the cutoff value indicated by the manufacturer. In my experience, the probe readings for stored stock cultures are slow to decline, and such cultures may be stored at 25°C in a usable condition for at least 2 months. In the extremely unlikely event of a below-range positive control reading, a new test of the unknown strain against fresh subcultures of the positive and negative controls may be needed, but the failure of the original test is otherwise harmless, in that it cannot give rise to a false test interpretation.

In any case where the laboratory intends to inoculate a subculture of the test isolate for probing after 48 or more hours, the positive and negative control isolates needed should also be subcultured at the same time. A subculture of the test isolate should be inoculated in all cases, since false-positive results may occasionally be caused by very different fungi not resembling the dimorphic fungi in question. For example, fungal melanin may chemiluminesce and cause dark, melanized fungi to give false-negative probe readings for *H. capsulatum* (27). Recently, Iwen et al. (11) reported repeated false-positive results in the *B. dermatitidis* probe for *Gymnascella hyalinospora,* a distantly related and morphologically distinct fungus. *P. brasiliensis* may also cross-react with the *Blastomyces* probe (17). Other limitations of this method are described elsewhere in this handbook.

The basic protocol for the AccuProbe test for mycelial material grown on plates is as follows.

a. Materials
  (1) Test isolate and positive and negative control cultures
  (2) Types of material utilized: DNA probe tubes containing labeled probe, lysis tubes with glass beads, lysis reagent (reagent I), hybridization buffer (reagent II), selection reagent (reagent III), and detection reagents connected to luminometer pumps

b. Set up procedure
  (1) Turn sonicator (Gen-Probe) on 15 min in advance to degas the water.
  (2) Turn on 95°C heating block, prepare 60°C water bath, turn on and prepare luminometer (Gen-Probe), and ensure that luminometer detection reagents are in sufficient supply.

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c. RNA isolation procedure

(1) Label sufficient lysis tubes for test isolate and both control isolates.

(2) Add 100 µl each of reagents I and II [*see* item a.(2) above] to each labeled lysis tube.

(3) In the biological safety cabinet, use a loop to take ~2 mm^2 of mycelium and transfer it to the appropriately labeled lysis tube.

(4) Grind the material for 20 to 30 s with a previously ethanol-flamed and cooled glass rod in order to disrupt the mycelium.

◩ **NOTE:** This step was added in my laboratory; it is not in the manufacturer's instructions.

(5) Briefly vortex each tube and then place in sonicator rack. Sonicate for 15 min at 40 kHz.

(6) After sonication, place tubes in a 95°C water bath for 10 min. The resulting solution should be strongly enriched in isolated RNA from the test strains.

d. Procedure for hybridization of DNA probe to RNA isolated from cultures

(1) Label sufficient probe tubes [material type a.(2) in above list] for the number of cultures tested. Add 100 µl of the lysed culture solutions obtained in step 2 (item c above). Vortex briefly.

(2) Incubate tubes at 60°C for exactly 15 min. It is important to be vigilant about the time. This is the step in which labeled DNA specifically binds to the RNA in solution. Incubation for too short a time could cause the value of a true-positive result to be lower than it should be in subsequent reading, while incubation for too long may allow nonspecific binding to occur.

(3) Add 300 µl of selection reagent [reagent III in item a.(2) above] to each tube and vortex briefly. This reagent hydrolyzes the acridinium ester fluorescent label on any DNA probe that has not bound to compatible RNA from a test isolate, thus minimizing most nonspecific background signal.

(4) Incubate tubes at 60°C for exactly 5 min.

(5) Remove tubes from the 60°C water bath and leave them at room temperature for 5 min. Remove the caps and read the results in the luminometer within 1 h.

(6) Compare the final values of the luminometer readings to acceptable cutoff values for positive results as given by the manufacturer. The positive control and positive test isolate readings should be above 1,500 photometric light units (PLU) in the Gen-Probe ACCULDR (formerly PAL)-type luminometer or 50,000 relative light units (RLU) in the Gen-Probe Leader-type luminometer, while the negative controls should be below 600 PLU or 20,000 RLU in the two meter types.

More detailed instructions and a greater range of procedural options are contained in the manufacturer's insert. The above, however, briefly represents the procedure my laboratory found most convenient to use routinely.

3. Conversion of dimorphic fungi to their particulate phases

The various morphologies seen in converted cultures are briefly summarized in Table 8.9–8. QC, including QC isolates for most species, is discussed in item V.D above, and details of media are given in Appendix 8.9–2.

a. *B. dermatitidis*

Since diagnostic conversion of *B. dermatitidis* occurs on special media within 48 to 72 h (the traditional BHI agar is usually significantly slower and is not recommended), conversion is appropriate for all laboratories

Table 8.9–8 Particulate phases of dimorphic fungi as seen in in vitro conversion and their differentiating characteristics

Species	Particulate phase in vitro	Organism(s) to distinguish
B. dermatitidis	Yeast, budding on a broad base; heavy ("double") walled, typically 9–15 μm with bud attachment	*Cryptococcus neoformans*, with narrow bud attachment, growing mixed with a subcultured test mould in conversion test at 37°C, so that the mixture of yeast and mould gives a false appearance of being a dimorphic fungus
C. immitis	Spherule, 10–80-μm diam, with many 2–5-μm-diam endoconidia	*Prototheca* spp., smaller, with <10 endospores at maturity
H. capsulatum	Yeast, budding on narrow base, 2–4 μm	*C. glabrata* and other small yeasts; distinction is by reconverting to the mould phase at 25°C
P. brasiliensis	Yeast, 30–60 μm, typically with multiple buds (cells with single buds also found)	*B. dermatitidis* in young stages, where only single buds are seen, especially outside endemic zone; *C. neoformans* overgrowing subcultured mould
P. marneffei	Fission yeast (self-replicating arthroconidia)	Overgrowth of another subcultured mould by *Trichosporon* spp. Do dilution plate from 37°C culture to 25°C culture if in doubt.
S. schenckii	Yeast, 3–10 by 1–3 μm, with initially long obclavate (cigar-shaped) buds; sympodially formed pairs of such buds ("rabbit ears") also typically seen	*C. glabrata* and other small yeasts overgrowing subcultured mould; look for rabbit ears and cigar-shaped buds; if in doubt, dilute and subculture to Sabouraud agar at 25°C

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with sufficient volume of *B. dermatitidis* to justify retaining special media and in all cases where a subculture needs to be made prior to confirmation study for any reason or where the etiology of the disease has already been presumptively ascertained on the basis of diagnostic direct microscopy or histopathology. See further test strategy notes in item VII.C.2 above.

(1) Purify culture of contamination, especially by *Candida* spp., as contaminants can cause failure to convert.

(2) Inoculate isolate onto either Blasto D medium (19) (*see* Appendix 8.9–2) or cottonseed agar (14) and incubate it at 37°C for 72 h, or for several weeks if an African variant is suspected.

(3) Examine for production of detached yeast cells budding from a broad base.

◩ **NOTE:** Chloramphenicol is inhibitory to the yeast phase of *B. dermatitidis* in vitro. If an antimicrobial agent is required in the medium, use tobramycin (100 μg/ml). To decontaminate cultures, use cycloheximide for susceptible yeasts and special media containing egg albumin (3) for cycloheximide-tolerant yeasts. See Appendix 8.9–2 for a recipe for yeast-inhibiting blood agar.

b. *C. immitis*

Although some strains of *C. immitis* can be converted to the spherule phase in vitro on Converse medium in a 20% CO_2 atmosphere, this is not sufficiently reliable to use as a diagnostic test. In developing nations, where exoantigen and probe kits may not be available, animal inoculation (typically guinea pig testis) may be necessary. This topic is beyond the

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scope of this procedure. Historically published methods may not be currently ethically approved, and anyone intending to implement these techniques must seek or devise an ethically approved procedure that meets the criteria of the investigator's institution, region, and nation.

c. *H. capsulatum*

Conversion of *H. capsulatum* to its yeast phase in vitro is time-consuming and highly vulnerable to bacterial and yeast contamination. It should be avoided wherever exoantigen or probe tests can be made available. See further test strategy notes in item VII.C.2 above. Various media may be used for conversion, including BHI-blood agar with cysteine or glutamine, blood-glucose-cysteine agar, and Kurung-Yegian medium. The simple but effective Kurung-Yegian medium recipe (21) is given in Appendix 8.9–2 for possible use in areas where advanced technologies are difficult to obtain. This agar-free medium is very inexpensive. Blood-glucose-cysteine agar, which may also be used for *P. brasiliensis* and *S. schenckii* conversion, is given as an alternative.

(1) Inoculate suspected *H. capsulatum* at 8 to 10 places on slant and incubate at 37°C.

(2) At 5-day intervals (or more frequently), examine a previously undisturbed inoculum point. The amount of converted yeast may be limited at first, and insufficient material may be left in previously picked inoculum points to show timely conversion.

(3) If conversion fails within 10 days, transfer some material from previous slant to a new slant, but leave some material on older slant and continue incubating both.

(4) If copious yeasts appear 2 to 5 days after initial inoculation, subculture to Sabouraud agar to investigate possible yeast contamination. In case of doubt about the status of budding cells, show reconversion to mould phase on Sabouraud agar at 25°C.

d. *P. brasiliensis*

No commercially available exoantigen or DNA probe test is yet available for *P. brasiliensis.* Diagnosis is often strongly supported by direct microscopy and serology, so culture confirmation may vary in importance from case to case. Conversion occurs slowly on BHI-blood agar (14) (*see* section 3 of this handbook), blood-glucose-cysteine agar (*see* Appendix 8.9–2), or Kelley agar (see reference 14 for the recipe) at 35 to 37°C. An exoantigen test performs well in confirmatory study (17) but is not commercially available. Formal confirmation at the research level is normally performed by demonstrating the characteristic 43-kDa glycoprotein gp43 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This is done because poorly characterized nonpathogenic mimics converting in vitro in a manner suggestive of *P. brasiliensis* have been isolated from soil, and these mimics lack gp43 (6). A possible QC strain for *P. brasiliensis* is CBS 372.73 = ATCC 32069 (same strain) or UAMH 8037.

e. *P. marneffei*

Since there are several *Penicillium* subgenus *Biverticillium* species producing red colony reverse pigment, and since *P. marneffei* may be difficult to distinguish in histopathology from *H. capsulatum,* confirmation by conversion to the particulate phase may be important in diagnosis.

(1) Inoculate isolate onto Sabouraud agar (without cycloheximide) and incubate at 37°C.

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(2) Conversion should be visible microscopically within 4 to 10 days. QC strains are given in item V.D.3 above.

◩ **NOTE:** Formulations for Sabouraud agar vary; I have used the recipe in Appendix 8.9–2 successfully on repeated occasions for *P. marneffei.*

f. *S. schenckii*

Since *S. schenckii* is often sparsely present in infected tissue and appears as a nondescript budding yeast-like organism, it may be missed or misidentified in direct microscopy or histopathology. Therefore, culture and the confirmation of identity are often critical in diagnosis.

Conversion is not formally necessary but should be practiced by all laboratories not thoroughly expert in morphological identification of this organism. *S. schenckii* can be distinguished from other *Sporothrix* species by its formation of sheaths of dark secondary conidia around substrate hyphae (5, 13). Dixon et al. (5) reported rare avirulent isolates that could be distinguished from *S. schenckii* by failure to grow at 37°C in vitro. However, I have occasionally worked with isolates that grew poorly or not at all at this temperature (despite growing well at 35°C), suggesting that further investigation of this characteristic is needed. Despite the distinctive micromorphology of the *S. schenckii* mould phase, confirmation by conversion to the yeast phase is normally done routinely as a precaution (i.e., to ensure that other types of structures formed by unrelated fungi have not been confused with secondary conidia), even though some of the uncharacterized mimics may also undergo partial or complete conversion under the same conditions.

(1) Inoculate isolate onto a slant of BHI and overlay with 2 drops of a sterile 10% aqueous solution of yeast extract. Moisture favors conversion in this species.

(2) Incubate at both 35 and 37°C to avoid temperature intolerances. Potential QC strains are ATCC 14284 and UAMH 3613.

D. Exoantigen testing (*see* Appendix 8.9–2)

Exoantigen testing is based on a specific reaction between two components: (i) purified antibodies obtained from animals injected with known species-specific fungal antigens and (ii) antigens extracted from cultured cells of the same species. This reaction is visible in an immunodiffusion test in which solutions of extracted antigens and antibodies are placed into adjacent wells cut into a plate of special agar medium. The antigen and antibody solutions diffuse through the agar at a predictable rate (at a preselected temperature), and where they meet, they precipitate to form a visible whitish band of material, generically called precipitin. If antigens from an incompatible fungus are used, they usually do not precipitate with the antibodies at all, and so no precipitin line is formed. In a few closely related species, somewhat different antigens may still possess enough similarity to the original antigens to cross-react with the antibodies. However, the precipitin line formed will lie at a different distance from the wells than that formed by the fully compatible antibody-antigen precipitation. This is detected by using known control antigens with each test and placing them in wells adjacent to those inoculated with the antigens from the test isolate.

It is imperative that these cultures be handled in a biological safety cabinet.

1. Procedure

Manufacturers' instructions for any kits or devices should be considered to supersede the methods given here unless sufficient controlled comparison trials have been done to ensure that the methodologies are functionally interchangeable.

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It is imperative that these cultures be handled in a biological safety cabinet.

a. Extracting and concentrating antigens

◩ **NOTE:** All procedures in this item should be carried out in a class II biological safety cabinet. Bear in mind that even though the extraction solution tends to kill the fungus, the impact of adding it to the culture will cause an ascent of aerosols, especially with *Coccidioides.* Also, the insides of the growth tubes, even after the extraction procedure, will contain many conidia not contacted by the extracting medium. Some procedures call for performance of a sterility test by inoculating extract onto Sabouraud glucose agar; however, the time required for minute amounts of viable inoculum to produce a visible colony is uncertain, and the procedure is not recommended here.

(1) Use a ≥6-day-old culture grown in a tube of Sabouraud glucose agar (a 20- by 150-mm tube with 10 ml of agar is recommended by McGinnis and Pasarell [15]). A colony diameter of at least 30 (along the slant) by 15 (across the slant) mm is preferred.

(2) Use a pipette to cover the culture with 8 to 10 ml of a 1:5,000 aqueous solution of Merthiolate (thimerosal; Sigma-Aldrich, St. Louis, MO). Breaking the mycelium up by scraping it with an inoculum spatula may be advantageous, especially with *Histoplasma* (*Coccidioides* material is generally manipulated as little as possible). Allow antigen extraction to proceed for at least 24 h at room temperature (not less than 22.5°C).

(3) After this 24-h period, swirl the extract gently to mix it and then pipette 5 ml into an Amicon Minicon macrosolute B-15 concentrator (Millipore, Bedford, MA).

(4) Concentrate extracts from suspected *Coccidioides* cultures 25-fold (levels of concentration are marked on the concentrator). I have found it advantageous to extract a second culture or to use the other half of an original 10 ml of extract in order to also prepare a 5-fold concentration of *Coccidioides* antigen, as the normally recommended 25-fold concentration may be overly concentrated for clear reading. Extracts from suspected *Histoplasma* or *Blastomyces* isolates should be concentrated 50-fold. Nonsporulating or atypical organisms that might be either *Coccidioides* or one of the other test organisms should be prepared so that sufficient extract is available to perform both 25- and 50-fold concentrations.

b. Performance of immunodiffusion

(1) Thaw any frozen antiserum or control antigen reagents and mix gently. Such reagents may be obtained as components of commercially available kits, e.g., the Exo-Antigen identification system (Immunomycologics, Norman, OK).

(2) Obtain a plate of immunodiffusion agar marked out with immunodiffusion grids. These can be obtained from commercial sources (e.g., Meridian Diagnostics, Cincinnati, OH) or made in-house (*see* Appendix 8.9–2). Pipette the reference antiserum so that it fills the center well of the immunodiffusion pattern but does not overflow from the sides of the well. After filling the well, allow the serum to diffuse for 1 h at room temperature.

(3) After the serum has diffused, place control antigen in the two end wells of the hexagonal grid. Place antigen of the unknown isolate in three to four of the remaining wells. One may be filled with blank Merthiolate reagent if desired as a negative control. Extracts of two different isolates should not be placed immediately side by side in adjacent wells, as a strong positive reaction in one of the two may

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prevent accurate reading of the other, potentially giving a weak false-positive reading.

(4) Place the immunodiffusion plate in a moist chamber or in a plastic bag (without jostling or slanting) and allow the antigen and antibody to diffuse together and react for 24 h at 25°C.

(5) Read the plates by using an immunodiffusion plate reader or by otherwise using diffusing backlighting. If reactions are still not clear, McGinnis and Pasarell (15) suggest washing the agar surface, covering it with distilled water, and reexamining it.

c. Results

The test is considered positive if the unknown isolate forms lines of identity with reference control antigens of a dimorphic fungus. When the antigen from the test isolate matches that of the positive control, a perfect hexagonal corner ("line of identity") is formed where the two precipitin lines from each pair of adjacent wells meet exactly and fuse end to end. In the event of a cross-reaction from another species, the precipitin line formed does not meet with the control line in a regular, closed corner, but instead meets so that one of the two lines overlaps the other or so that the lines cross over each other. With *Histoplasma,* since the H and M antigens do not form simultaneously in equal quantities in growing cultures, a certain line of identity with either of the two control antigens is sufficient to confirm the identification.

VIII. DERMATOPHYTE IDENTIFICATION

Identification of dermatophytes to the species level is important (i) to rule out confusion with nonpathogenic fungi both outside and within the genera *Trichophyton* and *Microsporum,* (ii) to allow control of dermatophytes acquired from animals by correctly indicating species with particular normal animal host ranges, (iii) to allow correct treatment in diseases such as tinea capitis, where drug dosage levels or treatment times may require species-specific adjustment, and (iv) to allow epidemiological connection with other affected persons and contagious, asymptomatic carriers in the family or in schools and other institutions, especially with *Trichophyton tonsurans, M. audouinii, Trichophyton violaceum,* and *Trichophyton soudanense.*

Dermatophytes are the only fungi specialized to cause contagious human and animal disease. Two fundamental features related to continuous pathogenicity strongly influence identification: (i) lack of recent evolutionary selection for normal types of fungal reproduction has caused many species to produce few or no conidia, and (ii) release from selection for ordinary fungal morphology has led to a conspicuous rise in macromorphological and micromorphological variability, meaning that a relatively high proportion of atypical to aberrant isolates are seen (25).

At the same time, however, it remains true that most of the isolates seen for a given species are typical, and many dermatophytes can easily be identified from primary growth medium. Below, a method to decontaminate cultures is presented; then, the useful characteristics in dermatophyte identification are outlined, and a scheme is put together for their best usage. This scheme is based on that outlined by Summerbell and Kane (26).

A. Decontamination of dermatophyte cultures contaminated with antimicrobial agent-polyresistant bacteria

Association with antimicrobial agent-polyresistant skin bacteria is common for *T. rubrum* and is occasionally seen with other dermatophytes. *T. rubrum* has been reported to produce penicillin and to select a lactam-resistant bacterial microbiota; also, it competes with highly polyresistant pseudomonads in nails, and these bacteria may be coisolated with it. Moreover, because some contam-

VIII. DERMATOPHYTE IDENTIFICATION *(continued)*

inating bacteria may adhere to aerial filaments in culture and not be fully exposed to antimicrobial agents in growth media, even some partially antimicrobial agent-susceptible bacteria may be carried over into subcultures. All these bacteria cause false-positive and false-negative reactions in physiological tests. The detection of cryptic contamination on BCP milk glucose agar is mentioned in item VIII.C.3. Decontamination of such bacterially contaminated cultures requires the primitive microbiological technique outlined below. Note that the series of medium tubes required is described in Appendix 8.9–2, and this must be consulted before reading the procedure below.

1. Culture and reisolation
   Take inoculum from the margin of the contaminated colony or area of least contamination and inoculate each of the four tubes (*see* Appendix 8.9–2). Incubate at 25°C and examine in 7 days.
2. Growth in tube 0 will frequently be turbid because of the presence of bacteria. Examine tubes 1 to 3 to detect both fungal growth and similar bacterial turbidities. Agitate slightly if necessary to visualize the latter. Mark all turbid tubes as contaminated.
3. Among the clear tubes, look for sufficient distinct new fungal growth to allow reisolation. Use only growth from within the medium, not from the surface. If clear new growth is insufficient on day 7 to allow reisolation from any tube, place tubes back into the incubator and reexamine on day 10 or 14 or until day 21, when growth can be recorded as negative. When growth is detected, reisolate the dermatophyte by subculturing from the tube with the highest acid concentration permitting purified growth.

B. Dermatophyte micromorphological characteristics

The special morphological features of various dermatophytes are listed in Table 8.9–9 and illustrated in Fig. 8.9–A15 through 8.9–A24 in Appendix 8.9–1. Note that *Trichophyton interdigitale* (a long-disused name newly revived by Gräser et al. [7] to include all zoophilic, as well as anthropophilic, *T. mentagrophytes*-like isolates in a phylogenetic cluster related to the teleomorph *Arthroderma vanbreuseghemii*) is not distinguished from *T. mentagrophytes* in the table. *T. interdigitale* as phylogenetically defined can only be identified by using molecular techniques or, in zoophilic isolates, by mating them, and this distinction currently has no medical significance. *T. mentagrophytes* as conventionally defined was already long known to be an aggregate of more than one biological species, and the name is used here in that collective sense. The names *Microsporum gypseum* and *Trichophyton terrestre* are also used as conventional aggregate names comprehending four and three known biological species, respectively.

C. Physiological and other special medium tests for identifying dermatophytes

Dermatophytes may be recognized by morphology alone without needing to resort to physiological testing. Among those which remain unidentified after initial morphological characterization, some may be suspected of being particular species based on the morphological characteristics seen, and special tests to aid these presumptive identifications are given below. For others, for which the species identification may be uncertain, a minimal set of physiological test media is recommended: vitamin-free Casamino Acids agar (*Trichophyton* agar no. 1) to screen for vitamin and growth factor requirements, Christensen's urea broth or agar, BCP milk glucose agar, and Sabouraud agar as a growth control (*see* Appendix 8.9–2 for medium recipes).

Always examine the isolate for colony and microscopic morphology. Since some species can be very similar, it can be useful to consider the results of direct specimen microscopy and any patient details, such as travel or association with animals, before deciding which growth factor test to perform.

Table 8.9–9 Dermatophyte micromorphological structures

Characteristic	Definition	Identification aid
Aerial arthroconidia	Hyphal branches forming conidia may also break up, forming single cells.	
Substrate arthroconidia	Chains of strongly swollen cells that break to form separate round cells—seen on the host or in fresh isolates	
Terminal chlamydospores	Chlamydospores produced near the ends of hyphae; variations include symmetrical and asymmetrical laterally distended chains	*M. audouinii, T. verrucosum, T. violaceum* (Fig. 8.9–A15 to 8.9–A17)
Pedicels (Fig. 8.9–A18)	"Matchstick" stalks connecting microconidia to hyphae	*T. tonsurans* and *M. persicolor*
En thyrse; sessile	Distribution of microconidia along a relatively unbranched hypha	*M. gypseum* complex
Racemose; en grappe	Microconidia on hyphae with densely rebranching pattern resembling branching in a bunch of grapes; formation is grape-like.	*M. racemosum*
Distoseptate (Fig. 8.9–A19)	Macroconidia that lack distinct cross walls and have cytoplasm broken into cellular compartments	*T. ajelloi, M. cookei, M. vanbreuseghemii*
Setae (Fig. 8.9–A20)	Also called spirals (see ascoma morphology [Table 8.9–3])	*T. mentagrophytes, M. persicolor*
Reflexive branching (Fig. 8.9–A21)	Radial thickenings on colony surface contain hyphae growing both forward and backward in respect to the direction of colony extension.	*T. soudanense*
Favic chandeliers (Fig. 8.9–A22)	Hyphae with terminal branching in an antler-like pattern with strongly swollen tips; sometimes called nailhead hyphae	*T. schoenleinii*
Balloon forms	Greatly swollen microconidial structures	*T. tonsurans*
Filiform	Abortive, spindly side branches arising from chains of slightly swollen cells; also called caterpillar form	*T. tonsurans*
Pectinate branching (Fig. 8.9–A23)	Short, rounded side branches in a comblike pattern on one side of the hypha	*M. audouinii, T. rubrum*
Diverticula (Fig. 8.9–A24)	Solitary, very wide, nonextending side branches; also called projections	*T. rubrum*
Nodular bodies	Tightly coiled, knotted-looking thickened hyphal structures in the submerged mycelium	*T. mentagrophytes* nodular variant (*T. krajdenii*)
Perforating organ	Robust, sharply pointed long cone, with a restricted waist near the point of origin; produced to perforate hair	*T. mentagrophytes* complex, *M. canis* complex, and soil isolates

VIII. DERMATOPHYTE IDENTIFICATION *(continued)*

1. Growth factor tests for identifying dermatophytes

a. Most dermatophytes do not require any external supply of vitamins or other special growth factors, but some *Trichophyton* spp. require one or more of the following: thiamine, inositol, nicotinic acid, and L-histidine. To perform all possible vitamin tests on an unknown isolate requires seven media, referred to as *Trichophyton* agars (the conventional phrase "*Trichophyton* agar no." is abbreviated as "T" below).

(1) T1, vitamin-free Casamino Acids agar (possible QC isolates: one each of the QC isolates suggested for media T2, T4, and T5 plus one isolate listed in item V.E.1)

Table 8.9–10 Phenotypic identification characteristics of common and occasionally seen dermatophytes, as well as rare species potentially used in QC trials

Dermatophyte	Growth[e]	Macromorphology	Micromorphology	Growth factor requirement	BCP milk glucose (pH results for 7–10 days)	Urease (7 days; broth)[f]	Hair perforation[f]	Other comments
E. floccosum[a]	Mod. rapid	Flat, slightly granular at first, soon developing white puffs of degeneration; sandy to olive brown; reverse pale to yellowish	Macroconidia abundant, club shaped with broadly rounded apex, usually with <6 cells; no microconidia formed; many chlamydospores in primary isolates	None	Alkaline	Pos	Neg	
M. audouinii[b]	Mod. rapid	Flat to velvety, thin; pale salmon to pale brownish reverse	Rare, deformed macroconidia, often with beak, constricted midregion, and at least trace granulation; drop-shaped microconidia and aerial arthroconidia may be present; pectinate branching, terminal chlamydospores often seen	None on *Trichophyton* agars (*see* "Other comments")	No pH change or alkaline	Neg	Neg	Poor growth and no or brownish pigment on polished-rice medium; usually connected with patient or index patient in or recently from Africa; only children typically infected
M. canis[a]	Rapid	Flat to velvety, thin, with yellow (rarely pale) reverse	Macroconidia thick walled, roughened, and beaked; microconidia drop shaped	None	No pH change; macroconidia often abundant	Pos	Pos	Good growth and yellow pigment on polished-rice medium; human infection usually from cat or dog
M. cookei[b]	Mod. rapid	Granular to velvety; reverse wine red	Macroconidia rough, thick walled, with cellular compartments rather than true cross walls; microconidia drop shaped	None	No pH change	Pos	Pos	Nonpathogenic
Microsporum ferrugineum[c]	Slow	Flat; reverse yellow, rusty, or pale	No conidia; coarse, straight "bamboo" hyphae may be present.	None	No pH change	Neg	Neg	Yellow colony on Lowenstein-Jensen medium (compare *T. soudanense*)

M. gallinae[c]	Mod. rapid	Flat to velvety; red pigment diffuses into agar	Macroconidia smooth to slightly rough, often bent and with thickest cells near the apex	None	No data	Neg	Neg	Rare; human infection usually from chicken
M. gypseum[a]	Rapid	Granular; sandy in color; reverse usually pale to brownish	Macroconidia abundant, thin walled, fusoid (tapered at both ends), roughened; microconidia drop shaped, mostly formed "en thyrse" (a feature only noted if *M. racemosum* is queried)	None	No pH change	Pos	Pos	Human infection usually from soil contact
M. nanum[b]	Mod. rapid	Powdery; sandy in color; reverse often reddish brown	Macroconidia rough, usually only 1–3 cells long, egg shaped to ellipsoidal	None	No data	Pos	Pos	Human infection usually from pig
M. persicolor[b]	Rapid	Powdery; sandy in color; reverse pale to yellowish, sometimes with rosy tones	Macroconidia fusoid (tapered on both ends); often absent or smooth walled on Sabouraud agar but usually common and rough walled on Sabouraud agar with added salt (3 or 5% NaCl); microconidia formed on pedicels (must be checked within 5 days)	None	No pH change	Pos	Pos	Usually poor growth at 37°C in vitro; rose to wine red reverse on sugar-free media, e.g., glucose-free Sabouraud agar; human infection usually from soil (fomites from voles)
M. praecox[b]	Rapid	Powdery; sandy in color; reverse yellow	As for *M. gypseum*	None	No pH change	Pos	Neg	Uncommon
M. racemosum[c]	Rapid	Powdery; sandy in color; reverse red	Macroconidia as for *M. gypseum;* microconidia mostly formed "en grappe"	None	No pH change	Pos	Pos	Rare
M. vanbreuseghemii[c]	Rapid	Powdery; pinkish or buff; pale to yellow reverse	Macroconidia rough, thick walled, cylindrical; often >8 cells long, with cellular compartments rather than true cross walls	None	No data	Pos	Pos	Rare

(continued)

Table 8.9–10 Phenotypic identification characteristics of common and occasionally seen dermatophytes, as well as rare species potentially used in QC trials *(continued)*

Dermatophyte	Growth[e]	Macromorphology	Micromorphology	Growth factor requirement	BCP milk glucose (pH results for 7–10 days)	Urease (7 days; broth)[f]	Hair perforation[f]	Other comments
T. ajelloi[b]	Mod. rapid	Powdery, rich tan in color; reverse pale, brownish, or with purple-black pigment	Macroconidia smooth, thick walled, cylindrical; often >7 cells long, with cellular compartments rather than true cross walls	None	No data	Pos	Pos	Nonpathogenic
T. concentricum[c]	Slow	Folded, honey brown colony	No conidia	50% of isolates show slight stimulation by thiamine (compare *Trichophyton* agars 1 and 4)	No data	Pos or neg	Neg	Only from indigenous Asian, Austronesian/Melanesian, or indigenous Central and South American people with distinct tinea imbricata infection
T. equinum[b]	Mod. rapid	Flat to velvety colony with yellow to red-brown reverse	Macroconidia uncommon; cylindrical to club shaped, smooth; microconidia abundant, on small pedicels (examine before 5 days)	Most isolates absolutely require nicotinic acid (compare *Trichophyton* agars 1 and 5); rare Australian *T. equinum* var. *autotrophicum* does not	Alkaline	Pos	Usually neg, sometimes pos	Human infection usually from horse
T. megninii[b]	Mod. rapid	Cottony, with white down sometimes suffused with rosy pigment; reverse red to red-brown	Macroconidia seldom seen, pencil shaped; microconidia drop shaped	Requires L-histidine (compare *Trichophyton* agars 6 and 7)	Alkaline	Pos or weak pos	Neg	Endemic in Portugal and nearby areas
T. mentagrophytes (zoophilic)[a]	Rapid	Granular to powdery; yellow-cream to buff surface; pale to red-brown reverse	Macroconidia uncommon; club shaped, smooth; microconidia nearly spherical, abundant, mostly produced en grappe in dense tufts; spiral appendages present	None	Alkaline (rarely weak)	Pos	Pos	Human infection usually from rodent or rabbit

T. mentagrophytes var. *erinacei*[b]	Rapid	Granular to powdery; yellow-cream to buff surface; yellow reverse	Macroconidia uncommon, club shaped, smooth; microconidia nearly spherical, abundant, mostly produced en grappe in dense tufts; spiral appendages present	None	Alkaline	Neg	Pos	Human infection usually from hedgehog or its fomites; therefore, mostly restricted to regions with wild hedgehogs or to pet hedgehog owners
T. mentagrophytes (anthropophilic)[a,d]	Rapid	Powdery to cottony; yellow-cream to buff or white surface; pale to red-brown reverse	Macroconidia uncommon, club shaped, smooth; microconidia nearly spherical or drop shaped, abundant, produced mainly en grappe when round and en thyrse when drop shaped; spiral appendages present but rare in very cottony isolates	None	Alkaline	Pos	Pos	
T. mentagrophytes (nodular variant = *T. krajdenii*)[b]	Mod. slow	Cottony; cream to white surface; intense yellow reverse	Macroconidia rare; microconidia usually drop shaped, sometimes also round; coiled, yellow "nodular bodies" and yellow pigment granules present in submerged mycelium; spiral appendages seldom seen	None	Alkaline	Pos	Pos	Although usually very different in morphology, this variant so far is not genetically distinguished from other anthropophilic anamorphs related to *A. vanbreuseghemii.*
T. rubrum[a]	Mod. slow	Cottony to velvety; white to reddish surface; typically wine red reverse, but yellow variants occasional; red color poorly formed in presence of common bacterial contamination	Macroconidia seldom seen, pencil shaped; microconidia drop shaped, abundant, scanty, or not formed; lateral hyphal projections often present	None	No pH change (alkalinity after 14 days)	Neg (rarely weak)	Neg	

(continued)

Table 8.9–10 Phenotypic identification characteristics of common and occasionally seen dermatophytes, as well as rare species potentially used in QC trials *(continued)*

Dermatophyte	Growth[e]	Macromorphology	Micromorphology	Growth factor requirement	BCP milk glucose (pH results for 7–10 days)	Urease (7 days; broth)[f]	Hair perforation[f]	Other comments
T. rubrum (Afro-Asiatic variant = *T. raubitschekii*)[b]	Mod. slow	Powdery to low velvety; cream to deep red; reverse wine red	Macroconidia abundant, club shaped, sometimes with "rat tail" extension; microconidia drop shaped to round; many chlamydospores in primary isolates	None	No pH change (alkalinity after 14 days)	Pos	Neg	Although usually very different in morphology, this variant so far is minimally genetically distinguished from typical *T. rubrum*. It is usually from upper-body infection (tinea corporis; tinea cruris).
T. schoenleinii[c]	Slow	Convoluted, slightly velvety whitish colony	No conidia seen; "favic chandeliers" or "nailhead hyphae" present	None	Alkaline	Variable	Neg	Very rare; associated with clinically recognizable "favus" lesions; now extirpated except in rural central Asia, rural Africa
Trichophyton simii[b]	Rapid	Granular to powdery; yellow-cream to buff surface; pale to red-brown reverse	Macroconidia abundant, often with some cells swollen as chlamydospores; microconidia drop shaped	None	Alkaline	Pos	Pos	Endemic to India and Africa; similar to zoophilic *T. mentagrophytes* but macroconidial number and shape are atypical; reference distinction is by mating or molecular study

T. soudanense[b]	Mod. slow	Flat, bright yellow to (less commonly) wine red colony with radial striations and starlike margin; uncommonly cottony; reverse yellow to wine red	Macroconidia not seen; microconidia drop shaped, scarce, or absent; reflexive hyphal branches in radial striations	Usually no growth on *Trichophyton* agar no. 1; a few isolates grow well. Stimulation on other *Trichophyton* agars usually not seen, but stimulation on no. 5 may occur rarely.	Alkaline, with small zone of clearing	Usually Neg, occasionally Pos	Neg	Endemic to sub-Saharan Africa but widely disseminated in cosmopolitan parts of Europe and Americas; dark colony on Lowenstein-Jensen medium (compare *M. ferrugineum*)
T. terrestre[b]	Mod. rapid	Powdery white to cream or pinkish surface; pale or rarely yellow to red reverse	Macroconidia numerous, mostly small (5 or fewer cells) intergrading with large club-shaped microconidia in a continuous series, so that 3-, 2-, and 1-celled conidia are present	None	Alkaline	Pos	Pos	No growth at 37°C in vitro; nonpathogenic
T. tonsurans[a]	Mod. slow	Powdery to velvety surface; reverse chestnut red-brown and/or sulfur yellow, rarely pale	Macroconidia uncommon, small, pencil or club shaped; microconidia abundant, often on broad "matchstick" pedicels; "balloon forms" and "filiform branches" may be seen	Strongly stimulated by thiamine (compare *Trichophyton* agars 1 and 4)	Alkaline, sometimes weak	Pos	Usually neg, sometimes pos	

(continued)

Table 8.9–10 Phenotypic identification characteristics of common and occasionally seen dermatophytes, as well as rare species potentially used in QC trials *(continued)*

Dermatophyte	Growth[e]	Macromorphology	Micromorphology	Growth factor requirement	BCP milk glucose (pH results for 7–10 days)	Urease (7 days; broth)[f]	Hair perforation[f]	Other comments
T. verrucosum[a]	Slow	Convoluted, slightly velvety whitish or tan colony	Macroconidia seldom seen, with rat tail extension; microconidia round to drop shaped	Strongly stimulated by thiamine and in most cases also inositol (compare *Trichophyton* agars 1, 3, and 4)	Alkaline (may be weak) with broad zone of clearing	Neg	Neg	Human infection usually from cattle; growth stimulated at 37°C
T. violaceum[b]	Slow	Glabrous (bald-looking), smooth or convoluted colony; purple-red, sometimes with white sectors; rarely whitish	Macroconidia seldom seen; microconidia not formed or formed mostly on thiamine medium or on sporulation media	Stimulated by thiamine (compare *Trichophyton* agars 1 and 4)	No pH change or weak alkaline with small to broad zone of clearing (always broad after 14 days)	Pos or weak	Neg	Endemic to north Africa and Middle East but widely disseminated in cosmopolitan parts of Europe, Americas, and South Africa

[a] Common.

[b] Uncommon, but likely to be seen in large laboratories in the Americas and Europe.

[c] Unlikely ever to be seen except in proficiency test or region of endemicity.

[d] Technical taxonomic note. This ecological category includes anthropophilic but not zoophilic forms both of *T. interdigitale* sensu Gräser et al. (7) and of *T. mentagrophytes* anamorphs phylogenetically clustered with *Arthroderma benhamiae*. These forms were previously collectively called by the nomenclaturally invalid name "*T. mentagrophytes* var. *interdigitale*" by some workers. Although this name was conceived as encompassing all anthropophilic *T. mentagrophytes*-like isolates, this heterogeneous group cannot be placed under any single name except *T. mentagrophytes* sensu lato (species aggregate concept of *T. mentagrophytes* as used in the present work). Recent phylogenetic work has not been clear on this point, because the existence of rat and chinchilla strains, as well as anthropophilic strains, within the concept of *T. interdigitale* sensu Gräser et al. was not explicitly mentioned.

[e] Mod., moderately.

[f] Pos, positive; Neg, negative.

VIII. DERMATOPHYTE IDENTIFICATION *(continued)*

(2) T2, T1 plus inositol; used for showing partial stimulation in ~80% of *Trichophyton verrucosum* isolates (possible QC isolate: UAMH 5666)

(3) T3, T1 plus thiamine plus inositol; used for showing stimulation in *T. verrucosum*

(4) T4, T1 plus thiamine; the most useful medium, used for showing stimulation in *T. tonsurans* and *T. violaceum* and slight stimulation in *T. verrucosum*, as well as ~50% of *Trichophyton concentricum* isolates (possible QC isolate: *T. tonsurans* ATCC 14002, CBS 118.65, or UAMH 8552)

(5) T5, T1 plus nicotinic acid; used for showing absolute requirement for nicotinic acid by *Trichophyton equinum* var. *equinum* (possible QC isolate: *T. equinum* ATCC 26365 = CBS 856.71 [same strain; listed as *T. tonsurans* in CBS database due to controversial proposal for taxonomic merger of *T. equinum* and *T. tonsurans*])

(6) T6, amino acid-free ammonium nitrate agar

(7) T7, T6 plus L-histidine; used for showing stimulation in *Trichophyton megninii* (possible QC isolate: *T. megninii* ATCC 12106 or CBS 500.48 [the latter is listed as *T. rubrum* in CBS database due to controversial proposal for taxonomic merger of *T. megninii* and *T. rubrum*])

b. If the isolate has a preliminary identification, use the *Trichophyton* agars that will prove or disprove the identification.

c. Inoculation and reading

Inoculate a small piece of fungus, being careful not to transfer any growth medium and to use cooled sterilized inoculation needles.

◩ **NOTE:** Only the nicotinic acid requirement of *T. equinum* is an absolute requirement leading to no growth on the vitamin-free T1. Other dermatophytes show weak growth on the media negative for the growth factor they "require" but abundant growth on the appropriate growth factor-supplemented medium.

d. Cultures should be incubated at 25°C for 7 days for moderate- to fast-growing isolates and for 10 to 14 days for slow-growing isolates. Cultures suspected of being *T. verrucosum, Trichophyton schoenleinii*, or *T. concentricum* may be incubated at 37°C to exploit the accelerated growth rate of *T. verrucosum* at that temperature.

e. Record results in a range from + (trace growth) to 4+ (control level growth). Species with thiamine, inositol, or L-histidine requirements ordinarily show ~2+ growth on growth factor-free medium. New readers unfamiliar with this system should compare the growth of appropriate QC strains (as listed above under item VIII.C.1.a) on growth factor-free medium and the appropriate control medium in order to gain an appreciation of the differences between nutrient-deprived and normal growth.

2. Urease test for identifying dermatophytes

Several dermatophyte species specialized for pathogenesis have lost the urease enzyme activity that is found in all soil-associated dermatophyte species. This difference is detected by means of the Christensen urea test. In practice, this test is most commonly used to distinguish the urease-negative *T. rubrum* from similar isolates in the *T. mentagrophytes* complex. This test is also useful to recognize occasional clinical contaminants that are cycloheximide tolerant, white, protractedly nonsporulating nondermatophytic moulds and some nonpathogenic *Chrysosporium* spp., such as *Chrysosporium georgiae*, that may resemble atypical *T. mentagrophytes* or *T. rubrum.*

The complete list of urease-negative and -positive species is given in Table 8.9–10. QC strains are given in item V.E.1 above.

VIII. DERMATOPHYTE IDENTIFICATION *(continued)*

Inoculation and reading are performed as follows.

a. Use only pure cultures, as contaminants can give false results. Simultaneous inoculation onto BCP agar will demonstrate the presence of contaminants (26).

b. Use a small amount of inoculum to inoculate the tubes so that acid or basic compounds are not transferred from prior medium. Incubate at 25°C for 14 days, reading positive results when visible (examine on day 7) and negative results on day 14.

c. Positive results consist of a change of the phenol red indicator to red-purple; negative results are seen as an unchanging yellow-orange color as found in the freshly prepared medium. A yellow, acid reaction indicates bacterial or yeast contamination.

3. BCP milk glucose test for identifying dermatophytes

Dermatophytes all raise the pH of most complex media, such as Sabouraud agar, by breaking down proteins to produce alkaline compounds. In many species, however, this action either is not seen or is strongly delayed when milk casein is used as a protein base and glucose is added as a carbon source. *T. rubrum* and most *Microsporum* spp. in particular show this repression of ammonifying proteolysis on milk glucose medium, as detected by the indicator BCP. On the other hand, *T. mentagrophytes, Epidermophyton floccosum, T. tonsurans,* and most soilborne *Trichophyton* species (e.g., *Trichophyton ajelloi*) rapidly ammonify milk glucose medium, producing a color change in the pH indicator.

In addition, most rapidly growing dermatophytes produce enzymes that break down opaque milk proteins to produce clear medium, but the action of these proteins usually does not extend beyond the colony margin. However, the slow-growing *T. verrucosum* and *T. violaceum,* and to a lesser extent the moderately slowly growing *T. soudanense,* have milk-clearing enzymes that diffuse outward more rapidly than the colony itself grows, producing a distinctive ring of clearing on milk media.

BCP milk glucose agar is antimicrobial agent free, as it favors heavy growth of bacteria, exposing their presence. It also indicates organic acid production by many contaminating bacteria and yeasts, with an indicator change to yellow. This then allows one to correctly interpret correlated false-positive (or acid) urease tests.

Finally, BCP milk glucose medium strongly favors production of macroconidia by *M. canis* isolates that remain nonsporulating on Sabouraud glucose agar, and it elicits strong production in *T. soudanense* of radial striae in which abundant reflexive branches may be found. QC strains for this test are given in item V.E.2 above.

Inoculation and reading are performed as follows.

a. BCP milk glucose agar should be inoculated near the middle of the slant with a small inoculum to make it easier for the differences in the growth rates of different species to be seen.

b. Record growth rates at 7 days, after comparing them with the control Sabouraud tube. Rates should be recorded as slow (meaning slow growing but essentially the same as on Sabouraud), restricted (meaning slow growth significantly slower than that seen on Sabouraud), or profuse (rapid growth as seen on Sabouraud).

c. Record medium pH at 7 and 10 days. The potential readings are as follows: unchanged (sky blue), indicating a negative reaction; alkaline (blue-purple to purple), indicating a positive reaction; and acid (yellow), indicating contamination.

d. Record distinct clearing of milk solids around the colony periphery on day 7. Ignore clearing beneath the colony.

VIII. DERMATOPHYTE IDENTIFICATION *(continued)*

◩ **NOTE:** Many *Trichophyton* cultures kept for >14 days will produce extensive clearing, and some bacterial contaminants may also produce clearing. Also, degenerated cultures of all dermatophytes, if they have been improperly stored in local or professional culture collections, will tend to have much stronger clearing reactions than fresh isolates. QC, therefore, must not be performed with such isolates.

e. Check the micromorphology of (i) cultures suspected to be *M. canis* but not sporulating on Sabouraud glucose, (ii) cultures suggestive of *T. soudanense* with radial striae, and (iii) any very atypical cultures. BCP milk glucose may stimulate formation of macroconidia in unusual isolates, such as degenerated *E. floccosum,* in situations in which other media do not.

4. Cornmeal glucose agar (cornmeal dextrose agar)

Cornmeal glucose agar (2) is one of several media used to elicit wine red pigment in *T. rubrum* and characteristic micromorphology in numerous dermatophytes. Others include Casamino Acids erythritol albumin agar (26), which has the advantage of nutritionally inhibiting yeast and bacterial contamination; modified Borelli's lactrimel agar (12); and potato flake agar. These media are to some extent interchangeable in this function, although the optimal incubation times may vary and the exact micromorphologies elicited may differ.

Stimulation of the pigment production of atypical *T. rubrum* isolates can facilitate their identification, while *T. violaceum, T. soudanense, T. megninii,* and *C. georgiae,* which also produce red pigments, can be distinguished by morphology or characteristics developed on other media.

◩ **NOTE:** Red-pigmented *T. terrestre* isolates are either rare or nonexistent, despite statements in the literature.

QC strains for this test are given in item V.E.3 above.

Inoculation and reading—inoculate an ~1-mm^2 piece centrally, incubate it at 25°C, and examine it on days 7, 10, and 14.

5. Polished-rice test for *Microsporum* spp.

The most common *Microsporum* spp. are *M. canis* and *M. gypseum.* Both can usually be identified by characteristic micromorphology on primary isolation medium, although *M. canis* produces a certain proportion of atypical isolates requiring subculture to the minimal set of physiological test media suggested earlier. The polished-rice test is mainly used to confirm suspected *M. audouinii,* an anthropophilic dermatophyte almost always seen in scalp ringworm in children. *M. audouinii* is quite variable but resembles many weakly pigmented, poorly conidial *M. canis* isolates and can best be distinguished from them on autoclaved polished rice. It has an uncharacterized growth factor deficiency that causes it to grow only weakly on this medium, sometimes with concomitant production of some brownish soluble pigment. *M. canis,* on the other hand, grows vigorously and usually produces lemon yellow pigmentation. Other dermatophytes also grow vigorously. QC strains for this test are given in item V.E.4 above.

Inoculation and reading—inoculate the surface of the rice with the test fungus, placing four pieces evenly around the bottle adjacent to the wall. This maximizes the reader's ability to see pigment formation and compensates in part for the tendency of *M. audouinii* to die out in culture. Simultaneously inoculate Sabouraud glucose growth control for the suspected *M. audouinii.*

6. Hair perforation test

Dermatophytes that are ecologically associated with soil and with animals denning in the soil are specialized to rapidly perforate and digest loose-lying shed hairs. A specialized "hair perforation organ" is produced to physically and enzymatically pierce them. Dermatophytes that have an ecological as-

VIII. DERMATOPHYTE IDENTIFICATION *(continued)*

sociation with animals not denning in the soil have strongly tended to lose this specialized hair-perforating ability. Typical animal hosts associated with at least some dermatophytes lacking hair perforation abilities are horses, cattle, fowl, and humans (25). The spectrum of dermatophytes growing on humans includes several specialized anthropophilic species, such as *T. rubrum* and *T. tonsurans,* that always or mostly lack hair perforation ability; some members of the *T. mentagrophytes* complex that have retained hair perforation ability; species such as *T. verrucosum* and *T. equinum,* acquired from non-soil-dwelling animals, that always or mostly lack hair perforation ability; and species from soil-dwelling or ancestrally soil-dwelling animals (rodents, rabbits, dogs, and cats) that always manifest hair perforation ability. The hair perforation test is mainly used to distinguish atypical *T. rubrum* from *T. mentagrophytes* but can also be used to distinguish *M. audouinii* from *M. canis,* the uncommon *Microsporum praecox* from *M. gypseum,* and so on (Table 8.9–10). QC strains are given in item V.E.5 above.

Preparation, inoculation, and reading are performed as follows.

a. Collect hair from a prepubertal child, ideally under 18 months old; some texts recommend blond hair if available. Place in a test tube and autoclave for 15 min at 121°C.

b. In a petri dish, add 25 ml of sterile distilled water and 2 or 3 drops (0.1 ml) of sterile 10% yeast extract solution, and add several (at least eight) pieces of sterile hair.

c. Place several inoculum pieces of the test fungus into the suspension, preferably so that they contact some of the hairs. Incubate at room temperature. Set up positive and negative control tests on separate plates.

d. Examine test fungus and controls on day 7 and at 7-day intervals thereafter until a definitive result is obtained. The cutoff for negative results is 4 weeks.

e. Remove a few conspicuously fungally colonized hairs; mount them in KOH, NaOH, or lactophenol cotton blue mounting medium; and examine their whole lengths under low-power microscopy. Search for the conspicuous perpendicular pits indicative of hair perforation. Artifacts, such as fractures of the hair, burns from overheated forceps, and especially elongated trapped air bubbles, must be distinguished. If in doubt, confirm the presence of the fungal perforating organ in the perforation pit. Positive and negative controls should be checked at the same time.

In recent years, as commercially available yeast extract has become more highly purified and as the diversity of antifungal inhibitors in shampoos has increased, it has become more difficult to obtain reliable performance of positive controls in at least some laboratories. There are alternative phenotypic tests for almost all decisions that can be made using the hair perforation test, except for the reference confirmation of *M. praecox* and *T. equinum* var. *autotrophicum,* which otherwise may be confused with *M. gypseum* and *T. mentagrophytes,* respectively.

D. Practical dermatophyte identification scheme

Below is a relatively cautious identification scheme that will serve to identify >99% of dermatophytes seen in laboratories in cosmopolitan areas. It is assumed that if contamination is detected, decontamination will be carried out, except in cases where identification of the isolate can be fully confirmed by examining characteristics whose formation is not affected by contamination (e.g., spiral appendages). The scheme will not suffice for all QC test isolates because some species seldom or never seen except in these tests—particularly *Microsporum nanum, T. schoenleinii,* and *Microsporum gallinae*—have been omitted. For these, see Table 8.9–10. *T. equinum* var. *autotrophicum* has also been omitted due to its extreme rarity outside of Australia and New Zealand.

VIII. DERMATOPHYTE IDENTIFICATION *(continued)*

1. Examine primary Sabouraud medium or other selective isolation medium at 7 and 14 days.

a. Cottony or deeply velvety white colonies with wine red reverse: confirm at least one typical drop-shaped microconidium and no spiral appendages or other atypical features; identify as *T. rubrum.*

b. Powdery to low-cottony cream to white colonies with pale to pale brownish reverse: confirm abundant round or broadly drop-shaped microconidia (not formed on long "matchstick" pedicels) plus at least one well-formed spiral appendage; identify as *T. mentagrophytes.*

c. Powdery to low-cottony whitish, yellow, or mahogany red-brown colonies with red-brown to sulfur yellow reverse: confirm abundant optically dense, broadly drop-shaped microconidia, often formed on broad matchstick pedicels, sometimes intermixed with balloon forms or filiform "caterpillar" branching clusters. If from tinea capitis or from known heavily infested geographic area, identify as *T. tonsurans.*

d. Flat to velvety thin colony with yellow reverse: confirm at least one thick-walled, roughened, and beaked macroconidium; identify as *M. canis.*

e. Granular, sandy colony with pale reverse: confirm abundant rough, thin-walled, fusoid macroconidia; identify as *M. gypseum* (if reverse is bright yellow, reference test for *M. praecox* is called for [reconsider *M. canis* if there is some doubt about whether macroconidial walls are thick or thin]; if red, reference test for *Microsporum racemosum* is called for).

f. Flat, olive-brown to olive-gray or dull yellowish brown colony with pale or yellowish reverse: confirm abundant broadly club-shaped macroconidia and no microconidia (if only chlamydospores are present, subculture and reexamine in 7 days); identify as *E. floccosum.*

g. Isolates with features suggestive of geophilic, nonpathogenic dermatophytoids, e.g., rough, thick-walled macroconidia with cellular compartments and colony with red reverse (*Microsporum cookei*); copious macroconidia intergrading with long microconidia in a continuous range from one- to six-celled (occasionally more) conidia [confirm no growth at 37°C and identify as *T. terrestre;* if 37°C growth occurs and colony reverse is red, go to item 2.b.(5) below for suspected Afro-Asiatic *T. rubrum* or *Trichosporon raubitschekii*]; isolates with long, smooth, thick-walled macroconidia with cellular compartments and usually purple colony reverse (*T. ajelloi*), confirm as in Table 8.9–10 where possible and otherwise do reference identification.

h. All other dermatophytes, including any suggestive of the first six species listed above but with any unusual appearance or features, subculture to minimal set of physiological test media suggested in the introductory paragraph of item VIII.C above. If any isolate is suggestive of a dermatophyte with a known growth factor requirement or standard auxiliary test (e.g., polished-rice test for *M. audouinii*) as outlined in Table 8.9–10, subculture to that test as well. Subculture very slow-growing, white to pale tan isolates suggestive of *T. verrucosum* and very slow-growing, deep purple-red isolates suggestive of *T. violaceum* to BCP milk glucose agar incubated at 37°C, as well as to the minimal physiological set and *Trichophyton* agars T3 and T4.

2. Examine minimal set of physiological test media after 7 to 10 days at 25°C.

a. Isolates with distinct zones of peripheral clearing on BCP milk glucose and no signs of contamination

(1) BCP milk glucose from 25 or 37°C incubation shows distinct zone of clearing; colony small, pale red or purple-red; poor growth on T1. Confirm T4 stimulation and red-purple colony for *T. violaceum;* con-

VIII. DERMATOPHYTE IDENTIFICATION *(continued)*

firm strongest *Trichophyton* agar growth on T3 and whitish to pale tan color for most *T. verrucosum* isolates, with equally strong growth on T4 in some isolates. Examine substrate mycelium from 37°C BCP milk glucose, and confirm chains of symmetrical chlamydospores for *T. verrucosum.* If asymmetric chlamydospores are seen instead *and* T4 growth is equal to T3 growth *and* especially if the patient may have a history of travel to east Africa (surrogate: Arabic, Eritrean, Ethiopian, or Somali surname) *and* especially if isolate is from endothrix tinea capitis, check with physician, rule out recent contact with cattle, and confirm atypical pale *T. violaceum.*

(2) BCP milk glucose shows small but consistent zone of clearing all around colony; pH alkaline; colony on this medium brownish, yellow, or red with radial striations; urea negative (rarely positive); no growth on T1: confirm reflexive branching in striations; typical *T. soudanense.*

(3) Other colonies with zones of clearing: check for bacterial contamination by streaking to blood agar at 37°C; if positive, purify and resubculture to minimal physiological medium set. If negative, reference examination is needed.

b. Isolates with no clearing and no pH change on BCP milk glucose and normal growth on T1

(1) Urease negative; BCP milk glucose colony restricted, cottony, with peripheral wine red pigment. Confirm at least one drop-shaped microconidium on T1 or sporulation medium (e.g., potato flake agar); *T. rubrum.*

(2) Urease positive; BCP milk glucose colony profuse, flat, often with yellow pigment. Confirm at least one thick-walled, roughened, and beaked macroconidium. Confirm that colony is not salmon colored or pale brownish with few and mostly distorted macroconidia *and* from a child; if it combines these features, do polished-rice test to rule out *M. audouinii.* Otherwise, identify as *M. canis.* If isolate tested to rule out *M. audouinii* produces vigorous growth on polished rice (yellow pigment will also usually, but not always, be formed), identify as *M. canis.* If isolate shows poor growth on polished rice (brownish pigment will also usually, but not always, be formed), identify as *M. audouinii.*

(3) Urease negative; BCP milk glucose colony profuse, flat. Sabouraud colony has salmon or pale brownish pigment, conidiation sparse or absent on both Sabouraud and BCP milk glucose. Distorted, beaked, partially rough-walled macroconidia may be seen, as may microconidia, small aerial arthroconidia, pectinate branches, or terminal chlamydospores. Isolate is from child, usually from scalp. Do polished-rice test. If isolate shows poor growth (brownish pigment will also usually, but not always, be formed), identify as *M. audouinii.* Otherwise, reference identification is needed.

(4) Urease positive; BCP milk glucose colony profuse, powdery, reminiscent of *T. mentagrophytes.* Subculture to Sabouraud and incubate at 37°C; also subculture to Sabouraud plus 3% NaCl *and* Sabouraud plus 5% NaCl (just add the stated proportion of salt to regular Sabouraud recipe while making up in-house). Confirm poor 37°C growth and abundant formation of rough-walled macroconidia on at least one of the two salt media; *Microsporum persicolor.*

(5) Urease positive; BCP milk glucose colony restricted, velvety, with wine red reverse pigment. Confirm copious macroconidia and micro-

VIII. DERMATOPHYTE IDENTIFICATION *(continued)*

conidia on Sabouraud, usually also on T1 (however, microconidia are more predominant on this medium); intense red colony reverse pigment on Sabouraud and on potato flake, cornmeal glucose, or lactrimel if used; Afro-Asiatic *T. rubrum*, considered by some authors to be separate species *T. raubitschekii*.

c. Isolates with no clearing and no pH change on BCP milk glucose and reduced growth on T1

Urease positive; BCP milk glucose colony powdery; abundant optically dense, broadly drop-shaped microconidia on BCP milk glucose and Sabouraud, often formed on broad matchstick pedicels, sometimes intermixed with balloon forms or filiform "caterpillar" branching clusters. Confirm growth stimulation on T4; *T. tonsurans.*

d. Isolates with no clearing but with pH change to alkaline (blue-violet to purple color) on BCP milk glucose and normal growth on T1

(1) Urease positive; BCP milk glucose colony profuse with granular, powdery, velvety, or cottony surface texture; copious round or drop-shaped microconidia present; wine red pigments not formed on any medium (chestnut red-brown pigments rarely formed); confirm at least one well-formed spiral appendage; *T. mentagrophytes.*

◩ **NOTE:** If copious macroconidia intergrading with long microconidia are present on Sabouraud glucose agar, a test for growth at 37°C should be done to rule out *T. terrestre.*

(2) Urease positive; BCP milk glucose colony yellow-brown or cottony white with or without yellow margin; no microconidia present; confirm at least a small number of broadly clavate, short macroconidia; atypical *E. floccosum.*

(3) Urease negative or positive; BCP milk glucose colony profuse, flat. Sabouraud colony has salmon or pale brownish pigment; conidiation sparse or absent on both Sabouraud and BCP milk glucose. Distorted, beaked, partially rough-walled macroconidia may be seen, as may microconidia, small aerial arthroconidia, pectinate branches, or terminal chlamydospores. Isolate is from child, usually from scalp. Do polished-rice test. If isolate shows poor growth (brownish pigment will also usually, but not always, be formed), identify as *M. audouinii.* Otherwise, reference identification is needed.

(4) Urease positive or negative; BCP milk glucose colony profuse but only moderately rapidly growing (same rate as on Sabouraud; not restricted with respect to Sabouraud growth rate); colony with wine red reverse on Sabouraud. Confirm absence of contamination by streaking a plate of blood agar and incubating it at 37°C; inoculate T6 and T7. If stimulation is seen on T7, indicating histidine requirement, call physician and confirm history of travel to Portugal, the west Mediterranean, or central Africa; *T. megninii.* Otherwise, reference identification is needed.

(5) Urease positive; possibly weak at 7 days but strong at 10 days. BCP milk glucose colony profuse but only moderately rapidly growing (same rate as on Sabouraud; not restricted with respect to Sabouraud growth rate); colony with yellow to yellow-orange reverse on Sabouraud and yellow peripheral growth on BCP milk glucose. Confirm yellow, coiled nodular bodies in subsurface mycelium, at least some broadly drop-shaped or round microconidia; *T. mentagrophytes* "nodular variant," sometimes considered a separate species, *Trichophyton krajdenii* (*see* comment in Table 8.9–10). The occasionally seen name "T. mentagrophytes var. nodulare" is apocryphal (no such taxon was ever formally described) and cannot be used.

VIII. DERMATOPHYTE IDENTIFICATION *(continued)*

(6) Urease negative; BCP milk glucose colony profuse, with granular surface texture; copious round or drop-shaped microconidia present; wine red pigments not formed on any medium; colony reverse yellow; spiral appendages present. Confirm that patient may have had contact with hedgehog or with soil in region inhabited by hedgehogs; *T. mentagrophytes* var. *erinacei.*

e. Isolates with no clearing but with pH change to alkaline (blue-violet to purple color) on BCP milk glucose and reduced growth on T1

(1) Urease positive; BCP milk glucose colony powdery; abundant optically dense, broadly drop-shaped microconidia on BCP milk glucose and Sabouraud, often formed on broad matchstick pedicels, sometimes intermixed with balloon forms or filiform "caterpillar" branching clusters. Confirm growth stimulation on T4; *T. tonsurans.*

(2) Urease positive; BCP milk glucose colony velvety, with colorful (yellowish to red-brown) reverse; drop-shaped conidia present, sometimes on short pedicels; no growth at all on T1. Confirm normal growth on T5; *T. equinum* var. *equinum.*

POSTANALYTICAL CONSIDERATIONS

IX. REPORTING AND INTERPRETATION

A. General considerations

Full reporting of mycological identifications may take time, and the problem of preliminary reporting, omnipresent in medical laboratory work, is particularly exacerbated. There are two nearly contradictory general principles that should be kept in mind: (i) anything that the laboratory knows about the case that the physician needs to know in order to treat the patient correctly should be reported, and (ii) laboratories should, as much as possible, avoid sending out preliminary reports that they will later have to contradict. These two exigencies call for the development of maximally accurate preliminary reporting systems.

B. Dimorphic fungi

The laboratory is often well aware before the formal confirmation of identity that a fungus is highly unlikely to be anything other than a dimorphic pathogen. This is especially the case when characteristic structures have been seen in direct bodily specimen microscopy. In some cases, rapid reporting may be essential to save the patient's life.

1. Report suspected diagnosis with any caveat (e.g., suggestive of . . . ; confirmation to follow) by telephone. Keep a record of notification, including name of person receiving report.

2. Send a fax only if it will be received immediately by suitable personnel and receipt is confirmed.

3. If confirmation using DNA probes can be achieved the same day, within normal hours, delay notification until the result is obtained.

C. Opportunistic and subcutaneous fungi from normally sterile sites

1. Reporting the growth of "a mould" as soon as such growth is evident but before identification structures are formed is important where immunocompromised patients are concerned but is not normally necessary in cases of subcutaneous infection (e.g., mycetoma) of immunocompetent patients. In the latter type of case, where direct microscopy or histopathology indicates the presence of fungal structures, such preliminary culture reports are redundant. Unless there are contraindications, it is more useful to identify the causal agent at least to the genus level before sending out a preliminary report. Genus level identification of moulds, in general, has a high correlation with appropriate antifungal drug selection today and should be carried out

IX. REPORTING AND INTERPRETATION *(continued)*

as soon as possible. Preliminary reporting of a few broad categories with potential treatment significance may also be useful when recognized

◩ **NOTE:** Recommended treatments may change over time or in different geographic areas; they are noted here only to substantiate the potential value of these preliminary reports.

- **a.** "A zygomycetous fungus with aseptate hyphae; identification to follow" (treatable mainly with amphotericin B)
- **b.** "A 'black yeast' fungus in the related genera *Exophiala, Wangiella, Phaeococcomyces,* or *Phaeoannellomyces;* identification to follow" (itraconazole and ketoconazole may be most commonly used)
- **c.** "A dematiaceous fungus; identification to follow" (treatment options often as for black yeasts, i.e., itraconazole or ketoconazole)

2. Generic reports of the following organisms are especially important as preliminary reports because of their important treatment ramifications. They concern agents that may be involved in rapidly progressing opportunistic infection.

- **a.** Zygomycetes (Mucormycotina): *Rhizopus, Rhizomucor, Absidia,* and *Cunninghamella*
- **b.** Hyalohyphomycetes (hyaline filamentous moulds): *Aspergillus, Fusarium, Scedosporium, Acremonium, Paecilomyces,* and *Trichoderma*
- **c.** Phaeohyphomycetes (melanized filamentous "dematiaceous" moulds): *Curvularia, Exserohilum, Bipolaris, Dactylaria* (*Ochroconis*), and *Scedosporium*

  ◩ **NOTE:** *Scedosporium* has been included with both the hyaline and melanized groups above because in preliminary examination it may appear either way, depending on the individual isolate and the growth medium.

3. For *Fusarium* spp. not immediately forming macroconidia, distinction from related fungi, such as *Acremonium* and *Cylindrocarpon,* may be delayed. It may be useful to preliminarily report these fungi collectively as "an atypical Hypocrealean fungus, e.g., *Fusarium, Acremonium,* or a related genus; identification to follow." Treatments of members of this group of fungi have many commonalities (e.g., poor susceptibility to amphotericin B is common). The fungal order name Hypocreales is not yet widely familiar in biomedicine but appears destined to become so soon as molecular biology reorganizes fungal taxonomy.

4. In tropical and subtropical areas, including the U.S. Gulf Coast area, aseptate mycelial cultures that may be the oomycete *Pythium insidiosum* should also be preliminarily recognized as rapidly as possible (very rapidly growing nonsporulating aseptate mycelium, usually from subcutaneous biopsy; sporulation with swimming zoospores may be induced by placing a block of colonized agar in water).

5. In addition, some species identifications are especially important for their urgent treatment ramifications and should be performed as soon as possible on fungi from normally sterile sites.

- **a.** Zygomycetes (Mucormycotina): *Apophysomyces elegans* and *Saksenaea vasiformis* (mainly agents of severe cutaneous, subcutaneous, and osteomyelitic zygomycosis sometimes necessitating limb amputation, especially if allowed to progress through diagnostic delay)
- **b.** Hyalohyphomycetes: *Paecilomyces variotii* (polyene [e.g., amphotericin B] susceptible), *Paecilomyces lilacinus* (often polyene resistant), and *Scedosporium apiospermum* (all agents of a wide variety of opportunistic mycoses)
- **c.** Phaeohyphomycetes: *Scedosporium prolificans* and *S. apiospermum* (difficult-to-treat agents of a wide variety of opportunistic phaeohyphomy-

IX. REPORTING AND INTERPRETATION *(continued)*

cotic manifestations, including disseminated infection; early identification may be critical in some cases)

D. Corneal scrapings or ocular aspirates

Growth of any mould whatsoever entails an immediate, telephoned preliminary report that a mould is growing. The same broad-category generic and species names given above as urgent when from normally sterile systemic sites are equally urgent for ocular outgrowths. The rapid recognition of *Paecilomyces lilacinus*, which is usually resistant to the commonly used pimaricin-natamycin and topical amphotericin B, is especially important. It should be recognized that many species, such as *Penicillium citrinum*, that are generally thought of as common contaminants are well-known and serious pathogens of mechanically traumatized corneas and that no outgoing report should suggest without qualification that they are insignificant.

Use the following note with all identifications of non-dimorphic-mould fungi from eyes where information about etiologic verification (e.g., a note that fungal filaments were seen in corneal scrapings) has not been included in the laboratory requisition.

"This species is a normally nonpathogenic, common contaminant, but an indefinite number of such fungi are capable of causing mycotic keratitis subsequent to traumatic inoculation. If keratitis is suspected in this case, confirmation can be accomplished by demonstrating fungal filaments directly in corneal scrapings."

Such a note is unnecessary when the report is directed to a recipient known to have expertise in ocular mycoses or when the isolate or specimen is stated to be from endophthalmitis or from an immunocompromised patient.

E. Nonsterile respiratory sites (except sinuses)

Screening that allows the most rapid recognition and preliminary or final reporting of dimorphic fungi from nonsterile respiratory sites is of urgent concern. This matter is dealt with in item IX.B above. It should be noted in addition, however, that any pale mould that grows out on cycloheximide-containing medium from a respiratory specimen should be under a high index of suspicion as a possible dimorphic fungus until microscopically demonstrated otherwise. Nonsporulating, whitish, cycloheximide-tolerant fungi remain suspect, and in areas where *Blastomyces* is known to be endemic they should be subcultured to specialized (48- to 72-h) conversion medium, such as Blasto D medium, immediately. It is the judgment call of the laboratory director, in consultation with the physician, as to when to do probe or exoantigen tests on such isolates and which species to test for. Even the normally heavily sporulating and easily preliminarily recognized *Coccidioides* is occasionally isolated as a form with strongly delayed and aberrant conidiation.

Equally important is the preliminary reporting of aspergilli, zygomycetes, and *Fusarium* and *Scedosporium* spp. from any patients who are or may be immunocompromised. These reports may be at the genus level or at the broad-category levels outlined above for fungi from normally sterile sites. Species level reporting should follow unless the isolate in question has been ruled out as a potential opportunist (e.g., shown to be a *Mucor* sp. not growing at body temperature or a *Fusarium* sp. not corresponding to any of the described opportunistic species in the genus and not suggested by case circumstances to be a novel opportunist).

The genus *Aspergillus* is complex in that it contains both aggressive opportunists and common, almost entirely nonpathogenic fungi. A preliminary genus level report may be necessary where species identification cannot be performed immediately, but it carries a relatively low level of medical resolution. The following species are potentially opportunistic and should be reported at the species level as soon as possible, not because the species name has particular

IX. REPORTING AND INTERPRETATION *(continued)*

treatment significance (except with *Aspergillus terreus*), but to show that the fungus has been confirmed as a potentially opportunistic *Aspergillus* sp.: *Aspergillus fumigatus, Aspergillus flavus, A. terreus, Aspergillus nidulans,* and *A. niger.* On the other hand, *Aspergillus ochraceus, Aspergillus versicolor, Aspergillus sydowii,* and members of the *Aspergillus glaucus* complex have very little opportunistic potential, even in severely immunocompromised patients. These species should also be reported at the species level, along with the comment "normally nonpathogenic."

The very significant fungus that has commonly been misidentified in past medical literature as *A. glaucus* and *A. versicolor* (as well as *Aspergillus restrictus, Aspergillus conicus, Aspergillus penicillioides,* and *Penicillium spinulosum*) is the host-adapted form of *A. fumigatus,* which is isolated either as a nonsporulating fungus or as a fungus with few and aberrant conidiogenous structures. This fungus can be rapidly preliminarily recognized by outgrowth of whitish, often dense and radially folded colonies from respiratory sources. Usually, although not always, isolation plates show several similar colonies. Subculture such colonies immediately to sporulation medium (e.g., PGA or Leonian's), and place these subcultures at 45 and 37°C. In almost all cases, growth at 45°C will be detectable within 24 h. I have routinely used the following preliminary report. "A nonsporulating fungus able to grow at 45°C in vitro has been isolated. Although such isolations from respiratory samples may be insignificant, they often indicate the host-adapted 'dysgonic' variant of *Aspergillus fumigatus.* Full identification of this organism may be delayed. Further report to follow."

Scedosporium spp. should be identified at the species level as soon as possible, because treatments may differ. Because *S. apiospermum* may or may not form its *Pseudallescheria boydii* teleomorph, report this species as "the *Scedosporium apiospermum* asexual state of *Pseudallescheria boydii*" even when only the asexual morph is seen. This report may appear cumbersome, but it gives the physician access to all relevant literature keywords. The mycology laboratory director's telephone number should be on the outgoing report in all mycology reporting in case clarification is needed. Be aware that the *Scedosporium* state of *Petriella setifera* may also be seen, and this fungus of very low virulence should not be reported as *S. apiospermum.* It is microscopically similar to the latter until the perithecial sexual state develops after 14 days, but it appears atypical macroscopically in young growth, presenting either as a heavily tufted (synnematous), flat colony or as a white colony with little conidiation. *S. apiospermum,* on the other hand, is rather constant as a heavily powdery or, more often, woolly mouse gray-brown colony, with some ropy synnemata or hyphal bundles but not with a consistent growth of short (1-mm), upright tufts on a flat background. When in doubt, report "*Scedosporium* species; atypical; full identification to follow."

Respiratory specimens intermix potential pathogens with the entire incident outdoor and indoor air spora, and it is common to isolate organisms such as *P. variotii, Fusarium oxysporum, Beauveria bassiana,* and *Engyodontium album* that are potentially opportunistic but that are far more commonly isolated from respiratory specimens as insignificant organisms. For these fungi, the following clarifying note is used in all cases of such isolation. "This normally nonpathogenic, common contaminant may uncommonly cause opportunistic infection, usually in the severely immunocompromised patient."

The reporting of nonpathogenic respiratory contaminants to physicians may give the false impression that a potential disease agent has been reported and that treatment should be considered. On the other hand, however, it remains possible that a species never before recognized as an opportunist may cause an

IX. REPORTING AND INTERPRETATION *(continued)*

opportunistic infection. Some of the fungi commonly growing from respiratory specimens, especially *Cladosporium* spp., are well known to have maximal growth temperatures well below body temperature and to have little or no realistic potential as opportunists. The quantity and diversity of such organisms growing out may make it very difficult for larger or busier laboratories to deal with them except by ruling out known opportunists, especially as ~10 to 20% of the insignificant fungi that grow out are persistently nonsporulating wood-decaying basidiomycetes or plant-associated ascomycetes that cannot be conventionally identified at the genus level in culture. It is convenient for mycologically sophisticated laboratories to recognize locally common insignificant fungi at the genus or broad-group level and simply not to report them. There are "gray area" fungi, such as *Penicillium chrysogenum, P. citrinum,* and *Alternaria alternata,* that grow from respiratory specimens on a daily basis but are also validly reported from a respiratory infection approximately once per decade worldwide. These fungi should be reported at least at the genus level if the patient is stated to be immunocompromised or if there is an extenuating circumstance signaling possible infection, in particular, filaments seen in direct microscopy or in biopsy histopathology that are not connected with a more likely opportunist. (Unfortunately, quantity of growth is not a reliable criterion, as prevalent airborne fungi may grow from respiratory specimens, including bronchoalveolar lavage fluid, in large numbers.)

F. Nasal and facial sinus scrapings

To avoid insignificant contaminants being isolated from nasal and facial sinus sites, an effort should be made to connect the species isolated with filaments seen in direct microscopy. Most genuine sinus colonizations are associated with heavy fungal colonization, yielding a typical direct microscopy presentation of a thickly interwoven, blanketing layer of fungal filaments. This heavy growth may not coat the entire sinus interior, but pockets containing such growth exist and are normally readily detected in typical surgically obtained specimens. These masses of filaments are frequently mostly or entirely dead, but if growth is obtained in culture at all, it is usually obtained as multiple consistent colonies. The following filamentous fungi are most commonly involved.

1. Zygomycetes (Mucormycotina): *Rhizopus* spp.
2. Hyalohyphomycetes: *A. flavus, A. fumigatus, A. nidulans, A. terreus, S. apiospermum,* and *Schizophyllum commune*
3. Phaeohyphomycetes: *A. alternata, Bipolaris* spp., *Curvularia* spp., *Exserohilum* spp., *S. apiospermum,* and *S. prolificans*

Other fungi well known as opportunists, such as *Scopulariopsis brevicaulis, P. lilacinus,* and *P. variotii,* may occasionally be significant, especially in immunocompromised patients.

Growth of *Rhizopus* can be correlated with aseptate filaments, aspergilli and *Scedosporium* spp. have septate filaments and sometimes produce typical conidia in situ (or ascomata with ascospores in the case of *A. nidulans*), the basidiomycetous mushroom *Schizophyllum* usually has clamp connections visible in direct microscopy, and the filaments of *Bipolaris, Curvularia,* and *Exserohilum* are usually conspicuously melanized. *Alternaria* filaments may or may not appear melanized (also, heme derived from blood may make *Aspergillus* filaments in sinus scrapings appear slightly brownish, so that they may be nearly as colored as *Alternaria* filaments) but will stain as melanized in the common histopathological Fontana-Masson melanin stain.

A publication arguing that all fungi isolated from partially lysed nasal mucus may be involved in causing sinusitis (18) overlooks or ignores the established presence of dormant air-derived conidia frequently trapped in this mucus. Such fungal spores are usually unable to grow at sinus cavity temperature and thus

IX. REPORTING AND INTERPRETATION *(continued)*

are highly unlikely to be involved in disease production, except in cases in which they can be confirmed as correlating with the presence of fungal elements in direct microscopy of scrapings. Confirming the insignificance of most cultures from nasal mucus is the finding in the same study (18) that similar fungi can be isolated from nasal-mucus lysates obtained from individuals without signs or symptoms of sinus infection. Thus, culture results from this technique should not be reported in a way that suggests causation of infection unless fungal establishment is confirmed by detecting vegetative structures (e.g., filaments, pseudohyphae, or budding yeast cells) in direct microscopy.

G. Ear canals

The isolation of insignificant contaminating inoculum from ear canals is common, and if possible, all isolations should therefore be correlated with direct microscopic evaluation before being reported. An exception should be made in apparent invasive infections of immunocompromised patients, in which case all fungi grown should be reported rapidly. Genuine otomycotic colonization of immunocompetent patients, when caused by moulds, features a very heavy investment of fungal filaments in the ear canal, and these filaments usually bear copious conidiogenous structures, especially when aspergilli are involved. The fungus isolated can normally, though not always, be correlated with that seen in direct microscopy by examining this in situ conidiation. The in situ conidiation leads to very heavy outgrowth on the primary isolation plate, so if information is not available on direct microscopy (e.g., where the fungus has been elucidated incidentally by a bacteriological swab study for culture only), the occurrence of confluent outgrowth of many dozens of colonies may be taken as suggestive of causality in otomycosis. Suspected agents of otomycosis may be preliminarily reported by genus name if species identification will take additional days, since otomycotic colonizations may be painful. The following species are most commonly involved: *A. niger, A. fumigatus, A. terreus, A. flavus, A. nidulans, S. apiospermum,* and *S. prolificans.* Occasionally, unusual fungi will be involved, and it should always be borne in mind that dimorphic fungi may be isolated from any body site.

H. Skin scrapings yielding nondermatophytes

For filamentous fungi other than dermatophytes obtained from dry dermatological skin scrapings (i.e., not from skin biopsies, punch biopsies, pus aspirates, wound swabs, burn eschar, etc.), only two organisms, *Scytalidium dimidiatum* (pycnidial synanamorph name, *Nattrassia mangiferae;* outdated name, *Hendersonula toruloidea*) and the possibly conspecific *Scytalidium hyalinum,* are likely to be etiologically significant. These two *Scytalidium* spp. may be reported directly as etiologic agents just as dermatophytes are *except in rare cases where all three of the following conditions apply:* (i) the fungus obtained is a "type 1" fast-growing, woolly *S. dimidiatum* isolate (16), (ii) no filaments were detected in direct microscopy of the skin specimen (reexamination of any residual skin scrapings is warranted if this was the initial finding prior to culture), and (iii) the laboratory is in a tropical or subtropical area where potential host plants of the wild-type type 1 *S. dimidiatum,* such as pecan or mango, occur. The two significant *Scytalidium* spp. are recognized by cycloheximide sensitivity in vitro (~5% of isolates are semitolerant as defined in item VII.A above) and by their production of branching chains containing both one-celled and two-celled arthroconidia that vary conspicuously in size. *S. dimidiatum* is dark gray, while *S. hyalinum* is white with a pale yellowish colony reverse (23).

Scytalidium isolations from skin and nails may be reported as follows. "*Scytalidium* [insert species name]. This species frequently causes dermatophytosis-like infections of nails, soles, and palms. It is usually resistant to antifungal drugs in vivo, despite appearing susceptible in in vitro tests."

IX. REPORTING AND INTERPRETATION *(continued)*

On rare occasions, especially in immunocompromised patients, the cornified skin epidermis may be colonized by moulds other than dermatophytes and *Scytalidium.* Such cases can be verified only by consistent repeated isolation from successive samples taken at different time points *in combination with* demonstration of compatible fungal elements in direct microscopy or histopathology.

I. Toenails and fingernails yielding nondermatophytes

1. Filamentous fungi other than dermatophytes obtained from toenails and fingernails present by far the most ecologically complex area of mycological diagnosis. *S. dimidiatum* and *S. hyalinum* are simple: they should be reported under the same conditions as given in item H above for dry skin scrapings.

Fungi other than *Scytalidium* spp. that have repeatedly been rigorously shown to cause onychomycosis in the absence of a concomitant dermatophyte are relatively few in number. The most commonly significant are listed in Table 8.9–11. All of these fungi may also be isolated purely as insignificant contaminants; in fact, in most cases, they are much more commonly seen as insignificant isolations than as significant isolations. In addition, there are a few gray-area fungi, such as *A. alternata* and *Chaetomium globosum,* that are abundant nail contaminants and that are only extremely rarely seen as etiologic agents. These fungi have been excluded from Table 8.9–11 and are discussed further below.

The fungi listed in Table 8.9–11 may grow in laboratory specimens under a bewildering variety of significant conditions. These conditions are listed below, marked with one to four asterisks for situations varying from uncommon (one asterisk) to common (four asterisks). All are regularly encountered in larger laboratories. The following describe fungi in Table 8.9–11 when grown in culture from nails, especially the hallux (large) toenail.

a. If grown out in cases with unusual, definitely nondermatophytic microscopic structures seen invading the nail tissues in direct microscopy or histopathology, they

(1) May be sole agents of onychomycosis, growing alone and connected with distinctive structures in direct nail microscopy (e.g., *Scopulariopsis*-like lemon-shaped conidia or *Aspergillus* heads with conidia)**

(2) May be agents of a mixed infection with a dermatophyte which failed to grow either because it was overgrown by the mould or because it was not viable or not present in the exact portion of nail that was sampled*

(3) May be agents of a mixed infection with a dermatophyte that also grows out*

b. If grown out in cases with ordinary, possibly dermatophytic or possibly nondermatophytic filaments seen in direct microscopy and *with no dermatophyte growing,* they

(1) May be sole agents of onychomycosis, growing alone and connected with the nondistinctive, septate filaments seen in direct microscopy**

(2) May be contaminants growing alone on isolation media and seemingly (although falsely) connected with the nondistinctive, septate filaments that in fact (as revealed by later repeat cultures or by specific immunohistochemistry) are dead dermatophyte filaments (the connected dermatophyte infection has living elements elsewhere in the nail, but the portion sampled contained only dead filaments)****

(3) May be contaminants that have overgrown a causal dermatophyte on isolation media***

(4) May be agents of mixed onychomycosis co-occurring with a causal dermatophyte that by chance is not isolated from the initial filaments or that has been overgrown*

Table 8.9–11 Most common fungi other than dermatophytes and *Scytalidium* repeatedly and rigorously implicated as causal agents of onychomycosis

Acremonium potronii
Acremonium strictum
Aphanoascus fulvescens
Aspergillus candidus
Aspergillus flavus
Aspergillus fumigatus
Aspergillus nidulans
Aspergillus niger
Aspergillus sydowii
Aspergillus terreus
Aspergillus versicolor
Fusarium oxysporum
Fusarium solani
Microascus cinereus
Onychocola canadensis
Scopulariopsis brevicaulis

IX. REPORTING AND INTERPRETATION *(continued)*

c. If grown out in cases with ordinary, possibly dermatophytic or possibly nondermatophytic filaments seen in direct microscopy and *with a dermatophyte growing*, they
  (1) May be contaminants insignificantly co-occurring with a causal dermatophyte****
  (2) May be agents of mixed onychomycosis significantly co-occurring with a causal dermatophyte*

d. If grown out in cases with no filaments seen in direct microscopy and *with a dermatophyte growing*, they
  (1) May be contaminants isolated along with a dermatophyte, and the filaments of the causal dermatophyte may be missed in direct microscopic analysis or histopathology**
  (2) May be agents of mixed infection growing along with a dermatophyte, and the filaments of both the dermatophyte and the nondermatophyte may have been missed in direct microscopic analysis or histopathology*

e. If grown out in cases with no filaments seen in direct microscopy and *with no dermatophyte growing*, they
  (1) May be contaminants growing purely from sedimented conidia with no correlated filaments in the nail****
  (2) May be sole agents of onychomycosis growing alone, but their filaments may be relatively sparse and may have been missed in direct microscopic analysis (especially a problem with *Acremonium* from superficial white onychomycosis)**

Additional details are given by Summerbell (24). Note that the well-demonstrated (1) reality that more than one nondermatophyte may co-occur etiologically in the same nail, in the presence or absence of a dermatophyte, has not been permutationally expatiated in the list in terms of the possible laboratory presentations that may ensue. Further permutations are generated when physicians mix materials from separate nails together in the same specimen packet, since individual nails may have separate nondermatophyte colonizers (unlike dermatophytes, these nondermatophytes cannot grow from nail to nail across contiguous skin; they also differ from dermatophytes in being unlikely to invade successive nails contagiously). For example, see reference 7, in which a patient with *A. terreus* established in one hallux nail and *Onychocola canadensis* in the other is documented. It is not surprising, therefore, that this complexity coincides with an area of diagnosis often proclaimed to be controversial, mainly because simplistic beliefs and solutions avoiding the above-mentioned complexity may be fervently adhered to despite abundant counterevidence.

2. After much experience and consultation with dermatologists and researchers, I developed the following system of reporting the fungi listed in Table 8.9–11 from nails.

a. If a fungus from Table 8.9–11 is grown in the presence of distinctive elements that explicitly confirm its nail invasion, the elements seen are characterized as nondermatophytic and are described in the direct microscopic report (e.g., “dark [melanized] fungal filaments seen”), and the fungus is identified by name and noted as consistent with the elements seen.

b. If a fungus from Table 8.9–11 is grown from a specimen for which direct microscopy shows nondistinctive fungal filaments, the species is identified and the following note is appended. “This common, normally nonpathogenic fungus, when isolated from filament-positive nail material, is usually a contaminant overgrowing nonviable dermatophyte inoculum.

IX. REPORTING AND INTERPRETATION *(continued)*

Uncommonly, it causes onychomycosis and can be confirmed as an etiologic agent by consistently repeated isolation in the absence of a dermatophyte. Rare mixed infections will show consistently repeated isolation in successive samples, along with at least one isolation of a dermatophyte."

c. If a fungus in Table 8.9–11 is grown from a specimen for which direct microscopy is negative, the species is identified, and the following note is attached (based on numerous incidents in which nondermatophytes first isolated in such cases later proved to be causal and to have localized filaments that were detectable after extensive searching). "In cases like the present one where direct microscopic examination shows no mycelium, this isolation would ordinarily be considered surface contamination. Since the above species does occasionally cause onychomycosis, however, in addition to being a common contaminant, this isolation is noted for your records."

d. If a fungus in Table 8.9–11 is grown along with a dermatophyte, it is reported beneath the dermatophyte report, and the following note is appended. "This common, normally nonpathogenic fungus, when isolated concurrently with a dermatophyte, is usually an insignificant contaminant. Uncommonly, it is involved in a mixed onychomycosis infection and can be confirmed as established in the nail by consistently repeated isolation in successive samples. Whether such fungi, if established in the nail, may be concomitantly eliminated by therapy directed against the dermatophyte is in many cases not reliably predicted by their antifungal susceptibilities in vitro."

e. If a species from Table 8.9–11 is received from another laboratory with a requisition stating that it is from a nail but with no indication of whether direct microscopy was positive or negative for fungal elements, the species is identified, and two successive notes are attached.

(1) If direct microscopy (hydroxide, calcofluor, or periodic acid-Schiff [PAS]) of the nail specimen is negative for fungal filaments, this isolate would conventionally be regarded as a contaminant. If direct microscopy is positive, the note in item (2) below applies.

(2) This common, normally nonpathogenic fungus, when isolated from filament-positive nail material, is usually a contaminant overgrowing nonviable dermatophyte inoculum. Uncommonly, it causes onychomycosis and can be confirmed as an etiologic agent by consistently repeated isolation in the absence of a dermatophyte. Rare mixed infections will show consistently repeated isolation in successive samples, along with at least one isolation of a dermatophyte.

The net effect of all these notes is to elicit repeat samples in situations where physicians judge that these samples are applicable, given accurate information about the opportunistic nature of the fungi isolated and their own knowledge about how patients are responding to therapy. Not all the species known to rarely cause opportunistic onychomycosis are listed in Table 8.9–11 (see reference 23 for others), and species are still being added to the list of rigorously confirmed agents of onychomycosis.

■ *Spurious reports based on inadequately confirmed cases are also relatively common.*

Especially in the case of fungi such as *C. globosum* that are ubiquitous as nail contaminants but very rarely confirmed as agents of onychomycosis, reporting all possible agents of opportunistic onychomycosis as Table 8.9–11 fungi would be an immense and unjustifiable generator of inconsequential reports. Non-*Scytalidium* moulds other than Table 8.9–11 fungi are best only

IX. REPORTING AND INTERPRETATION *(continued)*

reported as possibly implicated in opportunistic onychomycosis, using the notes for Table 8.9–11 fungi above, if no dermatophyte is isolated *and* if direct microscopy shows fungal elements that are unusual and that appear very likely not to belong to a nongrowing dermatophyte. *Chaetomium* and *Alternaria*, for example, when actually infecting nails, may have distinctive brown (melanized) hyphae in situ. As another example, very rare onychomycoses caused by *Exophiala (Wangiella) dermatitidis* show abundant dark yeast cells and filaments in nail tissue (23).

Some laboratories report all nondermatophyte isolations from nails, whether or not the organisms have ever been confirmed as causal agents of onychomycosis or possess the physiological prerequisites of such pathogenesis. This is sometimes represented as letting the physician decide the significance of these names. I have experienced a steady stream of calls from physicians and their staff for treatment advice for imagined infections caused by completely nonpathogenic fungi, such as *Epicoccum nigrum* and *Pithomyces chartarum*, in nails, frequently accompanied by comments that nothing can be found about these fungi in any book. Physicians in general practice and many specialties do not have access to large components of the mycology information base. The calls mentioned surely represent only a small portion of the medical confusion caused by this irresponsible reporting practice, and the probability that at least some patients will be treated, at considerable expense, for these nonexistent infections is very high.

Several recent publications in dermatology journals have recommended histopathology with fungus-specific stains (e.g., PAS) as a sole method of diagnosing onychomycosis. It should be noted that this technique is only able to distinguish dermatophytic from nondermatophytic elements in the rare cases in which the latter have highly distinctive features incompatible with a dermatophyte. This proposed technique is thus a recipe for misdiagnosing nondermatophytic onychomycosis as dermatophytosis and potentially for treating it with inappropriate drugs. Differential immunohistochemistry is theoretically able to solve this problem by distinguishing dermatophytic and nondermatophytic elements in nails with specific immunofluorescent antibody reactions, but to my knowledge this esoteric and laborious technique has only been utilized so far in a single research project (1). PCR is similar to culture in connection with nondermatophytic onychomycosis because it is unable to distinguish between etiologic elements and contaminating elements in a single specimen.

J. Wounds and ulcers sampled by swabs, and syringe-aspirated pus

For filamentous fungi other than potential dimorphic fungi obtained from wounds and ulcers of nonimmunocompromised patients sampled by bacteriological swabs or syringe aspirations of pus, several ecological situations need to be encompassed in reports. The most common cause of heavy fungal outgrowth from these samples is saprobic growth of moulds, especially *Fusarium* spp., on serous exudate on the surfaces of nonsterile open lesions. If a direct microscopic Gram smear or KOH mount is done, copious fungal filaments may be seen, but these are loose, not penetrating tissue. There are, however, some less likely possibilities.

1. A mycetoma in which grains were not observed either macroscopically or in direct microscopy but nonetheless were included in the sample and grew to yield the causal organism
2. An intact mycotic subcutaneous cyst sampled by syringe aspiration alone, or recently traumatically broken open and sampled by swab, with subsequent elucidation only of the material from loose filaments in the serous core of the cyst and not of the filaments penetrating the tissue of the cyst wall

IX. REPORTING AND INTERPRETATION *(continued)*

3. An opportunistic disseminated or primary cutaneous opportunistic infection in a patient whose immunocompromised status is unknown or has not been disclosed

Normally, nonpathogenic species from such samples are identified and given the following clarifying note. "This normally nonpathogenic, common organism often heavily colonizes surfaces of wounds and ulcers as a seemingly harmless saprobe (saprophyte) growing on serous exudate. In the strongly immunocompromised patient (usually neutropenic), invasion may be possible. If infection of an immunocompetent patient is long-lasting and involves deep tissue, then the possibility of a mycetoma or mycotic cyst may be considered. These conditions are demonstrable by biopsy histopathology showing fungal elements organized into granular structures in dermal tissue (mycetoma) or diffusely invading the dermal cyst wall (mycotic cyst). If the lesion extends to bone, mycotic osteoarthritis or, in compound fractures, osteitis may also be considered."

As with all the clarifying notes given above, this one is subject to modification by the laboratory director in cases where part of its contents may not be appropriate or on all occasions where new scientific knowledge renders it partially obsolete or incomplete. Medical mycology literature must be diligently followed to ensure that all such information remains up to date.

REFERENCES

1. **Arrese, J. E., C. Piérard-Franchimont, R. Greimers, and G. E. Piérard.** 1995. Fungi in onychomycosis. A study by immunohistochemistry and dual flow cytometry. *J. Eur. Acad. Dermatol. Venereol.* **4:**123–130.
2. **Bocobo, F. C., and R. W. Benham.** 1949. Pigment production in the differentiation of *Trichophyton mentagrophytes* and *Trichophyton rubrum*. *Mycologia* **41:**291–302.
3. **Chaturvedi, S., H. S. Chaturvedi, and Z. Khan.** 1990. Efficacy of brain heart infusion-egg albumen agar, yeast extract phosphate agar and peptone glucose agar media for isolation of *Blastomyces dermatitidis* from sputum. *Mycopathologia* **112:**105–112.
4. **Clinical and Laboratory Standards Institute.** 2004. Quality control for commercially prepared microbiological culture media; approved standard—3rd ed. CLSI Document M22-A3, Wayne, PA.
5. **Dixon, D., I. F. Salkin, R. A. Duncan, N. J. Hurd, J. H. Haines, M. E. Kemna, and F. B. Coles.** 1991. Isolation and characterization of *Sporothrix schenckii* from clinical and environmental sources associated with the largest U.S. epidemic of sporotrichosis. *J. Clin. Microbiol.* **29:**1106–1113.
6. **Franco, M., E. Bagagli, S. Scapolio, and C. S. Lacaz.** 2000. A critical analysis of isolation of *Paracoccidioides brasiliensis* from soil. *Med. Mycol.* **38:**185–191.
7. **Gräser, Y., A. F. Kuijpers, W. Presber, and G. S. De Hoog.** 1999. Molecular taxonomy of *Trichophyton mentagrophytes* and *T. tonsurans*. *Med. Mycol.* **37:**315–330.
8. **Gupta, A. K., C. B. Horgan-Bell, and R. C. Summerbell.** 1998. Onychomycosis associated with *Onychocola canadensis:* ten case reports and a review of the literature. *J. Am. Acad. Dermatol.* **39:**410–417.
9. **Hibbett, D. S., M. Binder, J. F. Bishcoff, et al.** 2007. A higher-level phylogenetic classification of the Fungi. *Mycol. Res.* **111:**509–547.
10. **Hoffman, K., S. Discher, and K. Voigt.** 2007. Revision of the genus *Absidia* (*Mucorales*, Zygomycetes) based on physiological, phylogenetic, and morphological characters; thermotolerant *Absidia* spp. form a coherent group, *Mycocladiaceae* fam. nov. *Mycol. Res.* **111:**1169–1183.
11. **Iwen, P. C., L. Sigler, S. Tarantolo, D. A. Sutton, M. G. Rinaldi, R. P. Lackner, D. I. McCarthy, and S. H. Hinrichs.** 2000. Pulmonary infection caused by *Gymnascella hyalinospora* in a patient with acute myelogenous leukemia. *J. Clin. Microbiol.* **38:**375–381.
12. **Kaminsky, G. W.** 1985. The routine use of modified Borelli's lactritmel agar (MBLA). *Mycopathologia* **91:**57–59.
13. **Mackinnon, J. E., I. A. Conti-Diaz, E. Gezuele, E. Civila, and S. da Luz.** 1969. Isolation of *Sporothrix schenckii* from nature and considerations on its pathogenicity and ecology. *Sabouraudia* **7:**38–45.
14. **McGinnis, M. R.** 1980. *Laboratory Handbook of Medical Mycology.* Academic Press, New York, NY.
15. **McGinnis, M. R., and L. Pasarell.** 1992. Mould identification, p. 6.11.1–6.11.17. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook*, vol. 1. American Society for Microbiology, Washington, DC.
16. **Moore, M. K.** 1988. Morphological and physiological studies of isolates of *Hendersonula toruloidea* Nattrass cultured from human skin and nail samples *J. Med. Vet. Mycol.* **26:**25–39.

REFERENCES *(continued)*

17. **Padhye, A. A., G. Smith, P. G. Standard, D. McLaughlin, and L. Kaufman.** 1994. Comparative evaluation of chemiluminescent DNA probe assays and exoantigen tests for rapid identification of *Blastomyces dermatitidis* and *Coccidioides immitis*. *J. Clin. Microbiol.* **32:**867–870.
18. **Ponikau, J. U., D. A. Sherris, E. B. Kern, H. A. Homburger, E. Frigas, T. A. Gaffey, and G. D. Roberts.** 1999. The diagnosis and incidence of allergic fungal sinusitis. *Mayo Clin. Proc.* **74:**877–884.
19. **Sewell, D. L.** 1992. Quality control, p. 13.2.1–13.2.35. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook*, vol. 2. American Society for Microbiology, Washington, DC.
20. **Sigler, L.** 1997. *Chrysosporium* and molds resembling dermatophytes, p. 261–311. *In* J. Kane, R. C. Summerbell, L. Sigler, S. Krajden, and G. Land (ed.), *Laboratory Handbook of Dermatophytes.* Star Publishing, Belmont, CA.
21. **St.-Germain, G., and R. C. Summerbell.** 1996. *Identifying Filamentous Fungi.* Star Publishing, Belmont, CA.
22. **Summerbell, R. C.** 1993. The benomyl test as a fundamental diagnostic method for medical mycology. *J. Clin. Microbiol.* **31:**572–577.
23. **Summerbell, R. C.** 1997. Nondermatophytic molds causing dermatophytosis-like nail and skin infection, p. 213–259. *In* J. Kane, R. C. Summerbell, L. Sigler, S. Krajden, and G. Land (ed.), *Laboratory Handbook of Dermatophytes.* Star Publishing, Belmont, CA.
24. **Summerbell, R. C.** 1997. Epidemiology and ecology of onychomycosis. *Dermatology* **194**(Suppl. 1)**:**32–36.
25. **Summerbell, R. C.** 2000. Form and function in the evolution of dermatophytes, p. 30–43. *In* R. K. S. Kushwaha and J. Guarro (ed.), *Biology of Dermatophytes and Other Keratinophilic Fungi.* Revista Iberoamericana de Micología, Bilbao, Spain.
26. **Summerbell, R. C., and J. Kane.** 1997. Physiological and other special tests for identifying dermatophytes, p. 45–77. *In* J. Kane, R. C. Summerbell, L. Sigler, S. Krajden, and G. Land (ed.), *Laboratory Handbook of Dermatophytes.* Star Publishing, Belmont, CA.
27. **Walsh, T. J., D. H. Larone, W. A. Schell, and T. G. Mitchell.** 2003. *Histoplasma, Blastomyces, Coccidioides*, and other dimorphic fungi causing systemic mycoses, p. 1781–1797. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, DC.

SUPPLEMENTAL READING

De Hoog, G. S., J. Guarro, J. Gené, and M. J. Figueras. 2000. *Atlas of Clinical Fungi*, 2nd ed. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Domsch, K.-H., W. Gams, and T.-H. Anderson. 1993. *Compendium of Soil Fungi.* IHW-Verlag, Eching, Germany.

Kane, J., R. C. Summerbell, L. Sigler, S. Krajden, and G. Land. 1997. *Laboratory Handbook of Dermatophytes.* Star Publishing, Belmont, CA.

Larone, D. H. 2002. *Medically Important Fungi*, 4th ed. ASM Press, Washington, DC.

Midgley, G., Y. N. Clayton, and R. J. Hay. 1997. *Diagnosis in Color Medical Mycology*. Mosby, St. Louis, MO.

Murray, P. R., E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.). 2007. *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.

Odds, F. C. 1991. Sabouraud('s) agar. *J. Med. Vet. Mycol.* **24:**355–359.

Samson, R. A., E. S. Hoekstra, J. C. Frisvad, and O. Filtenborg. 2001. *Introduction to Food- and Airborne Fungi*, 6th ed. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Sandin, R. L., G. S. Hall, D. L. Longworth, and J. A. Washington. 1993. Unpredictability of commercially available exoantigen culture confirmation tests in confirming the identity of five *Blastomyces dermatitidis* isolates. *Am. J. Clin. Pathol.* **99:**542–545.

St.-Germain, G., and R. C. Summerbell. 1996. *Identifying Filamentous Fungi.* Star Publishing, Belmont, CA.

Summerbell, R. C., and J. Kane. 1997. The genera *Trichophyton* and *Epidermophyton*, p. 131–191. *In* J. Kane, R. C. Summerbell, L. Sigler, S. Krajden, and G. Land (ed.), *Laboratory Handbook of Dermatophytes.* Star Publishing, Belmont, CA.

Summerbell, R. C., R. A. Haugland, A. Li, and A. K. Gupta. 1999. rRNA gene internal transcribed spacer 1 and 2 sequences of asexual, anthropophilic dermatophytes related to *Trichophyton rubrum. J. Clin. Microbiol.* **37:**4005–4111.

APPENDIX 8.9–1

Figures

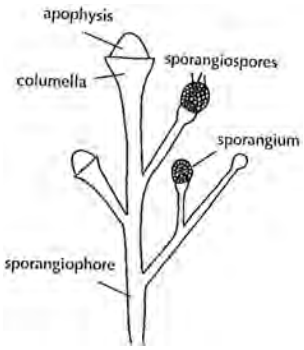


Figure 8.9–A1 Sporangiophores and sporangia of *Absidia* (schematic).

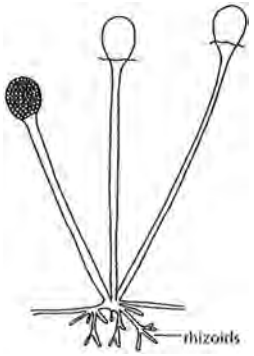


Figure 8.9–A2 Sporangiophores and rhizoids of *Rhizopus* (schematic).

Figure 8.9–A3 Simple arthroconidia of *Geotrichum* (schematic).

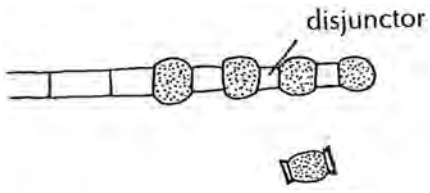


Figure 8.9–A4 Alternate arthroconidia of *Coccidioides* (schematic).

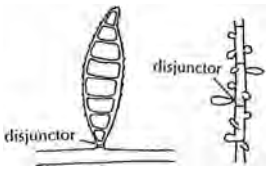


Figure 8.9–A5 Macroconidium (left) and microconidia (right) of *Microsporum* (schematic).

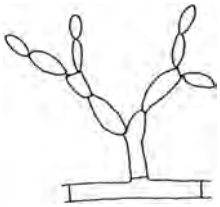


Figure 8.9–A6 Blastoconidia of *Cladosporium* (schematic).

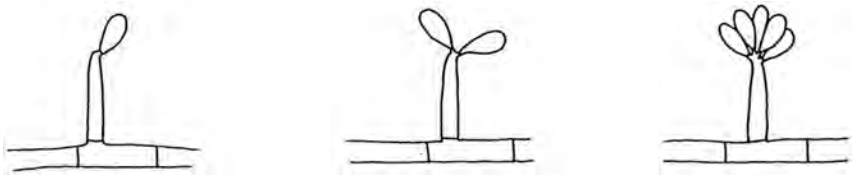


Figure 8.9–A7 Sympodially proliferating conidiogenous cell with blastoconidia of *S. schenckii* in rosette (schematic).

APPENDIX 8.9–1 *(continued)*

Figure 8.9–A8 Phialide of *Phialophora* (schematic).

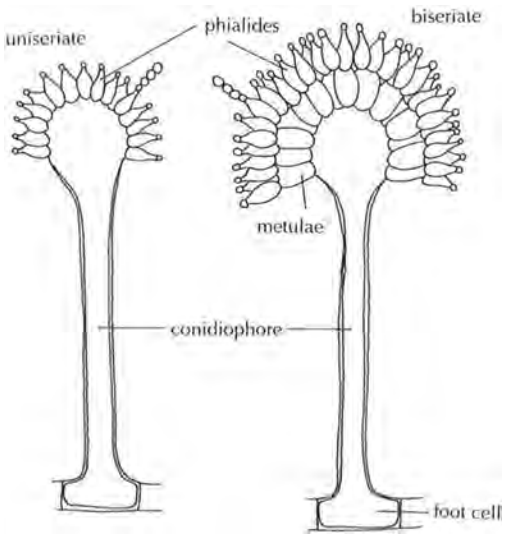
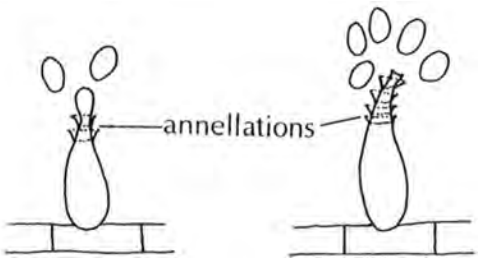


Figure 8.9–A9 *Aspergillus* structures.

Figure 8.9–A10 Annellides of *Exophiala* (schematic).



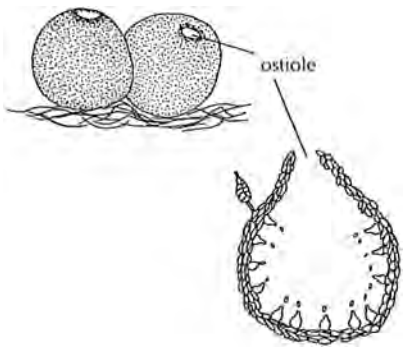


Figure 8.9–A11 Pycnidia of *Phoma,* face view and cross section (schematic).

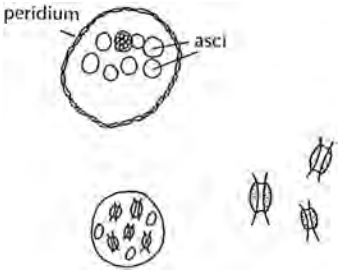


Figure 8.9–A12 Ascoma structures of *Eurotium* (one of several sexual states of *Aspergillus* spp.) (schematic) as seen at different magnifications (cleistothecium with asci, as seen at $\times 100$; ascus with ascospores, as seen at $\times 400$; released ascospores, as seen at $\times 1{,}000$).

APPENDIX 8.9–1 *(continued)*

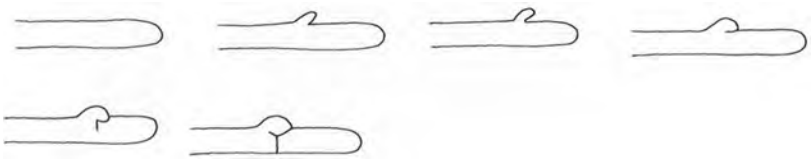


Figure 8.9–A13 Cell division in a dikaryotic basidiomycetous hypha, showing development of a clamp connection.

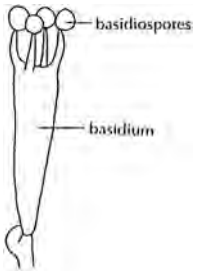


Figure 8.9–A14 Basidium and basidiospores.

Figure 8.9–A15 Terminal chlamydospore.

Figure 8.9–A16 Chain of symmetrical chlamydospores.

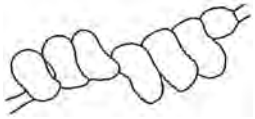


Figure 8.9–A17 Chain of asymmetrical chlamydospores (cow's teeth).

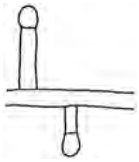


Figure 8.9–A18 Pedicels (matchstick stalks) of *T. tonsurans* (schematic).

Figure 8.9–A19 Macroconidia of *M. cookei* (schematic) with cytoplasmic compartments indicated by dotted lines.

Figure 8.9–A20 Helical seta (spiral appendage) of *T. mentagrophytes* (schematic).

Figure 8.9–A21 Reflexive branching of *T. soudanense* (schematic).

Figure 8.9–A22 Swollen nailhead hyphae of *T. schoenleinii* (schematic).

APPENDIX 8.9–1 *(continued)*

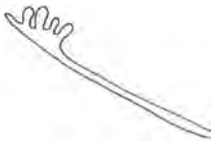


Figure 8.9–A23 Hypha with pectinate branching as seen in *M. audouinii* (schematic).

Figure 8.9–A24 Lateral hyphal "projection" or diverticulum of *T. rubrum* (schematic).

APPENDIX 8.9–2

Include QC information on reagent container and in QC records.

Media and Reagents, Sources, and Recipes

◩ **NOTE:** QC of most of the materials summarized in this appendix is detailed in appropriate parts of items 8.9.V, 8.9.VII, and 8.9.VIII.

A. Potato glucose agar (PGA)

potato flour*	39 g
yeast extract	2.5 g
glucose	10 g
agar (Difco**)	15 g
distilled water	1,000 ml

Autoclave at 121°C for 15 min. Pour into tubes or plates.

*No known difference among many potential commercial sources available from grocery store or wholesaler; therefore, no supplier is specified.

**Difco: BD Biosciences, Sparks, MD. The manufacturer is mentioned because the quantity of agar necessary varies by manufacturer.

B. Leonian's agar (Cain modification)

malt extract (Difco)	12.5 g
maltose	12.5 g
monopotassium phosphate (KH_2PO_4)	2.5 g
yeast extract	2.0 g
magnesium sulfate · $7H_2O$	1.25 g
Bacto peptone (Difco)	1.25 g
agar (Difco)	40 g
distilled water	2,000 ml

Autoclave at 121°C for 15 min. Pour into tubes or plates. The double-size (2,000-ml) batch is prepared to render the quantities of the minor ingredients convenient to work with.

◩ **NOTE:** The complex biologicals malt extract and Bacto peptone could possibly be obtained from other manufacturers; no comparison data are available.

C. Potato flake agar

Potato flake agar in its cycloheximide-free, colorless form can be used generically to promote sporulation or conidiation in a wide variety of clinical fungi. It has become most widely used, however, for dermatophyte identification, and for this use it is supplemented with cycloheximide and a pH indicator. This specialized version is available commercially as Potato Flakes Agar (Hardy Diagnostics, Santa Maria, CA) or as Rapid Sporulation Medium (Bacti-Lab, Mountain View, CA). It may elicit more rapid formation of conidia in dermatophytes than can be obtained on Sabouraud agars. Also, according to Aly (1), its colored pH indicator does not obscure the red pigment of *Trichophyton rubrum*. This indicator allows dermatophyte growth to be tentatively confirmed. Dermatophytes produce alkaline by-products during growth on proteinaceous materials; in this case, the result is a change of the bromthymol blue (BTB) indicator (Difco) from yellow to blue-green. This is a relatively specific response, although uncommonly isolated nonpathogenic dermatophytoids like *Trichophyton terrestre* and onygenalean chrysosporia like the *Chrysosporium* state of *Arthroderma cuniculi* will produce the same effect (5). The recipe below is for the dermatophyte version of the medium; for the generic version, omit the cycloheximide in step 2 and all of step 3. The volume of distilled water in step 1 should be adjusted to 900 ml. For work with known pure cultures, step 2 can be omitted and the water in step 1 can be adjusted to 1,000 ml.

APPENDIX 8.9–2 *(continued)*

1. Step 1. To a 2-liter flask, add the following:

agar (Difco)	25 g
glucose (Difco)	10 g
Betty Crocker Potato Buds*	20 g
distilled water	850 ml

Heat and stir until boiling commences.

2. Step 2. To a 250-ml flask, add the following:

distilled water	100 ml
chloramphenicol**	0.5 mg
cycloheximide***	0.5 mg

Heat and stir just until particles dissolve.

3. Step 3. To 15 ml of 0.1 N NaOH
   a. Add 0.5 g of BTB and then make up to 100 ml with distilled water (stock solution).
   b. Use 40 ml of BTB stock solution for each 1 liter of medium.
   c. Prepare 0.8 N solution of HCl for pH adjustment.
4. Step 4. Mixing
   a. Add 100 ml of antimicrobial agent solution to agar solution just as boiling commences.
   b. Add BTB solution as indicated in step 3.b; stir.
   c. Adjust pH to 5.8 with 0.8 N HCl.
   d. Autoclave at 121°C for 15 min.
   e. Pour into tubes or plates.

*General Mills, Minneapolis, MN; variants use only 15 g of Potato Buds; Instant Mashed Potato Puffs (General Mills) is given by Rinaldi (4) as an alternative.
**Parke Davis Pharmaceuticals, Detroit, MI (division of Pfizer Inc., New York, NY)
***Calbiochem: CN Biosciences, San Diego, CA; Merck KG AA, Darmstadt, Germany

D. Cereal agar

Mead Johnson Mixed Cereal Pablum (for babies) (Mead Johnson Nutritionals, Evansville, IN)	25 g
agar	5 g
water	250 ml

Add water to dry ingredients and autoclave at 121°C for 20 min (15 lb/in^2; 20 min). Pour into plates or tubes. The small volume is to ensure good heat penetration and prevent survival of spore-forming bacteria. If more is wanted, make multiple small batches, not large batches. Kane et al. (3) comment that mixed cereal is superior to baby oatmeal, although the latter has also been used in similar media.

E. Tap water agar

tap water	1,000 ml
agar	15 g

Autoclave at 121°C for 15 min. Pour into tubes or plates.

F. QC isolates—maintenance

For PGA, if the same isolate is used repeatedly, stocks should be carefully conserved, ideally as multiple subcultures at −20 to −80°C to ensure that pleomorphic degeneration does not distort results. No *Microsporum canis* subculture that has remained on growth medium at room temperature or above for >30 days should be considered reliable.

APPENDIX 8.9–2 *(continued)*

G. Dimorphic-fungus identification—media for conversion of dimorphic fungi

1. Blasto D medium

glucose	7 g
Tween 80	0.2 ml
potassium sulfate (K_2SO_4)	0.5 g
magnesium citrate	1.5 g
dipotassium phosphate (K_2HPO_4)	5.0 g
asparagine	5.0 g
Casamino Acids	3.0 g
sodium chloride (NaCl)	0.85 g
agar	15 g
distilled water	1,000 ml

   a. Step 1. Mix ingredients, adjust to pH 6.6, bring to a boil to dissolve completely, and autoclave at 121°C for 15 min.
   b. Step 2. Apportion into tubes and slant.
   Antimicrobial agent-supplemented versions are advantageous for dealing with bacterially contaminated cultures. Chloramphenicol, which is a commonly used antimicrobial agent addition to media, is inhibitory to yeast phase *Blastomyces dermatitidis* in vitro, and another antimicrobial agent, such as tobramycin (100 µg/ml), should be used.

2. For *Histoplasma capsulatum*
   a. Kurung-Yegian medium

potato flour*	5 g
deionized water	500 ml
whole egg*	250 ml
egg yolk* (50% enrichment)	500 ml

   *No known difference among many potential commercial sources available (grocery store or wholesaler); therefore, no supplier is specified.
      (1) Step 1. Mix potato flour and water, adjust pH to 5.0, and then heat to dissolve. Autoclave at 121°C for 15 min and then cool to 50°C in a water bath.
      (2) Step 2. Surface disinfect whole eggs by standing them in 70% ethanol for 15 to 30 min. Break open eggs with a sterile knife and prepare egg yolk enrichment by draining off sufficient egg white. Break open additional eggs and blend both egg fractions together thoroughly, in a sterile blender if available. Add egg mixture to potato mixture from step 1 and mix. Apportion into tubes and slant. Allow to set for 25 min at 82°C in the autoclave (isothermal cycle).
   b. Blood-glucose cysteine agar

tryptose blood agar base (Acumedia; Neogen, Lansing, MI [catalog no. 7282])	33 g
L-cysteine (hydrochlorate)	1 g
defibrinated sheep blood	50 ml
penicillin (100,000 U/ml)	10 ml
distilled water	990 ml

   Mix tryptose blood agar base, cysteine, and water and bring to a boil so that dissolution occurs. Autoclave at 121°C for 15 min. Cool to 50°C and add blood and penicillin. Apportion into tubes and slant.

3. For *Penicillium marneffei*
   Sabouraud agar (Sabouraud formulation, with original glucose level and animal protein)

distilled water	1,000 ml
glucose	40 g
Bacto peptone*	10 g
Difco agar*	15 g

   Heat to near boiling to dissolve. Adjust pH with NaOH to 7.0. Autoclave at 121°C for 15 min. Apportion into tubes and slant.

   *Difco: BD Biosciences.

APPENDIX 8.9–2 *(continued)*

H. Exoantigen test—immunodiffusion agar

sodium chloride	0.9 g
sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)	0.4 g
phenol (aqueous, 90% [wt/wt])	0.25 ml
glycine	7.5 g
purified agar (Difco)	1.0 g
distilled water	to 100 ml

Mix sodium chloride and sodium citrate in 50 ml of distilled water in a 125-ml Erlenmeyer flask, dissolve, and then thoroughly mix in the phenol and glycine. Add agar, bring level of ingredients up to 100 ml with additional water, and autoclave for 10 min at 120°C. Final pH should be 6.3 to 6.4; adjust if necessary.

Preparing immunodiffusion plates

1. Aseptically pipette 6.5 ml of immunodiffusion agar (after autoclaving it and when it has cooled to 60 to 65°C) into each plastic petri dish (diameter, 100 mm). Allow to gel (30 min); this forms the basal layer of medium. Retain some agar at 60°C in a water bath so that an upper layer can be overlaid later.
2. After the basal layer is well gelled, add 3.5 ml of the retained 60°C medium on one side of each plate. Immediately lower and press a microimmunodiffusion template (Quality House, Cartersville, GA) into the hot agar so that air bubbles are not trapped. The template should be securely appressed to the basal layer. Again allow the medium to cool for at least 30 min to gel. After this point, the plates can be used immediately if needed.
3. After the upper medium layer has gelled, remove extra agar from the template with a 1-mm-wide spatula. This is most easily done if the plates have first been cooled in a refrigerator for 5 to 10 min. Plates may be stored for up to 4 weeks if retained in a moist chamber at 4°C. The expiration date should be recorded on the plate and in the laboratory's work records. It should be noted that commercially obtained identification plates (Meridian Diagnostics, Cincinnati, OH) will simply have precut wells forming an immunodiffusion grid and will already bear their expiration date on the plate or bag label.

I. Growth factors for dermatophytes

1. *Trichophyton* agars

Trichophyton agars are commercially available (Difco [BD Biosciences]). If the media are to be made in-house, the following recipes apply. Prepare vitamin-free Casamino Acids agar.

Casamino Acids, vitamin free	2.5 g
glucose	40 g
magnesium sulfate $\cdot 7H_2O$	0.1 g
monopotassium phosphate (KH_2PO_4)	1.8 g
agar	15 g
distilled water	to 1,000 ml

Autoclave at 10 lb/in^2 for 10 min (110°C). Adjust pH to 6.8. Add nutrient solutions as outlined below, pour into tubes, and slant.

For *Trichophyton* agar no. 2 (T2), add 20 ml of a sterile 250-μg/ml aqueous stock solution of inositol to the vitamin-free medium while still molten and mix well prior to pouring. For T3, add inositol as for T2, as well as 20 ml of a sterile 10-μg/ml aqueous stock solution of thiamine. For T4, add 20 ml of thiamine stock solution alone. For T5, add 20 ml of a sterile 10-μg/ml aqueous stock solution of nicotinic acid. For T6, substitute 1.5 g of ammonium nitrate (NH_4NO_3) for the Casamino Acids in the basal formulation. For T7, add 20 ml of a 150-μg/ml aqueous stock solution of L-histidine to T6. (To prepare *y*-μg/ml aqueous stock solution, add 0.1*y* mg of growth factor to 100 ml of distilled water. Sterilize by micropore filtering [0.22-μm pore size] or by gentle autoclaving at 10 lb/in^2 for 10 min. There is some controversy about whether such growth factors survive autoclaving well; QC tests that should be done on growth factor media in any case should detect any problems that may arise.)

All these media can be prepared in 100-ml volumes if small quantities are used. For the rarely used nicotinic acid test, the stock solution can simply be retained in the refrigerator, and 2 or 3 drops can be pipetted across the medium surface of a T1 slant in order to obtain a functional T5 test. This convenience is possible only for the nicotinic acid test because of its absolute (growth or no growth) nature.

APPENDIX 8.9–2 *(continued)*

2. Christensen's urea agar and broth
Christensen's urea broth or agar slants are commercially available from numerous manufacturers. A recipe for broth is given for in-house production. Due to the high cost of agar and quicker yield of test results in broth, broth is somewhat preferable to agar.
   a. Step 1. Prepare sterile urea solution.

      distilled water ..... 100 ml
      urea ..... 20 g
      glucose ..... 1 g

      Dissolve and sterilize by micropore filtration.
   b. Step 2. Prepare remaining ingredients.

      Bacto peptone ..... 1 g
      sodium chloride ..... 5 g
      anhydrous monopotassium phosphate (KH_2PO_4) ..... 2 g
      phenol red (0.25% stock in ethanol) ..... 8 ml

      Dissolve, adjust pH to 6.9, and autoclave at 15 lb/in^2 for 15 min (121°C). Cool to ca. 55 to 60°C and add urea solution. Readjust pH to 6.9 if indicator color shows any change. Dispense aseptically as 5-ml aliquots into, for example, 16- by 125-mm screw-cap tubes.
3. BCP agar
BCP milk glucose medium is available commercially from numerous sources (e.g., Hardy Diagnostics, Santa Maria, CA, or as Dermatophyte Milk Medium [Biomedia Unlimited, Toronto, Canada] or as Dermatophyte Differentiation Agar [Quelab Laboratories, Montreal, Canada]). A recipe for in-house production is given below. Note that although the recipe has glucose separately autoclaved, this was originally instituted as a precaution and may not be entirely necessary. Laboratories could, if desired, experiment to determine if steps 2 and 3 below could be combined, using appropriate QC isolates to judge the results.
   a. Step 1

      Difco agar ..... 7.5 g
      distilled water ..... 100 ml

      Soak agar for 15 min; autoclave at 15 lb/in^2 for 15 min (121°C). Retain at 60°C in a water bath unless steps 2 and 3 can be done at the same time. These steps have a shorter autoclave time, so two autoclaves are required to do all three steps simultaneously.
   b. Step 2

      skim milk powder ..... 20 g
      BCP (1.6% stock solution in ethanol) ..... 0.5 ml
      distilled water ..... 250 ml

      Dissolve and autoclave at 10 lb/in^2 for 8 min.
   c. Step 3 (done at same time as step 2)

      D-glucose ..... 10 g
      distilled water ..... 500 ml

      Dissolve and autoclave at 10 lb/in^2 for 8 min.
   d. Step 4
      Combine the autoclaved products of steps 1 to 3 and adjust pH to 6.6. Dispense aseptically and slant, e.g., 5-ml aliquots into 16- by 125-mm screw-cap tubes, with the longest slant allowed by the tube. Production QC: the medium should be sky blue.
4. Cornmeal glucose agar
Available commercially from Remel Inc. (Lenexa, KS)
   a. Step 1

      cornmeal ..... 40 g
      distilled water ..... 1,000 ml

      Make an infusion from cornmeal as follows. Mix cornmeal with water; heat for 1 h at 52°C or autoclave for 10 min at 121°C. Filter through cheesecloth and then through Whatman no. 2 filter paper. Bring up liquid to 1,000 ml with additional distilled water as necessary.

APPENDIX 8.9–2 *(continued)*

b. Step 2

glucose ……20 g
agar ……15 g

Add agar to infusion from step 1 and boil to dissolve. Add glucose. Bring liquid up to 1,000 ml with additional distilled water as necessary. Autoclave for 15 min at 121°C. Pour into tubes and slant. Final pH, 6.0.

Other agars that may be used to elicit the wine red pigment of *Trichophyton rubrum* include Casamino Acids erythritol albumin (CEA) agar (6) (commercially available as Candida Inhibitory Agar [Biomedia Unlimited, Toronto, Canada]) and modified Borelli's lactrimel agar (2) (available from Excel Laboratory Products, Belmont, West Australia).

5. Polished-rice medium

polished (white) rice* ……4.5 g
distilled water ……14 ml

*No known difference among many potential commercial sources available from grocery stores; therefore, no supplier is specified. However, ordinary long-grain white rice is generally used in preference to products with potentially higher or lower nutrient levels, such as converted rice, parboiled rice, instant rice, jasmine rice, or sticky rice.

Add ingredients to a 1-oz. universal bottle or to a suitable Erlenmeyer flask. Autoclave for 15 min at 121°C.

6. Sabouraud dermatophyte decontamination broth
   a. Step 1. Make Sabouraud broth with selective agents.

glucose ……40 g
Bacto peptone (Difco) ……10 g
distilled water …… 1 liter
chloramphenicol (10-mg/ml stock) ……10 ml
cycloheximide (in 2 ml of acetone) …….1 g
gentamicin (10 mg/ml) ……5.0 ml

Dissolve and adjust pH to 7. Autoclave for 15 min at 121°C. Dispense in 10-ml aliquots into tubes.

   b. Step 2. Prepare a stock solution of 0.5 N HCl.
   c. Step 3. Prepare an acidification series.

Take four tubes of broth from step 1. Label them 0, 1, 2, and 3. Dispense 1 drop of HCl stock solution into tube 1, 2 drops into tube 2, and 3 drops into tube 3. Batches of such tube series can be made up and stored in the 5°C refrigerator, with screw caps tightened, for at least 6 months.

QC isolates have not yet been designated for this technique; therefore, it is recommended to screen several isolates of *T. rubrum* and find one that grows well in tube 2 and shows at least some growth in tube 3. Ideally, a polyresistant bacterial isolate should be selected on the basis of showing visible inhibition in growth within the range of acid concentrations used. It should be monitored with newly made batches of decontamination broth series to be certain that it is constantly inhibited at the same point in the series.

J. Yeast-inhibiting blood agar

Yeast-inhibiting blood agar is used for selective isolation and partial purification of cultures of dimorphic and other cycloheximide-tolerant fungi. Egg white inhibits growth of all biotin-requiring yeasts, e.g., all common clinical *Candida* spp. except *C. krusei.*

1. Step 1. Prepare egg whites.

Sterilize 250-ml blender cylinder, and prepare eggs as follows.

   a. Clean eggs and then soak in 95% ethyl alcohol for 15 min.
   b. Clean hands with alcohol.
   c. Crack eggs with a sterile knife, and drain egg white into sterile blender cylinder. Discard egg yolk.
   d. Switch the blender on and then off almost immediately to make an even consistency of the egg whites. (Do not leave it on any longer, or too much froth will be formed. Too much oxygenation destroys activity.)
   e. Pour egg white into a sterile flask. Store any excess at 4°C.

APPENDIX 8.9–2 *(continued)*

2. Step 2. Prepare heart infusion agar.

   distilled water ……1,000 ml
   heart infusion agar (Difco) ……40 g

   Autoclave at 15 lb/in² for 25 min.
3. Step 3. Cool heart infusion agar and add the following.

   sterile defibrinated sheep blood ……53 ml
   chloramphenicol (1.6% stock solution) ……3 ml
   cycloheximide (2.0% stock solution) ……25 ml
   gentamicin (5% stock solution) ……0.4 ml
   prepared egg white ……30 ml

4. QC strains are *Candida albicans* ATCC 10231 = CBS 6431 = IFO 1594 (same strain in all collections), negative (no or pinpoint growth), and *B. dermatitidis* ATCC 60916 = CBS 673.78, positive (normal growth).

References

1. **Aly, R.** 1994. Culture media for growing dermatophytes. *J. Am. Acad. Dermatol.* **31:**S107–S108.
2. **Kaminsky, G. W.** 1985. The routine use of modified Borelli's lactrimel agar (MBLA). *Mycopathologia* **91:**57–59.
3. **Kane, J., R. C. Summerbell, L. Sigler, S. Krajden, and G. Land.** 1997. *Laboratory Handbook of Dermatophytes*, p. 324. Star Publishing, Belmont, CA.
4. **Rinaldi, M. G.** 1982. Use of potato flakes agar in clinical mycology. *J. Clin. Microbiol.* **15:**1159–1160.
5. **Sigler, L.** 1997. *Chrysosporium* and molds resembling dermatophytes, p. 261–311. *In* J. Kane, R. C. Summerbell, L. Sigler, S. Krajden, and G. Land (ed.), *Laboratory Handbook of Dermatophytes.* Star Publishing, Belmont, CA.
6. **Summerbell, R. C., and J. Kane.** 1997. Physiological and other special tests for identifying dermatophytes, p. 45–77. *In* J. Kane, R. C. Summerbell, L. Sigler, S. Krajden, and G. Land (ed.), *Laboratory Handbook of Dermatophytes.* Star Publishing, Belmont, CA.

8.10.1 Broth Dilution Antifungal Susceptibility Testing

PREANALYTICAL PROCEDURES

I. PRINCIPLE

Until recently, antifungal susceptibility testing (AFST) has lagged behind its antibacterial counterpart. A number of important achievements helped propel the antifungal susceptibility field. These achievements include publication of the approved reference method for broth dilution AFST of yeast, document M27-A3 of the Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards [NCCLS]) (1, 2); publication of interpretive breakpoints using this method (10); the recommended use of AFST during selection of therapy of patients with candidemia and hematogenously disseminated candidiasis as outlined by the published guidelines for the treatment of candidiasis (6); publication of a reference method for broth dilution AFST of conidium-forming filamentous fungi, CLSI document M38-A2 (3); and recent development and publication of AFST methods for dermatophytes (3). The procedures described below are based on CLSI documents M27-A3, M27-S3, and M38-A2.

The CLSI AFST method for both yeasts and moulds adopts a broth dilution MIC methodology that quantitatively measures the in vitro activity of an antifungal agent against a fungal isolate. Test tubes (or multiwell microdilution plates) containing various concentrations of an antifungal agent are inoculated with a standardized suspension of test organisms. The MIC is determined after 24 to 48 h of incubation at 35°C for yeasts and 96 h for moulds.

II. MATERIALS

A. Test media

The most suitable medium for AFST is determined by the organism being tested. The medium will be either RPMI 1640 (buffered with MOPS [morpholine propanesulfonic acid]) for yeasts and filamentous fungi or yeast nitrogen broth (YNB) for *Cryptococcus neoformans*. In addition, Sabouraud glucose agar or potato dextrose agar is required to subculture the organisms for inoculum preparation.

B. Antifungal agents

The list of antifungal agents may include amphotericin B, ciclopirox, flucytosine (5-FC), fluconazole, ketoconazole, itraconazole, terbinafine, voriconazole, anidulafungin, caspofungin, micafungin, posaconazole, and ravuconazole.

C. Equipment

1. Filter sterilization apparatus for preparation of RPMI 1640
2. 12- by 75-mm test tubes or 96-well U-shaped microdilution plates, and a microdilution plate reader
3. McFarland suspension standards and spectrophotometer at 530 nm
4. Volumetric pipettes (1 and 10 ml), multichannel pipettes (adjustable, 40 to 200 μl)

III. PREPARATION OF ANTIFUNGAL STOCK SOLUTIONS

A. Test medium

The synthetic medium RPMI 1640, with MOPS, hereafter referred to as RPMI, has been selected as the optimal growth medium for testing the susceptibility of *Candida* spp. to amphotericin B, 5-FC, and the azoles. However, studies have demonstrated that RPMI may not be adequate to support the growth of some *C. neoformans* strains (4). Instead, YNB is proposed as an alternative for this

III. PREPARATION OF ANTIFUNGAL STOCK SOLUTIONS *(continued)*

organism (3). RPMI is a broth medium without sodium bicarbonate which is supplemented with L-glutamine and a pH indicator (04-525Y [American Bioorganic Company, Inc., Niagara Falls, NY, and BioWhittaker, Walkersville, MD] and R-6504 from Sigma Chemical Co., St. Louis, MO]). Reconstituted RPMI powder should be filter sterilized, with a final pH between 6.9 and 7.1 at 25°C.

B. Stock solutions of antifungal agents

1. The susceptibility of yeasts to the following antifungal agents is tested: amphotericin B (Bristol Myers Squibb, Wallingford, CT); 5-FC (Sigma Chemical Co.); azoles, including fluconazole (Pfizer Pharmaceuticals, New York, NY) and voriconazole (Pfizer Pharmaceuticals) and ketoconazole and itraconazole (Janssen Pharmaceuticals, Titusville, NJ); and echinocandins, including anidulafungin (Pfizer Pharmaceuticals), caspofungin (Merck & Co., Inc., Whitehouse Station, NJ), and micafungin (Astellas Pharma US, Inc., Deerfield, IL). These drugs are obtained as pure reference powders directly from the manufacturers or the U.S. Pharmacopeia (Rockville, MD). Use of pharmaceutical preparations of these agents is not recommended. However, if pure powders are not available, as is often the case with amphotericin B, pharmaceutical preparations (Fungizone for injection [Bristol Myers Squibb]) may be substituted.
2. Stock solutions of antifungal agents do not commonly support the growth of contaminating organisms and can be considered sterile.
3. Caspofungin, fluconazole, 5-FC, and micafungin are prepared in sterile distilled water and normal saline, respectively. Stock solutions of amphotericin B, anidulafungin, itraconazole, ketoconazole, posaconazole, ravuconazole, and voriconazole are prepared in dimethyl sulfoxide (DMSO). Pharmaceutical preparations of amphotericin B are dissolved in sterile distilled water.
4. Because the assay units of a particular antifungal powder can differ widely from the actual weight of the powder, the antifungal solutions must be standardized according to the assays of each batch lot. One of the following formulae is used to determine the amount of powder or diluent needed: weight = (volume [in milliliters] × concentration [in micrograms per milliliter])/assay potency (in micrograms per milligram) or volume = (weight [in milligrams] × assay potency [in micrograms per milligram])/concentration (in micrograms per millilliter). If possible, >100 mg of powder should be weighed initially. For example, to prepare 100 ml of a stock solution at a concentration of 1,280 μg/ml using a powder that has a potency of 750 μg/mg, use the first formula to establish the weight of powder needed: weight = (target volume [100 ml] × desired concentration [1,280 μg/ml])/potency (750 μg/mg) = 170.7 mg. Using the second formula, and a weight in excess of that required (e.g., 182.6 mg), the volume of diluent is determined, as in this example: volume = (weight [182.6 mg] × potency [750 μg/mg])/desired concentration (1,280 μg/ml) = 107.0 ml. Thus, in the above example, 182.6 mg of powder is to be dissolved in 107.0 ml of diluent.

C. Working solutions of antifungal agents

1. Working antifungal solutions are prepared at 10 times the highest concentration to be tested for water-soluble drugs (e.g., fluconazole, 5-FC, and pharmaceutical preparations of amphotericin B) and 100 times the highest concentration for water-insoluble drugs (amphotericin B, itraconazole, and ketoconazole).
2. Dilution procedures for water-soluble drugs and for water-insoluble drugs are shown in Tables 8.10.1–1 and 8.10.1–2, respectively. Each of the intermediate concentrations should be diluted 1:10 in RPMI. This procedure will prevent precipitation of the compound, which would lead to dilution error.

Table 8.10.1–1 Dilution procedure for water-soluble drugs

Step	Concn (μg/ml)	Source	Vol (ml)	+	Medium (ml)	=	Intermediate concn (μg/ml)	=	Final concn at 1:10 (μg/ml)
1	5,120	Stock	1		7		640		64
2	640	Step 1	1		1		320		32
3	640	Step 1	1		3		160		16
4	160	Step 3	1		1		80		8
5	160	Step 3	0.5		1.5		40		4
6	160	Step 3	0.5		3.5		20		2
7	20	Step 6	1		1		10		1
8	20	Step 6	0.5		1.5		5		0.5
9	20	Step 6	0.5		3.5		2.5		0.25
10	2.5	Step 9	1		1		1.25		0.125
11	2.5	Step 9	0.5		1.5		0.625		0.0625
12	2.5	Step 9	0.5		3.5		0.3125		0.03125

III. PREPARATION OF ANTIFUNGAL STOCK SOLUTIONS *(continued)*

3. The concentrations tested should encompass the breakpoint concentrations and the expected results for the QC strains. The following drug concentration ranges should be used: amphotericin B, 0.0313 to 16 μg/ml; 5-FC, 0.125 to 64 μg/ml; ketoconazole, 0.0313 to 16 μg/ml; itraconazole, 0.0313 to 16 μg/ml; fluconazole, 0.125 to 64 μg/ml; posaconazole and voriconazole, 0.0313 to 16 μg/ml; and anidulafungin, caspofungin, and micafungin, 0.015 to 8 μg/ml.

IV. STORAGE OF ANTIFUNGAL STOCK SOLUTIONS

A. Small volumes of stock antifungal solutions can be stored in sterile plastic vials, sealed, and frozen at a temperature between −80 and −20°C for up to 6 months. Vials are thawed on the day of testing, and any unused drug must be discarded.

B. Prepared microdilution plates may be sealed in plastic bags and stored between −80 and −20°C for up to 6 months.

ANALYTICAL CONSIDERATIONS

V. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Yeast isolates and MIC ranges

Inclusion of QC isolates in susceptibility testing will allow monitoring of the precision and accuracy of the susceptibility test procedure, performance of the reagents, the testing conditions, and the performance of testing personnel (4).

Table 8.10.1–2 Dilution procedure for water-insoluble drugs

Step	Concn (μg/ml)	Source	Vol (ml)	+	DMSO amt (ml)	=	Intermediate concn (μg/ml)	=	Final concn at 1:100 (μg/ml)
1	1,600	Stock					1,600		16
2	1,600	Stock	0.5		0.5		800		8
3	1,600	Stock	0.5		1.5		400		4
4	1,600	Stock	0.5		3.5		200		2
5	200	Step 4	0.5		0.5		100		1
6	200	Step 4	0.5		1.5		50		0.5
7	200	Step 4	0.5		3.5		25		0.25
8	25	Step 7	0.5		0.5		12.5		0.125
9	25	Step 7	0.5		1.5		6.25		0.0625
10	25	Step 7	0.5		3.5		3.13		0.0313

V. QUALITY CONTROL *(continued)*

QC strains are to be incorporated into each daily run of patient testing. Acceptable examples of MIC ranges for QC isolates are outlined in Table 8.10.1–3.

B. Media

Ensure that all media and drugs are used within their expiration dates.

C. Equipment

Regularly clean and calibrate pipettes and the spectrophotometer to maintain accuracy and reduce the likelihood of contamination.

VI. PROCEDURES

A. Testing yeast isolates

1. Inoculum preparation

a. All organisms should be subcultured onto Sabouraud glucose agar or potato dextrose agar and incubated at 35°C for 24 h (*Candida* spp.) or 48 h *(C. neoformans).*

b. The inoculum should be prepared by picking five well-isolated colonies >1 mm in diameter and suspending the organisms in 5 ml of sterile saline (8.5 g of NaCl/liter; 0.85% saline) or sterile water.

c. A suspension equal to a 0.5 McFarland standard (spectrophotometer reading at a λ of 530 nm) is prepared by adding sterile saline or sterile water. This procedure will yield a concentration of 1×10^6 to 5×10^6 cells per ml.

d. A working suspension is prepared by adding 0.1 ml of the suspension from item c above to 9.9 ml of RPMI. This is further diluted 1:20 with RPMI to give a concentration of 5×10^2 to 2.5×10^3 cells per ml.

2. Test inoculation and incubation (macrodilution method)

a. Add 0.1 ml of each antifungal (at the intermediate concentration for water-soluble drugs and the intermediate concentration diluted 1:10 for water-insoluble drugs) to a sterile 12- by 75-mm test tube.

b. A growth control tube should receive 0.1 ml of drug diluent without antifungal agent. A second tube of drug diluent should remain uninoculated to serve as a sterility control.

Table 8.10.1–3 Recommended 24-h MIC limits for two QC strains for broth microdilution

Organism	Antifungal agent	MIC range (μg/ml)
Candida parapsilosis ATCC 22019	Amphotericin B	0.25–2.0
	Fluconazole	0.5–4.0
	Itraconazole	0.12–0.5
	Ketoconazole	0.03–0.25
	5-FC	0.06–0.25
	Voriconazole	0.016–0.12
	Posaconazole	0.06–0.25
	Anidulafungin	0.25–2.0
	Caspofungin	0.25–1.0
	Micafungin	0.5–2.0
Candida krusei ATCC 6258	Amphotericin B	0.5–2.0
	Fluconazole	8.0–64
	Itraconazole	0.12–1.0
	Ketoconazole	0.12–1.0
	5-FC	4.0–16
	Voriconazole	0.06–0.5
	Posaconazole	0.06–0.5
	Anidulafungin	0.03–0.12
	Caspofungin	0.12–1.0
	Micafungin	0.12–0.5

VI. PROCEDURES *(continued)*

c. Within 15 min of inoculum preparation, 0.9 ml of the inoculum is added to each tube in the series (with the exception of the sterility control). This brings the antifungal agents to their final concentrations.

d. Tubes should be incubated without agitation at 35°C for 24 to 48 h (*Candida* spp.) or 70 to 74 h *(C. neoformans).*

3. Reading results

a. The amount of growth in each tube is compared to that of the growth control using the following numerical scale: 0, optically clear; 1, slightly hazy; 2, prominent decrease (~50%) in turbidity; 3, slight reduction in turbidity; and 4, no reduction in turbidity.

b. For amphotericin B, the MIC is read as the lowest drug concentration that prevents any visible growth (score of 0). Trailing endpoints with amphotericin B are usually not encountered.

c. For 5-FC and azole antifungals, the MIC is read as the lowest drug concentration that yields 50% inhibition of growth (score of 2) relative to the growth control. The azoles will often show trailing endpoints, whereby turbidity persists in all drug concentrations above the MIC. To reduce variability in reading these endpoints, the amount of allowable turbidity can be demonstrated by diluting 0.2 ml of the growth control in 0.8 ml of RPMI.

d. For echinocandins, the MIC is determined after 24 h of incubation and read as the lowest drug concentration to produce a prominent decrease in turbidity (score of 2), which translates to a 50% reduction in growth relative to the growth control.

4. Microdilution modifications

a. Stock solutions of antifungal agents and their intermediate concentrations are prepared in the manner outlined above. The intermediate concentrations are diluted 1:5 with RPMI to achieve the 2× concentration needed for microdilution method.

b. A stock yeast suspension (1×10^6 to 5×10^6 cells per ml) is prepared as outlined above. This suspension is then diluted 1:50 and further diluted 1:20 with RPMI to achieve the 2× test inoculum (1×10^3 to 5×10^3 CFU/ml). When combined with drug, the final concentration will be 0.5×10^3 to 2.5×10^3 CFU/ml.

c. Using a multichannel pipette, dispense 100 μl of the 2× antifungal concentrations into columns 1 to 10 of sterile disposable 96-well (U-shaped) microdilution plates. Column 1 will contain the highest concentration and column 10 will contain the lowest concentration of drug. Columns 11 and 12 should receive 100 μl of diluent.

d. Using a multichannel pipette, dispense 100 μl of working yeast suspension into each well of columns 1 to 11. Column 11 will serve as the growth control. Column 12 should remain uninoculated and is used as a sterility control. QC organisms are to be incorporated into each daily run.

e. Incubate the plates as described above.

f. Plates are placed on a microdilution plate reader with a magnifying mirror; MICs are read in the same manner as described for macrodilution tubes.

B. Testing filamentous fungi and dermatophytes

1. Test medium, antifungal stock, and working solutions

a. The test medium of choice for filamentous moulds and dermatophytes is RPMI.

b. The preparation of stock solutions of antifungal agents remains the same as described in item III above.

c. Allylamine terbinafine, ciclopirox, and griseofulvin can be added to the list of antifungal agents, in particular when testing dermatophytes. Ciclo-

Table 8.10.1–4 Dilution procedure for water-insoluble drugs used in broth microdilution susceptibility tests for nondermatophyte isolates.

Step	Concn (μg/ml)	Source	Vol (ml)	+	DMSO amt (ml)	=	Intermediate concn (μg/ml)	=	Final concn at 1:50 (μg/ml)[a]
1	1,600	Stock					1,600		32
2	1,600	Stock	0.5		0.5		800		16
3	1,600	Stock	0.5		1.5		400		8
4	1,600	Stock	0.5		3.5		200		4
5	200	Step 4	0.5		0.5		100		2
6	200	Step 4	0.5		1.5		50		1
7	200	Step 4	0.5		3.5		25		0.5
8	25	Step 7	0.5		0.5		12.5		0.25
9	25	Step 7	0.5		1.5		6.25		0.125
10	25	Step 7	0.5		3.5		3.13		0.0625

[a] 2× concentration.

VI. PROCEDURES *(continued)*

pirox, terbinafine, and griseofulvin are water-insoluble drugs and should be dissolved in DMSO.

d. Working antifungal solutions are prepared at 10 times the highest concentration to be tested for water-soluble drugs (e.g., fluconazole, 5-FC, and pharmaceutical preparations of amphotericin B) and 100 times the highest concentration for water-insoluble drugs (ketoconazole, itraconazole, and amphotericin B).

e. Dilution series of water-insoluble drugs to be used in broth dilution susceptibility tests for nondermatophyte isolates are shown in Table 8.10.1–4.

f. Dilution series of water-insoluble drugs to be used in broth dilution susceptibility tests for dermatophyte isolates are shown in Table 8.10.1–5.

g. Dilution series for water-soluble drugs to be used in broth dilution susceptibility tests for dermatophyte and nondermatophyte isolates are shown in Table 8.10.1–6.

Table 8.10.1–5 Dilution procedure for water-insoluble drugs used in broth dilution susceptibility tests for dermatophyte isolates

Step	Concn (μg/ml)	Source	Vol (ml)	+	DMSO amt (ml)	=	Intermediate concn (μg/ml)	=	Final concn at 1:50 (μg/ml)[a]
1	6,400	Stock					6,400		128
2	6,400	Stock	0.5		0.5		3,200		64
3	6,400	Stock	0.5		1.5		1,600		32
4	6,400	Stock	0.5		3.5		800		16
5	800	Step 4	0.5		0.5		400		8
6	800	Step 4	0.5		1.5		200		4
7	800	Step 4	0.5		3.5		100		2
8	100	Step 7	0.5		0.5		50		1
9	100	Step 7	0.5		1.5		25		0.5
10	100	Step 7	0.5		3.5		12.5		0.25
11	12.5	Step 10	0.5		0.5		6.25		0.125
12	12.5	Step 10	0.5		1.5		3.125		0.0625
13	12.5	Step 10	0.5		3.5		1.56		0.0313
14	1.56	Step 13	0.5		0.5		0.78		0.0156
15	1.56	Step 13	0.5		1.5		0.39		0.0078
16	1.56	Step 13	0.5		3.5		0.195		0.0039
17	0.195	Step 16	0.5		0.5		0.0975		0.0019

[a] 2× concentrations.

Table 8.10.1–6 Dilution procedure for water-soluble drugs

Step	Concn (μg/ml)	Source	Vol (ml)	+	Medium amt (ml) =	Intermediate concn (μg/ml)	=	Final concn at 1:5 (μg/ml)
1	5,120	Stock	1		7	640		128
2	640	Step 1	1		1	320		64
3	640	Step 1	1		3	160		32
4	160	Step 3	1		1	80		16
5	160	Step 3	0.5		1.5	40		8
6	160	Step 3	0.5		3.5	20		4
7	20	Step 6	1		1	10		2
8	20	Step 6	0.5		1.5	5		1
9	20	Step 6	0.5		3.5	2.5		0.5
10	2.5	Step 9	1		1	1.25		0.25
11	2.5	Step 9	0.5		1.5	0.625		0.125
12	2.5	Step 9	0.5		3.5	0.3125		0.0625

VI. PROCEDURES *(continued)*

h. The concentrations tested should encompass the breakpoint concentrations and the expected results for the QC strains. For nondermatophyte moulds, the following drug concentration ranges are usually used: amphotericin B, 0.0313 to 16 μg/ml; 5-FC, 0.125 to 64 μg/ml; ketoconazole, 0.0313 to 16 μg/ml; itraconazole and new triazoles (posaconazole, ravuconazole, and voriconazole), 0.0313 to 16 μg/ml; fluconazole, 0.125 to 64 μg/ml; and echinocandins (anidulafungin, caspofungin, and micafungin), 0.015 to 8 μg/ml. Suitable drug concentration ranges for testing dermatophytes are as follows: ciclopirox, 0.06 to 32 μg/ml; griseofulvin, 0.125 to 64 μg/ml; itraconazole, 0.001 to 0.5 μg/ml; posaconazole, 0.004 to 8 μg/ml; voriconazole, 0.001 to 0.5 μg/ml; fluconazole, 0.125 to 64 μg/ml; and terbinafine, 0.001 to 0.5 μg/ml.

2. Inoculum preparation

Nongerminated conidial or sporangiospore suspensions can be prepared by a spectrophotometric procedure. Concentrations of viable conidial or sporangiospore test inocula in a range of 0.4×10^4 to 5×10^5 CFU/ml provided the most reproducible MIC data.

a. Nondermatophyte species

(1) Organisms should be grown on potato dextrose slants at 35°C for 7 days or until good sporulation is obtained; good sporulation may be obtained after 48 h of incubation for some isolates (Zygomycetes and *Aspergillus* spp). *Fusarium* spp. need to be incubated for 48 to 72 h at 35°C and then until day 7 at 25 to 28°C.

(2) Slants are covered with sterile saline, and conidia are harvested by agitation with a sterile Pasteur pipette. Addition of 1 drop (0.01 ml) of Tween 20 facilitates the preparation of *Aspergillus* sp. inocula. The resulting suspension is removed to a sterile conical tube and allowed to settle for 3 to 5 min. The upper layer is transferred to a second vial and vortexed. The optical density ($\lambda = 530$ nm) of this suspension is measured and adjusted with sterile saline according to the following guidelines.

(a) *Aspergillus* spp., *Paecilomyces lilacinus, Paecilomyces variotii, Exophiala dermatitidis,* and *Sporothrix schenckii:* 0.09 to 0.13 optical density (OD) unit

(b) *Fusarium* spp., *S. apiospermum, Ochroconis gallopava, Cladophialophora bantiana, R. oryzae,* and other zygomycetous spp.: 0.15 to 0.17 OD unit

VI. PROCEDURES *(continued)*

(c) *Bipolaris* spp. and *Alternaria* spp.: 0.25 to 0.3 OD unit

(3) Stock suspensions of conidia should then be diluted 1:50 in RPMI for microdilution testing and 1:100 in RPMI for macrodilution testing. The 1:50 inoculum dilutions will be two times more concentrated than the density needed (approximately 0.4×10^4 to 5×10^4 CFU/ml).

b. Dermatophyte species

(1) Certain dermatophyte isolates pose special problems, as conidiation is often absent on potato dextrose agar. *Trichophyton rubrum* isolates can be induced to form conidia on cereal agar plates (oatmeal agar) incubated at 30°C for 4 to 5 days or until good conidial growth is present (5). Conidia can be harvested to sterile saline by agitation with a sterile cotton-tipped swab. Allow the resulting suspension to settle for 5 to 10 min.

(2) Count conidia with a hemacytometer and adjust the concentration as needed. The final suspension should be made twice more concentrated than the density needed for testing (1×10^3 to 3×10^3 CFU/ml).

3. Test inoculation and reading (microdilution method)

a. The procedures for setting up the microdilution tests for dermatophytes and nondermatophyte organisms are the same as described above for yeast isolates (item VI.A). Briefly, inoculate each well on the day of the test with 0.1 ml of the 2× conidial or sporangiospore inoculum suspension. This step will dilute the drug concentrations, inoculum densities, and solvent, if used, to the final desired test concentrations.

b. Incubate all microdilution trays at 35°C without agitation.

4. Test inoculation and reading (broth macrodilution modification)

The steps and testing conditions relevant to the broth macrodilution test have not been evaluated with the echinocandins or for the dermatophytes. The procedures for setting up the macrodilution tests for nondermatophyte organisms are the same as described above for yeast isolates.

a. Prepare and adjust the stock inoculum suspensions as described for the broth microdilution test.

b. Mix the stock conidia or sporangiospore suspension for 15 s with a vortex and dilute 1:100 with medium to obtain the test inoculum (0.4×10^4 to 5×10^4 CFU/ml).

c. Add 0.1 ml of each antifungal (at the intermediate concentration for water-soluble drugs and the intermediate concentration diluted 1:10 for water-insoluble drugs) to a sterile 12- by 75-mm test tube.

d. A growth control tube should receive 0.1 ml of drug diluent without antifungal agent. A second tube of drug diluent should remain uninoculated to serve as a sterility control.

e. Add 0.9 ml of the inoculum to each tube in the series (with the exception of the sterility control). This brings the antifungal agents to their final concentrations.

f. Incubate the plates at 35°C as described above.

5. Reading results

a. Filamentous fungi, including *Fusarium* spp., *Aspergillus* spp., and *Sporothrix schenckii,* are evaluated after 46 to 50 h of incubation.

b. *Rhizopus* spp. are examined after 21 to 26 h of incubation.

c. *Scedosporium* spp. are evaluated after 70 to 74 h of incubation.

d. Dermatophyte isolates are evaluated after 4 days of incubation.

e. For echinocandins, isolates are evaluated after 21 to 26 h *(Aspergillus* spp. and *Paecilomyces variotii)* and 46 to 72 h (*Scedosporium* spp.), or

VI. PROCEDURES *(continued)*

the first day when sufficient growth (covering the bottom of the well) is present in the growth control well.

f. For echinocandins, the minimal effective concentration (MEC) corresponds to the lowest concentration of drug that leads to the growth of small, rounded, compact hyphal forms compared to the hyphal growth seen in the growth control well.

g. For amphotericin B, the MIC is defined as the lowest drug concentration that causes 100% inhibition of growth compared to the growth control. No trailing pattern should be seen in wells.

h. MIC reading endpoints are not well defined for fluconazole, 5-FC, or ketoconazole. For nondermatophyte isolates, a 50% inhibition in growth compared to the growth control is predictive of the MIC. For dermatophyte isolates, the turbidity allowed corresponds to 80% growth inhibition compared to the growth in the control well.

i. The MIC is read visually at the lowest drug concentration that causes 100% growth inhibition for the following agents: itraconazole, posaconazole, ravuconazole, and voriconazole. Trailing effect is usually not seen. When testing dermatophyte isolates against itraconazole, posaconazole, and voriconazole, the turbidity allowed corresponds to an 80% reduction in growth compared to the growth control well.

j. Endpoints are not well defined for ciclopirox, griseofulvin, or terbinafine. The turbidity allowed corresponds to an 80% or more reduction in growth compared to the growth control.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING AND INTERPRETATION OF RESULTS

A. General considerations

In vitro susceptibility testing is designed to aid in the prediction of the in vivo response to treatment. However, low MICs for fungi do not necessarily predict clinical success. So far, breakpoints have been established for oropharyngeal candidiasis due to *Candida* species for fluconazole, itraconazole, and 5-FC, enabling selection of a suitable therapeutic agent (see section below) (2). MIC testing for these organisms is merited if there are contraindications or refractory infection. Interpretive MIC breakpoints for echinocandins were recently added (2, 7, 8). Susceptibility data on echinocandins and voriconazole are based substantially on experience with nonneutropenic patients with candidemia, and their clinical relevance in other settings is uncertain (9). The MIC breakpoints for other drug-organism combinations and for different disease settings are yet to be determined. Therefore, the sensitivity of an organism to a specific drug should be compared to the MIC range for other isolates of that species, which indicate the likelihood of sensitivity or resistance.

B. Interpretation of MICs for yeast isolates

1. Amphotericin B

Interpretive breakpoints for amphotericin B have not been established. However, experience has shown that isolates for which the MIC is >1 μg/ml are likely to be resistant.

2. Ketoconazole

The MICs of ketoconazole for yeasts vary between 0.313 and 16 μg/ml. However, interpretive endpoints have not yet been established for this drug.

3. Other antifungal drugs

a. Interpretive breakpoints for fluconazole, 5-FC, itraconazole, and voriconazole for *Candida* spp. are summarized in Table 8.10.1–7.

Table 8.10.1–7 Interpretive breakpoints for *Candida* spp.

Antifungal agent	Breakpoint (μg/ml)				
	Susceptible	Susceptible—dose dependent	Intermediate	Resistant	Nonsusceptible
Anidulafungin	≤2				≥2
Caspofungin	≤2				≥2
Fluconazole	≤8	16–32		≥64	
5-FC	≤4		8–16	≥32	
Itraconazole	≤0.125	0.25–0.5		≥1	
Micafungin	≤2				≥2
Voriconazole	≤1	2		≥4	

VII. REPORTING AND INTERPRETATION OF RESULTS *(continued)*

b. Interpretive breakpoints have not yet been established for posaconazole or ravuconazole. Yeast MICs for both agents varied between 0.03 and 16 μg/ml, with the majority of isolates inhibited by ≤1 μg/ml.

c. Interpretive breakpoints for echinocandins (anidulafungin, caspofungin, and micafungin) have recently been adopted and are still considered tentative. MICs of ≤2 μg/ml are predictive of susceptibility. Isolates for which the MICs are >2 μg/ml are described as nonsusceptible. There is no resistance category assigned for the echinocandins.

C. Interpretation of MICs for filamentous fungi and dermatophytes

1. Amphotericin B
MICs for most filamentous moulds fall between 0.5 and 2.0 μg/ml, although the MICs for *Aspergillus terreus, Acremonium strictum, Scedosporium apiospermum,* and *Scedosporium prolificans* can be ≥2.0 μg/ml. Correlation of MIC and outcome of clinical treatment cannot as yet be predicted for the filamentous fungi.

2. Ciclopirox
Most ciclopirox MICs are ≤0.12 μg/ml for *T. rubrum*. Interpretive guidelines for nondermatophyte moulds have not been established.

3. Echinocandins (anidulafungin, caspofungin, micafungin)
Work to date has focused principally on testing of *Aspergillus* isolates for which the MECs are ≤1 μg/ml. Interpretive guidelines for filamentous moulds and dermatophytes have not been established.

4. Fluconazole
Most MICs are >64 μg/ml, and filamentous fungi are generally not susceptible. Exceptions include dermatophytes and some isolates of the dimorphic fungi.

5. 5-FC
Most MICs are >64 μg/ml, and filamentous fungi are generally not susceptible. Exceptions include some *Aspergillus* spp. and certain dematiaceous fungi.

6. Ketoconazole
MICs for filamentous moulds range from 16 to 0.03 μg/ml; however, correlation of MIC and outcome of clinical treatment cannot yet be predicted.

7. Itraconazole, posaconazole, ravuconazole, and voriconazole
MICs for filamentous moulds range from 16 to 0.03 μg/ml, with MICs for dermatophytes as low as 0.001 μg/ml. Interpretive breakpoints have not yet been established for itraconazole and the new triazoles and nondermatophyte moulds.

8. Terbinafine
Most terbinafine MICs are ≤0.01 μg/ml for the dermatophytes. Interpretive guidelines for other filamentous moulds have not been established.

VII. REPORTING AND INTERPRETATION OF RESULTS *(continued)*

9. Griseofulvin

MICs of griseofulvin are ≤1 μg/ml for the dermatophytes. Interpretive guidelines for other filamentous moulds have not been established.

VIII. ACKNOWLEDGMENT

We thank Jacqueline Herbert for her careful review and valuable comments.

REFERENCES

1. **Clinical and Laboratory Standards Institute.** 2008. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts*, 3rd ed. Approved standard M27-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
2. **Clinical and Laboratory Standards Institute.** 2008. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts*, 3rd ed. Informational supplement M27-S3. Clinical and Laboratory Standards Institute, Wayne, PA.
3. **Clinical and Laboratory Standard Institute.** 2008. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi.* Approved standard M38-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Ghannoum, M. A., A. S. Ibrahim, Y. Fu, M. C. Shafiq, J. E. Edwards, Jr., and R. S. Criddle.** 1992. Susceptibility testing of *Cryptococcus neoformans:* a microdilution technique. *J. Clin. Microbiol.* **30:**2881–2886.
5. **Jessup, C. J., J. Warner, N. Isham, I. Hasan, and M. A. Ghannoum.** 2000. Antifungal susceptibility testing of dermatophytes: establishing a medium for inducing conidial growth and evaluation of susceptibility of clinical isolates. *J. Clin. Microbiol.* **38:**341–344.
6. **Pappas, P. G., J. H. Rex, J. D. Sobel, S. G. Filler, W. E. Dismukes, T. J. Walsh, and J. E. Edwards.** 2004. Guidelines for treatment of candidiasis. *Clin. Infect. Dis.* **38:**161–189.
7. **Pfaller, M. A., L. Boyken, R. J. Hollis, J. Kroeger, S. A. Messer, S. Tendolkar, and D. J. Diekema.** 2008. In vitro susceptibility of invasive isolates of *Candida* spp. to anidulafungin, caspofungin, and micafungin: six years of global surveillance. *J. Clin. Microbiol.* **46:**150–156.
8. **Pfaller, M. A., D. J. Diekema, L. Ostrosky-Zeichner, J. H. Rex, B. D. Alexander, D. Andes, S. D. Brown, V. Chaturvedi, M. A. Ghannoum, C. C. Knapp, D. J. Sheehan, and T. J. Walsh.** 2008. Correlation of MIC with outcome for *Candida* species tested against caspofungin, anidulafungin, and micafungin: analysis and proposal for interpretive MIC breakpoints. *J. Clin. Microbiol.* **46:**2620–2629.
9. **Pfaller, M. A., D. J. Diekema, J. H. Rex, A. Espinel-Ingroff, E. M. Johnson, D. Andes, V. Chaturvedi, M. A. Ghannoum, F. C. Odds, M. G. Rinaldi, D. J. Sheehan, P. Troke, T. J. Walsh, and D. W. Warnock.** 2006. Correlation of MIC with outcome for *Candida* species tested against voriconazole: analysis and proposal for interpretive breakpoints. *J. Clin. Microbiol.* **44:**819–826.
10. **Rex, J. H., M. A. Pfaller, J. N. Galgiani, M. S. Bartlett, A. Espinel-Ingroff, M. A. Ghannoum, M. Lancaster, F. C. Odds, M. G. Rinaldi, T. J. Walsh, and A. L. Barry.** 1997. Development of interpretive breakpoints for antifungal susceptibility testing: conceptual framework and analysis of in vitro-in vivo correlation data for fluconazole, itraconazole, and *Candida* infections. Subcommittee on Antifungal Susceptibility Testing of the National Committee for Clinical Laboratory Standards. *Clin. Infect. Dis.* **24:**235–247.

8.10.2 Antifungal Disk Diffusion Susceptibility Testing

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

To make antifungal susceptibility testing simple, rapid, and cost-effective, the Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards [NCCLS]) subcommittee on antifungal susceptibility testing has developed recommendations for disk diffusion testing for antifungal agents. This method provides qualitative results 24 h sooner than the standard method for testing yeasts used in CLSI document M27. This technique is intended for testing *Candida* spp. except for *Candida krusei.* Moreover, disk diffusion interpretive criteria are available only for fluconazole and voriconazole, and the clinical relevance of testing any other drug-organism combination remains uncertain. Although disk diffusion methods for testing filamentous fungi are not addressed in this document, a proposal to develop such a document, M51, is underway. The procedures described below are based on CLSI documents M44-A and M44-S2 (1, 2).

II. MATERIALS

A. Test media

Mueller-Hinton agar is the preferred medium for routine susceptibility testing of yeasts because it is readily available and shows acceptable batch-to-batch reproducibility. Moreover, it is easily supplemented with glucose and methylene blue (GMB). Sabouraud glucose agar or potato dextrose agar is required to subculture the organisms for inoculum preparation.

B. Antifungal disks

Antifungal disks include fluconazole and voriconazole disks.

C. Equipment

1. Incubator set at 35°C with ambient air
2. McFarland 0.5 turbidity standard and spectrophotometer at a 530-nm wavelength
3. Sterile cotton (not synthetic polyester fiber) swabs
4. Sterile physiological (8.5 g of NaCl/liter; 0.85%) saline
5. Electronically digital caliper

III. PREPARATION OF MEDIUM

Mueller-Hinton agar medium for the disk diffusion test should be supplemented with 2% glucose and 0.5 μg of methylene blue dye per milliliter. The medium can be prepared and poured as the complete medium with supplement, or the supplement can be added to commercially prepared Mueller-Hinton agar plates.

A. Preparation of supplemented Mueller-Hinton agar

1. Mueller-Hinton agar should be prepared from a commercially available dehydrated Mueller-Hinton agar base according to the manufacturer's instructions.
2. Dissolve 0.1 g of methylene blue dye in 20 ml of distilled water and warm gently to dissolve. Add 100 μl of this solution per liter of agar suspension.

III. PREPARATION OF MEDIUM *(continued)*

3. Add 20 g of glucose per liter of agar suspension and autoclave as directed by the manufacturer's instructions.
4. Allow the agar solution to cool in a 45 to 50°C water bath. Agar medium pH should be between 7.2 and 7.4 at 25°C.
5. Pour the medium into plastic, flat-bottomed petri dishes.
6. Allow the agar medium to cool at room temperature. Wrap plates in plastic bags to minimize drying of agar. Store at refrigeration temperature (2 to 8°C). Plates should be used within 7 days of preparation.
7. If excess surface moisture is present on the agar surface or the petri dish cover, the agar plates should be dried in an incubator or laminar-flow hood for 10 to 30 min or until the excess moisture has evaporated.
8. A representative sample of each batch of plates should be examined for sterility by incubation at 30 to 35°C for 24 h.
9. The pH of each batch of prepared Mueller-Hinton agar should be checked. The agar medium should have a pH between 7.2 and 7.4 at 25°C after gelling.

B. Preparation of GMB supplementation of commercially prepared Mueller-Hinton agar

1. Commercially prepared Mueller-Hinton agar plates can be obtained directly from manufacturers.
2. Dissolve 0.1 g of methylene blue dye in 20 ml of distilled water and warm gently to dissolve. Add 100 μl of this solution per liter of agar suspension.
3. Prepare a 0.4-g/ml stock solution of glucose by dissolving 40 g of glucose in 100 ml of distilled water. Heat gently and mix to dissolve.
4. Make a GMB stock solution with a final concentration of 40% glucose and 10 μg of methylene blue per ml by adding 200 μl of methylene blue dye stock to 100 ml of glucose stock solution.
5. Dispense GMB stock solution in vials containing 3.5-ml aliquots for 150-mm plates or 1.5-ml aliquots for 90- to 100-mm plates.
6. Autoclave for 15 min at 121°C.
7. Pour 3.5 ml of GMB supplement onto the surface of a 150-mm plate with a 70-ml fill or 1.5 ml onto a 90- to 100-mm plate with a 30-ml fill. Spread the GMB supplement with a sterile spreader or a sterile bent glass rod across the surface of the agar plate.
8. Allow the GMB solution to completely absorb, for 4 to 24 h, before inoculating the plate. Plates can be dried at room temperature or in an incubator. Plates can be stored in a refrigerator until used.

IV. STORAGE

A. Prepared agar plates should be wrapped in plastic bags and stored in a refrigerator at 2 to 8°C. Plates should be used within 7 days of preparation.

B. GMB stock solution is stored at room temperature. Do not refrigerate, as this may cause precipitation. A 1-year shelf life is usually assumed.

C. Disk containers should be kept in a non-frost-free freezer until needed. Disk containers should always be refrigerated at 8°C or frozen at −14°C. Always refer to the manufacturer's instructions for use and expiration date.

D. Disk containers should be removed from the refrigerator or freezer 1 to 2 h before use to warm at 25°C. Once removed from its sealed packaging, the disk cartridge should be placed in a sealed desiccated container and stored at the proper temperature.

ANALYTICAL CONSIDERATIONS

V. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Yeast isolates and zone diameter breakpoint ranges

Inclusion of QC isolates in susceptibility testing will allow monitoring of the precision and accuracy of the susceptibility test procedure, performance of the reagents, the testing conditions, and the performance of testing personnel. QC strains are to be incorporated into each daily run of patient testing. Acceptable disk diffusion zone diameter breakpoint ranges for QC isolates are outlined in Table 8.10.2–1.

B. Media

Ensure that all media and disks are used within their expiration dates and stored at the appropriate temperature.

C. Equipment

Regularly clean and calibrate the pipettes and spectrophotometer to maintain accuracy and reduce the likelihood of contamination.

VI. PROCEDURES

A. Inoculum preparation

1. All organisms should be subcultured onto blood agar, Sabouraud glucose agar, or potato dextrose agar and incubated at 35°C for 24 h (*Candida* spp.).
2. The inoculum should be prepared by picking five well-isolated colonies >1 mm in diameter and suspending the organisms in 5 ml of sterile saline (8.5 g of NaCl per liter; 0.85% saline) or sterile water.
3. A suspension equal to a 0.5 McFarland standard (spectrophotometer reading at λ of 530 nm) is prepared by adding sterile saline or sterile water. This procedure will yield a concentration of 1×10^6 to 5×10^6 cells per ml.

B. Test inoculum and incubation

1. Dip a sterile cotton swab into the suspension prepared as described above. The swab should be pressed firmly against the inside wall of the tube to remove excess fluid before use.
2. Inoculate the plate with Mueller-Hinton plus GMB agar by evenly streaking the swab over the entire agar surface. The rim of the agar is swabbed as the final step.

Table 8.10.2–1 Disk diffusion zone diameter breakpoint ranges for QC isolates

Antifungal agent	Disk content (μg)	Breakpoint range (μg/ml)			
		C. albicans ATCC 90028	*C. parapsilosis* ATCC 22019	*C. tropicalis* ATCC 750	*C. krusei* ATCC 6258
Fluconazole	25	28–39	22–33	26–37	NA[a]
Voriconazole	1	31–42	28–37	NA	16–25
Posaconazole	5	24–34	25–36	23–33	23–31
Caspofungin	5	18–27	14–23	20–27	18–26

[a] NA, not applicable.

Table 8.10.2–2 Zone diameter interpretive standards and corresponding MIC breakpoints for *Candida* spp.[a]

Antifungal agent	Disk content (μg)	Zone diam (nearest whole mm)			Equivalent MIC breakpoint (μg/ml)		
		R	S-DD	S	R	S-DD	S
Fluconazole	25	≤14	15–18	≥19	≥64	16–32	≤8
Voriconazole	1	≤13	14–16	≥17	≥4	2	≤1

[a] S, susceptible; R, resistant; S-DD, susceptible—dose dependent; R, resistant.

VI. PROCEDURES *(continued)*

3. Allow the inoculated agar surface to partially dry by keeping the lid ajar for 3 to 5 min before applying the drug-impregnated disks.
4. Place antimicrobial disks onto the surface of the inoculated agar plate. Disks should be firmly in contact with the agar surface and distributed evenly so they are no closer than 24 mm from center to center. As a rule, no more than 12 disks should be placed on a 150-mm plate or more than 4 disks on a 100-mm plate.
5. Plates are inverted and placed in incubator at 35°C for 24 h before reading.

C. Reading results

1. The plate should be held a few inches above a black, nonreflecting background illuminated with reflected light. Measure the zone diameter to the nearest whole millimeter at the point at which there is a prominent reduction in growth. The zone of inhibition is uniformly circular, with a semiconfluent lawn of growth.
2. Disk diffusion zone diameters correlate inversely with MICs from standard dilution tests.

POSTANALYTICAL CONSIDERATIONS

VII. INTERPRETATION OF RESULTS

Refer to Table 8.10.2–2 for result interpretation.

VIII. ACKNOWLEDGMENT

We thank Jacqueline Herbert for her careful review and valuable comments.

REFERENCES

1. **Clinical and Laboratory Standards Institute.** 2004. *Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts.* Approved standard M44-A. Clinical and Laboratory Standards Institute, Wayne, PA.
2. **Clinical and Laboratory Standards Institute.** 2007. *Zone Diameter Interpretive Standards, Corresponding Minimal Inhibitory Concentration (MIC) Interpretive Breakpoints, and Quality Control Limits for Antifungal Disk Diffusion Susceptibility Testing of Yeasts,* 2nd ed. Informational supplement M44-S2. Clinical and Laboratory Standards Institute, Wayne, PA.

SECTION 9

Parasitology

SECTION EDITOR: *Robyn Y. Shimizu*

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9.1 Introduction

PART 1 Equipment

If necessary, maintenance procedures can be performed and documented more often than the minimal recommendations presented in this section.

I. MICROSCOPE

Good microscopes and light sources are mandatory for the examination of specimens for parasites. Identification of the majority of organisms depends on morphologic differences, most of which must be seen by using stereoscopic or regular microscopes. An explanation of how to calibrate the microscope can be found later in this section.

A. Stereoscopic

A stereoscopic microscope should be available for larger specimens (arthropods, tapeworm proglottids, and various artifacts). The total magnification usually ranges from ×10 to ×45. Some microscopes have a zoom capacity from ×10 to ×45, and others have fixed objectives (0.66×, 1.3×, 3×) that can be used with 5× or 10× oculars. Use the light source either from under the specimen or directed onto the top of the specimen.

B. Regular

The light microscope should be equipped with the following parts.

1. Head

A monocular head can be used, but a binocular type is recommended to reduce fatigue during lengthy examinations. The binocular head should contain a diopter adjustment to compensate for variation in focus between the eyes. A microscope equipped with a tilting ergonomic head allows for maximum user comfort.

2. Oculars

10× are required; 5× can be helpful but are not essential

3. Objectives

10× (low power), 40× to 45× (high power), and 97× to 100× (oil immersion)

4. Stage

A mechanical stage for X and Y movement is necessary. Graduated stages can be helpful and are recommended for recording the exact location of an organism in a permanently stained slide. This capability is essential for consultation and teaching responsibilities.

I. MICROSCOPE *(continued)*

5. Condenser
   - **a.** A bright-field condenser equipped with an iris diaphragm is required. The numerical aperture (N.A.) of the condenser should be equal to or greater than the N.A. of the highest objective.
   - **b.** An adjustable condenser is not required with the newer microscopes.
6. Filters
   Both clear blue glass and white ground-glass filters are recommended.
7. Light source
   - **a.** The light source, along with an adjustable voltage regulator, should be contained in the microscope base. Align this light source as directed by the manufacturer.
   - **b.** If the light source is external, the microscope must be equipped with an adjustable mirror and a condensing system containing an iris diaphragm that can be released or lowered.
   - **c.** The light source should be a 75- to 100-W bulb.

C. Microscope maintenance

1. Remove dust from all optical surfaces with a camel hair brush.
2. Remove oil and finger marks on the lenses immediately with a clean soft cloth or several thicknesses of lens tissue. Single-thickness lens tissue may permit corrosive acids from the fingers to damage the lens. *Do not use any type of tissue other than lens tissue; otherwise you may scratch the lens.* Use very little pressure to clean the lenses in order not to remove the coatings on the external surfaces of the lenses.
3. Use water-based cleaning fluid for normal cleaning. Use organic solvents sparingly and only if absolutely necessary to remove oil from the lens. Since microscope manufacturers do not agree on a common solvent, the manufacturer of each microscope should be consulted. One recommended solvent is 1,1,1-trichloroethane; it is a good solvent for immersion oil and mounting media, but it will not soften the lens sealers and cements. Do not use xylene, any alcohols, acetone, or any other ketone.
4. Clean the lamp (cool, in off position) with lens tissue moistened in 70% isopropyl or ethyl alcohol (to remove oil transferred from the fingers to the lamp) after the lamp has been installed in the lamp holder.
5. Clean the stage with a small amount of disinfectant (70% isopropyl or ethyl alcohol) when it becomes contaminated with fecal material.
6. Cover the microscope when not in use. In extremely humid climates (a relative humidity of more than 50%), good ventilation is necessary to prevent fungal growth on the optical elements.
7. Clean and lubricate the substage condenser slide as needed. Petroleum jelly or light grease can be used as a lubricant.
8. Schedule a complete general cleaning and readjustment at least annually to be performed by a factory-trained and authorized individual. Record all data related to preventive maintenance and repair.

II. CENTRIFUGE

A. Requirements

1. Either a table or floor model centrifuge, which should be able to accommodate 15-ml centrifuge tubes (for concentration procedures), is acceptable.
2. Regardless of the model, a free-swinging or horizontal head type is recommended. With this type of centrifuge, the sediment will deposit evenly on the bottom of the tube. The flat upper surface of the sediment facilitates the removal of the supernatant fluid from a loosely packed pellet.
3. The centrifuge should have adjustable speed settings.

II. CENTRIFUGE *(continued)*

B. Maintenance of the centrifuge

1. Check the carrier cups, trunnions, and rotor for corrosion and cracks before each run. If found to be defective, replace immediately.
2. Check for the presence of the proper cup cushions before each run.
3. Check the speed at all regularly used speeds at least quarterly by using a stroboscopic light to verify the accuracy of a built-in tachometer or speed settings. Record results.
4. Use 10% household bleach or phenolic solution to disinfect the centrifuge bowl, buckets, trunnions, and rotor following a breakage or spill and at least monthly. Following disinfection, rinse the parts in warm water, and give a final rinse in distilled water. It is imperative to thoroughly dry the parts with a clean absorbent towel to prevent corrosion.
5. Clean the inside of the cups in mild, warm soapy water, and scrub with a nylon brush. Use fine steel wool to remove stubborn deposits. The part should be rinsed in distilled water and thoroughly dried. This should be done as needed and at least quarterly.
6. Lubricate bearings (if not permanently lubricated) as specified by the manufacturer at least quarterly.
7. Check brushes, and replace if worn to 1/4 in. of the spring. Check semiannually.
8. Check autotransformer brush, and replace if worn to 1/4 in. of the spring. Check semiannually.
9. Remove the head and dust cover, and add lubricant as specified by the manufacturer to fill the grease cup. Do this semiannually.
10. Replace grease in grease cup as specified by the manufacturer annually.
11. Record all data related to preventive maintenance and repair.

III. FUME HOOD

A. Requirements

Although a fume hood is not required, many laboratories prefer to keep their staining reagents and formalin in a fume hood. Even with the substitution of dehydrating reagents other than xylene, fume hoods may be preferred in order to eliminate odors. In keeping with good laboratory practice, the placement of reagents and equipment into a hood should not interfere with the proper operation of the hood.

B. Maintenance of the fume hood

1. Check the air velocity with the sash fully open and the cabinet empty with a thermoanemometer (minimum acceptable face velocity is 100 ft [ca. 30 m]/min) (3, 20) at least quarterly.
2. Perform a smoke containment test with the cabinet empty to verify proper directional face velocity. Check at least quarterly.
3. Lubricate the sash guides as needed to provide for ease of operation.
4. See Appendix 9.10.7–1 at the end of this section for formalin exposure guidelines.

IV. BIOLOGICAL SAFETY CABINET

A. Requirements

A biological safety cabinet is not required for processing routine specimens in a diagnostic parasitology laboratory. However, some laboratories use class I (open-face) or class II (laminar-flow) biological safety cabinets to process all unpreserved specimens.

B. Maintenance of the biological safety cabinet

1. Disinfect the work area after each use. Do not depend on UV irradiation to decontaminate the work surface. UV irradiation has very limited penetrating powers (21).

IV. BIOLOGICAL SAFETY CABINET *(continued)*

2. Clean UV lamps (in the off position) with 70% isopropyl or ethyl alcohol at least weekly.
3. Monitor UV output (UV intensity meter; any major laboratory supplier) quarterly to confirm that the effective radiation (excess of 40 $\mu W/cm^2$ at a wavelength of 253.7 nm) is present at the work surface (20). If not, replace the lamp.
4. Have class I biological safety cabinets certified after installation but before use, after they have been relocated or moved, and at least annually. The certification should include the following.
   a. Measurements of air velocity at the midpoint height approximately 1 in. behind the front opening. Make these measurements approximately every 6 in.
   b. The average face velocity should be at least 75 linear ft/min. Use a thermoanemometer with a sensitivity of ± 2 linear ft/min (20).
   c. Perform a smoke containment test with the cabinet containing the routine work items, such as a Bunsen burner, test tube rack, bacteriological loop and holder, etc., to determine the proper directional velocity.
   d. Record the date of recertification and the name of the individual or company recertifying the cabinet.
5. Replace the filters as needed.
6. Have a class II biological safety cabinet certified to meet Standard 49 of the National Sanitation Foundation (Ann Arbor, MI) when it is installed (20).
7. Recertify the cabinet to meet Standard 49 when the cabinet is moved, after filters are replaced, after the exhaust motor is repaired or replaced, or after any gaskets are removed or replaced (20). Recertify at least annually, and record the date of service recertification along with the name of the individual or company performing the service.
8. Keep all airflow parts completely clear at all times.

V. REFRIGERATOR-FREEZER

A. Requirements

A general-purpose laboratory (non-explosion proof) or household-type refrigerator-freezer is adequate for use in the parasitology laboratory. The temperature should be approximately 5°C. Do not store solvents with flash points below refrigeration temperature even in modified (explosion-proof) refrigerators.

B. Maintenance of refrigerators-freezers

1. Monitor and record the temperature of the refrigerator on a daily basis. Place the thermometer into a liquid to permit stable temperature recording.
2. Monitor and record the temperature of the freezer on a daily basis. Place the thermometer into antifreeze (any brand with a freezing point below that of the freezer, such as ethylene glycol-water solutions, glycerol-water solutions, Prestone) to permit stable temperature recording.
3. Check the operation of the fan (if so equipped) inside the cabinet.
4. Monthly, check the door gasket for deterioration, cracks, and proper seal.
5. Semiannually, clean the condenser tubing and air grill with a vacuum cleaner.
6. Semiannually, check to ensure that the drain tubes are kept open.
7. Annually, wash the interior with a warm solution of baking soda and water (approximately 1 tablespoon/qt [ca. 13 to 14 g/0.946 liter]). Rinse with clean water, and dry.
8. Annually, decontaminate the interior as needed.
9. Annually, wash the door gasket and water collection tray with a mild soap and water. If the gasket accumulates a black mold, scrub with a 50% household bleach solution and a small brush. Rinse with clean water, and dry.

PART 2

Safety

The standard safety considerations of any clinical laboratory (3–7, 11, 13–17, 19, 21) should be practiced in a parasitology laboratory. Specific attention should be given to personal practices, specimen and reagent handling, and equipment (*see* section 15).

I. PERSONAL PRACTICES

Since many parasitic infections are acquired via the oral route, it is imperative that the parasitologist practice good personal hygiene. Hand washing with soap and water and adherence to laboratory technique standards are two of the most important ways to guard against infection in a clinical laboratory. Gloves, while not mandatory, are recommended, and standard precautions must be followed at all times.

II. IMMUNIZATIONS

In addition to the routine childhood immunizations against polio and rubella, an immunization against hepatitis B is recommended for those individuals processing fresh unpreserved fecal specimens. At present, there are no effective immunizations for parasitic diseases.

III. SPECIMENS

Observe standard precautions.

A. Collection

1. Collect fecal specimens and transport them to the laboratory in such a way that no one handling the container comes in direct contact with the specimen. Bag the test request slip separately from the specimen.
2. Usually, the specimen is placed in a waxed cardboard container with a tightly fitting lid.
3. If polyvinyl alcohol (PVA) fixative (containing mercuric chloride) is included in the collection kit, the following is strongly recommended.
   a. The word "Poison," the skull-and-crossbones symbol, and the statement "Causes Severe Burns" must be included on the specimen container label.
   b. It is also recommended that the following first aid instructions be included on the collection kit insert:

      > Contains mercuric chloride. Avoid contact with eyes or mucous membranes or prolonged contact with skin. Wash hands thoroughly after use. If swallowed, give milk or egg whites. Call physician immediately. For contact with eyes or skin, flush thoroughly with water. For eyes, get prompt medical attention. Keep out of reach of children.

4. Blood specimens should be collected in a manner consistent with standard precautions (17).

B. Handling

1. The unpreserved stool specimen should be considered potentially infectious; gloves are recommended. All of the following may be infective: protozoan cysts; *Cryptosporidium* oocysts; microsporidial spores; eggs of *Taenia solium*, *Enterobius vermicularis*, and *Hymenolepis nana;* and larvae of *Strongyloides stercoralis.*
2. The fresh specimen may also contain *Salmonella* spp., *Shigella* spp., or other bacterial pathogens, and bloody stools may pose a special hazard as potential carriers of hepatitis A and B viruses, human immunodeficiency virus, and enteric non-A, non-B viruses (17).
3. Because the unpreserved specimen may contain these infectious agents, the specimen must be collected, handled, and shipped to the laboratory so

III. SPECIMENS *(continued)*

the handlers are prevented from coming in direct contact with the specimen.

4. A 5% solution of formalin may permit *Ascaris lumbricoides* (6, 7, 10) eggs to become embryonated in stool specimens and thereby become infective. A 10% solution of formalin reduces this potential hazard. Personnel are urged to use standard precautions at all times.

C. Processing

1. In processing the unpreserved specimen, avoid direct contact between the specimen and the parasitologist and/or the equipment being used. Use of a biological safety cabinet is often recommended.
   - **a.** For example, use a 3- by 2-in. microscope slide for preparing direct wet mounts.
   - **b.** Sealing the stool preparation with clear nail polish or petroleum jelly will reduce the possibility of direct contact, although this approach is often not practical for routine clinical laboratories.
   - **c.** Be careful when handling the preserved specimen. Maintain pressure on the stopper while shaking the specimen in the defatting step of the concentration procedure (use of ethyl acetate). Built-up pressure may cause an uncontrolled release of the contents.

D. Disposal

1. Unpreserved fecal and blood specimens can be safely discarded into a sanitary sewage system if local health codes permit (17).
2. Specimens preserved in formalin or PVA fixative may pose a unique disposal problem because of regulations regarding formalin and mercury-based compounds. PVA fixative contains Schaudinn's fluid (mercuric chloride base) to which PVA plastic powder is added. Consult state public health officials for proper disposal guidelines.

IV. REAGENTS

Know what chemical hazards are in the workplace (2). One way to make the hazards known is to make available the MSDS on the chemical(s) in use. The availability and location of these sheets should be well known by all laboratory personnel. Usually they are kept filed in a notebook rather than being posted.

A. Use

There are certain basic considerations when chemicals are used in the laboratory.

1. Use the smallest container of flammable solvents (flash point below 37.8°C) compatible with the work.
2. Do not use flammable solvents near open flames or microincinerators.

B. Storage

1. Because the parasitology laboratory is usually one of many sections within the clinical laboratory, the storage of reagents must be considered in terms of the complete clinical laboratory.
2. Be sure that the quantities of flammable solvents stored comply with the Code of the National Fire Protection Association, Quincy, MA, and the relevant regulations of the OSHA (1), and store solvents so that incompatible chemicals are segregated.
3. Do not store flammable solvents having a flash point below 4°C in a refrigerator.
   - **a.** Evaporation can occur in these reagents even at this low temperature and cause dangerous levels of vapors to accumulate inside the refrigerator.
   - **b.** Refrigeration temperatures also cause solvents to pick up atmospheric moisture faster than does room temperature, and refrigeration does not retard the formation of dangerous peroxides in ethers.

IV. REAGENTS *(continued)*

c. It is better to store these solvents in stoppered containers on well-ventilated open shelves away from open flames.

d. Some laboratories have elected to store these solvents in an explosion-proof fume hood.

4. As in the general laboratory, do an inventory of reagents at least annually, and discard all reagents that show signs of deterioration or that are beyond their expiration dates.

C. Disposal

Most water-soluble chemicals can safely be discarded in the sanitary sewer.

1. Neutralize acids and bases to within a pH range of 2 to 12.5, and then dilute them 1:20 with tap water prior to disposal (13).

2. Dilute small quantities of flammable solvents similarly prior to disposal in the sewer.

3. Since permissible substances and quantities will vary, contact state and local authorities for proper disposal guidelines.

D. Substitution of less hazardous reagents

The financial impact of various state and federal regulations governing the use and disposal of hazardous laboratory reagents serves as the impetus to search for less hazardous reagents. Some of the reagents for which there are substitutes are as follows.

1. Ethyl acetate (22) with a higher flash point (4.0°C) replaced ether (flash point of −45°C) in the formalin-ether concentration technique. Hemo-De (Thermo Fisher Scientific, Waltham, MA) can also replace ethyl acetate in the concentration procedure (18). This reagent has a flash point of 57.8°C and is generally regarded as safe by the Food and Drug Administration.

2. Xylene, as used in the trichrome or iron hematoxylin staining of PVA-fixed fecal smears, is a potential toxic and fire hazard. Again, Hemo-De has successfully replaced xylene in both the carbol-xylene and xylene steps of the trichrome procedure (6, 7). There are other substitutes available as well (Hemo-Sol; Fisher Scientific). Check with your local pathology departments or reagent suppliers for other alternatives.

3. Mercuric chloride, used in PVA fixative and Schaudinn's fixative, presents both a toxic hazard and a disposal problem. Copper sulfate has been suggested as a substitute for mercuric chloride (6, 8); however, protozoan morphology will not be as clear and precise if this formula is used. Many single-vial systems are now available; most fixatives use a zinc base. However, the total formulas for these stool preservatives are proprietary (6).

4. Before any of these substitute reagents are incorporated into the routine procedures, perform comparative studies.

5. Consult section 15 for further cautions.

V. EQUIPMENT

A. Microscope

1. If the stage of the microscope becomes contaminated with fecal material, wipe it clean with a disinfectant (70% isopropyl or ethyl alcohol).

2. Observe the general safety precautions governing the use of electrical equipment; i.e., do not use near water, check for bare wires, turn off power before servicing, etc.

B. Centrifuge

1. Use plastic tubes to reduce the hazards of breakage during centrifugation. If glass centrifuge tubes are used, inspect them, and use only those free of defects. To prevent aerosols, cap tubes prior to centrifugation.

2. Always balance the centrifuge prior to use.

V. EQUIPMENT *(continued)*

3. Close the centrifuge lid when the centrifuge is in operation. Should noise or vibration develop, stop the centrifuge and check for symmetrical loading of the head.

C. Fume hood

1. If a fume hood is used to store flammable solvents such as ethyl acetate, ether, or xylene, keep the hood in operation to prevent the buildup of dangerous or toxic levels of these reagents. An adequate face velocity of 60 to 100 ft/min is recommended (3).
2. If the hood is used for other purposes, place reagents so that they do not interfere with the proper operation of the hood.

PART 3

Quality Control

I. INTERNAL QUALITY CONTROL

The internal QC component of a QC program generally consists of documenting the proper functioning of reagents and equipment at prescribed intervals and evaluating performance on split samples (day to day, batch to batch) and within-batch reproducibility studies (*see* section 14).

A. Check the permanent staining procedure with known positive control specimens when the stain is prepared (or a new lot number is purchased) and at least weekly. Record all QC results.

B. If positive specimens are not available, use smears of feces containing epithelial cells or pus cells (6, 9, 16).

C. Check stains for blood parasites for staining quality at the time of preparation, when put into use, and quarterly. If positive blood smears are not available, use a negative smear.

D. Introduce previously identified specimens as blind specimens to evaluate the overall performance of the parasitology laboratory. The use of these samples is of particular value in those laboratories that process few specimens and in those that receive a small number of positive specimens or lack a variety of specimens.

II. EXTERNAL QUALITY CONTROL PROFICIENCY TESTING

A. Every parasitology laboratory should subscribe to a proficiency testing program to provide an unbiased evaluation of its performance.

B. For tests for which there is no commercially available proficiency testing, an alternative performance or assessment system must be established to determine the reliability of testing.

III. REFERENCE MATERIALS

A. Reference materials should be available for comparison with unknown organisms, refresher training, and the training of additional personnel.

B. Ideal reference materials include formalin-preserved specimens of helminth eggs, larvae, and protozoan cysts; stained fecal smears of protozoan oocysts, spores, cysts, and trophozoites; and positive blood smears.

C. Color slides and atlases are recommended, although the level of microscopic focus cannot be changed.

D. Reference books and manuals from a number of publishers are available, and selected ones should be part of the parasitology library.

PART 4

Quality Assurance

I. QUALITY ASSURANCE

QA is the sum of all the activities necessary to produce consistently accurate test results. These include preanalytical, analytical, and postanalytical activities (*see* section 14).

I. QUALITY ASSURANCE *(continued)*

A. Preanalytical activities

These are all activities up to but not including the actual laboratory manipulation of the specimen that influence the quality of laboratory results.

1. Training of personnel

a. The person advising the patient or medical staff must be adequately trained in all facets of specimen collection (preparation of the patient, specimen collection times, sample quality and volume, condition of specimen container, use of preservatives, and proper labeling).

b. The person performing the parasitologic examination must be familiar with appropriate technical procedures to be used for each type of specimen and morphological recognition and differentiation of parasites.

2. Preparation of the patient

This involves the use of proper laxatives and/or enemas and the proper method for handling the patient prior to collecting a pinworm specimen, i.e., before bowel movement and bathing.

3. Specimen collection

a. Consideration must be given to the sequence of collecting multiple specimens, i.e., the time interval between administering antibiotics, X-ray dyes (barium), or nonabsorbable antidiarrheal preparations and the collection of the specimen and the time between subsequent collections.

b. Blood collections for blood-borne parasites should be optimized for the suspected parasites (15).

4. Specimen quality and volume (*see* individual protocols)

5. Specimen handling and labeling (*see* individual protocols)

B. Analytical activities

1. These include the technical or laboratory procedures necessary to produce accurate test results.

a. A procedure manual should include a detailed description of the procedures necessary to process the specimens and to identify the various parasites and their diagnostic stages.

b. Description of procedures must be part of a written procedure manual (12) that is reviewed and, when approved by the laboratory director, becomes the legal protocol for the laboratory by which each laboratorian is to be held responsible for performing tests *exactly* as described.

c. The procedure manual should be reviewed at least annually and before procedural changes are adopted by the laboratory director.

2. Also included are all aspects of QC and proficiency testing, including a corrective action plan when the appropriate results are not obtained. Preventive maintenance and equipment calibration data are also required.

C. Postanalytical activities

1. These activities include information that is transferred verbally, in writing, or by electronic means from the laboratory to the clinician and that provides the clinician with the meaningful laboratory report necessary to optimally manage the patient. This report includes the extent of laboratory procedures performed (i.e., complete workup or limited to direct smear), presence of abnormalities seen in the specimen, excessive amounts of blood or yeast cells, etc., in addition to the identity of the parasite(s) found.

2. Include qualifying statements regarding the quality of the specimen such as "inadequately preserved when received in the laboratory" or "contaminated with water or urine."

II. QUALITY ASSURANCE DOCUMENTATION

All QA monitors must be properly documented. Use of the 10-step protocol developed by the JCAHO is recommended (5).

REFERENCES

1. **Code of Federal Regulations.** 1989. Title 29 part 1910.106. U.S. Government Printing Office, Washington, DC.
2. **Code of Federal Regulations.** 1989. Title 29 part 1910.1200. U.S. Government Printing Office, Washington, DC.
3. **Crane, J. T., and J. Y. Richmond.** 1995. Design of biomedical laboratory facilities, p. 171. *In* D. O. Fleming, J. H. Richardson, J. J. Tulis, and D. Vesley (ed.), *Laboratory Safety: Principles and Practices,* 2nd ed. ASM Press, Washington, DC.
4. **Fleming, D. O.** 1995. Laboratory biosafety practices, p. 203. *In* D. O. Fleming, J. H. Richardson, J. J. Tulis, and D. Vesley (ed.), *Laboratory Safety: Principles and Practices,* 2nd ed. ASM Press, Washington, DC.
5. **Fromberg, R. (ed.).** 1987. *Monitoring and Evaluation of Pathology and Medical Laboratory Services.* Joint Commission on Accreditation of Healthcare Organizations, Chicago, IL.
6. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology,* 5th ed. ASM Press, Washington, DC.
7. **Garcia, L. S.** 2009. *Practical Guide to Diagnostic Parasitology,* 2nd ed. ASM Press, Washington, DC.
8. **Horen, W. P.** 1981. Modification of Schaudinn's fixative. *J. Clin. Microbiol.* **13:**204–205.
9. **Isenberg, H. D. (ed.).** 1998. *Essential Procedures for Clinical Microbiology.* ASM Press, Washington, DC.
10. **Melvin, D. M., and M. M. Brooke.** 1985. *Laboratory Procedures for the Diagnosis of Intestinal Parasites,* p. 163–189. U.S. Department of Health, Education, and Welfare publication no. (CDC) 85-8282. U.S. Government Printing Office, Washington, DC.
11. **National Institutes of Health.** 1979. *NIH Guidelines for Recombinant DNA Research Supplement: Laboratory Safety Monograph.* National Institutes of Health, Bethesda, MD.
12. **NCCLS.** 2002. *Clinical Laboratory Technical Procedure Manuals,* 4th ed. Approved guideline GP2-A4. NCCLS, Wayne, PA.
13. **NCCLS.** 2002. *Clinical Laboratory Waste Management.* Approved guideline GP5-A2. NCCLS, Wayne, PA.
14. **NCCLS.** 1996. *Clinical Laboratory Safety.* Approved guideline GP17-A. NCCLS, Wayne, PA.
15. **NCCLS.** 2000. *Laboratory Diagnosis of Blood-Borne Parasitic Diseases.* Approved guideline M15-A. NCCLS, Wayne, PA.
16. **NCCLS.** 2005. *Procedures for the Recovery and Identification of Parasites from the Intestinal Tract.* Approved guideline M28-A2. NCCLS, Wayne, PA.
17. **NCCLS.** 2002. *Protection of Laboratory Workers from Occupationally Acquired Infections.* Approved guideline M29-A2. NCCLS, Wayne, PA.
18. **Neimeister, R., A. L. Logan, B. Gerber, J. H. Egleton, and B. Kleger.** 1987. Hemo-De as substitute for ethyl acetate in formalin-ethyl acetate concentration technique. *J. Clin. Microbiol.* **25:**425–426.
19. **Sewell, D. L.** 1995. Laboratory-associated infections and biosafety. *Clin. Microbiol. Rev.* **8:**389–405.
20. **U.S. Department of Health and Human Services.** 1993. *Biosafety in Microbiological and Biomedical Laboratories,* 3rd ed., p. 43. U.S. Government Printing Office, Washington, DC.
21. **Vesley, D., and J. Lauer.** 1995. Decontamination, sterilization, disinfection, and antisepsis in the microbiology laboratory, p. 219. *In* D. O. Fleming, J. H. Richardson, J. J. Tulis, and D. Vesley (ed.), *Laboratory Safety: Principles and Practices,* 2nd ed. ASM Press, Washington, DC.
22. **Young, K. H., S. L. Bullock, D. M. Melvin, and C. L. Sprull.** 1979. Ethyl acetate as a substitute for diethyl ether in the Formalin-ether sedimentation technique. *J. Clin. Microbiol.* **10:**852–853.

9.2.1 Collection of Fresh Specimens

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

One of the most important steps in the diagnosis of intestinal parasites is the proper collection of specimens (1–4). Improperly collected specimens can result in inaccurate results. Fresh specimens are mandatory for the recovery of motile trophozoites. However, unless strict collection and delivery times are adhered to, the specimen may have little value for diagnostic testing.

II. SPECIMEN

Observe standard precautions.

A. Collect all fecal specimens prior to the administration of antibiotics or antidiarrheal agents. Avoid the use of mineral oil, bismuth, and barium prior to fecal collection, since all of these substances may interfere with the detection or identification of intestinal parasites (1). The examination of purged specimens is less frequently performed and will not be discussed in this procedure. However, the same time limits for fixation and/or examination of diarrheic stools can be used for purged specimens submitted for examination.

B. Collect the fecal specimen in a clean, widemouthed container or on newspaper, and transfer it to a container with a tight-fitting lid.

C. Avoid contamination with urine or water from the toilet.

D. Transport the specimen to the laboratory as soon as possible, or keep it refrigerated until transport is possible. Obviously, dried specimens (diarrheic, semiformed, or formed) are not acceptable for fecal examination.

III. MATERIALS

A. Reagents
None

B. Supplies
1. Collection container(s)
2. Newspaper (optional)

C. Equipment
Standard

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. There is no maximum limit on the amount of stool collected.

B. As a minimum amount, collect several grams (or teaspoon amounts). Smaller amounts can be examined, but the specimen is likely to dry out before examination (unacceptable for testing).

C. Reject any specimen that appears to be dry on the surface or edges.

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. Sample areas of the feces that appear bloody, purulent, or watery for examination as direct wet smears, fecal concentration, and permanent stained smears.

C. For adequate sampling of a formed fecal specimen, collect and examine material from the sides, ends, and middle by using fecal concentration procedures and permanent stained smears (4).

POSTANALYTICAL CONSIDERATIONS

VI. PROCEDURE NOTES

A. To ensure the recovery of parasitic organisms that are passed intermittently and in fluctuating numbers, the examination of a minimum of three specimens collected over a 7- to 10-day period is recommended (1–4). Refer to Appendix 9.2.1–1 for other collection algorithms and options.

B. Infections with *Entamoeba histolytica*/*E. dispar* or *Giardia lamblia* may require the examination of up to six specimens before the organism is detected.

C. Liquid specimens should be received and examined or preserved by the laboratory within 30 min of passage, soft or semiformed specimens within 1 h of passage, and formed specimens on the same day of passage.

VII. LIMITATIONS OF THE PROCEDURE

A. Protozoan trophozoites will not survive if the stool specimen begins to dry out. Cysts will not form once the specimen has been passed.

B. Unless guidelines for delivery times are monitored and inappropriate specimens are replaced with appropriate ones, laboratory results may be incorrect. Include in the laboratory report a statement indicating that the results may be incorrect.

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
2. **Garcia, L. S.** 2009. *Practical Guide to Diagnostic Parasitology*, 2nd ed. ASM Press, Washington, DC.
3. **Melvin, D. M., and M. M. Brooke.** 1985. *Laboratory Procedures for the Diagnosis of Intestinal Parasites*, p. 163–189. U.S. Department of Health, Education, and Welfare publication no. (CDC) 85-8282. U.S. Government Printing Office, Washington, DC.
4. **NCCLS.** 2005. *Procedures for the Recovery and Identification of Parasites from the Intestinal Tract.* Approved guideline M28-A2. NCCLS, Wayne, PA.

APPENDIX 9.2.1–1

Fecal specimens for parasites: options for collection[a]

Option	Pros	Cons
Rejection of stools from inpatients who have been in-house for >3 days	Data suggest that patients who begin to have diarrhea after they have been inpatients for a few days are symptomatic not from parasitic infections but generally from other causes.	There is always the chance that the problem is related to a health care-associated (nosocomial) parasitic infection (rare), but *Cryptosporidium* and microsporidia may be possible considerations.
Examination of a single stool (O&P examination). Data suggest that 40–50% of organisms present will be found with only a single stool exam.	Some feel that most intestinal parasitic infections can be diagnosed from examination of a single stool. If the patient becomes asymptomatic after collection of the first stool, then subsequent specimens may not be necessary.	Diagnosis from a single stool examination depends on experience of the microscopist, proper collection, and the parasite load in the specimen. In a series of three stool specimens, frequently not all three specimens are positive and/or may be positive for different organisms.
Two O&P exams (concentration and permanent stained smear) are acceptable but not always as good as three specimens (may be a relatively cost-effective approach); any patient remaining symptomatic would require additional testing.	The examination of two specimens is more sensitive than a single examination. Different organisms may be found in the two specimens. One might be negative, while the other is positive.	May not be as cost-effective as examination of a single specimen but is more clinically relevant
Examine a second stool only after the first is negative and the patient is still symptomatic.	With additional examinations, the yield of protozoa increases (*Entamoeba histolytica,* 22.7%; *Giardia lamblia,* 11.3%; *Dientamoeba fragilis,* 31.1%)[b]	Assumes the second (or third) stool is collected within the recommended 10-day time frame for a series of stools; protozoa are shed periodically. May be inconvenient for patient. If the 10-day time frame is not met, then additional stools will represent a new sequence of 10 days.
Examination of a single stool and an immunoassay (EIA, FA, and lateral or vertical flow cartridge). This approach is a mix. One immunoassay may be acceptable; however, immunoassay testing of two separate specimens may be required to confirm the presence of *Giardia* antigen. One O&P exam is not the best approach (review last option below).	If the examinations are negative and the patient's symptoms subside, then probably no further testing is required.	Patients may exhibit symptoms (off and on), so it may be difficult to "rule out" parasitic infections with only a single stool and one fecal immunoassay. If the patient remains symptomatic, then even if two *Giardia* immunoassays are negative, other protozoa may be missed (the *Entamoeba histolytica*/*E. dispar* group, *Entamoeba histolytica*, *Dientamoeba fragilis*, *Cryptosporidium* spp., or the microsporidia). Normally, there are specific situations where fecal immunoassays *or* O&P exams should be ordered. It is not recommended to automatically perform both the O&P and fecal immunoassay as a stool exam for parasites. *Depending on the patient's history and clinical symptoms, either the O&P exam* ***or*** *a fecal immunoassay may be recommended,* ***but generally not both.***
The laboratory will pool three specimens for examination; perform *one* concentration and examine *one* permanent stain.	Three specimens are collected by the patient (three separate collection vials) over 7–10 days; pooling by the laboratory may save time and expense.	Organisms present in low numbers may be missed due to the dilution factor once the specimens are pooled.

(continued)

APPENDIX 9.2.1–1 *(continued)*

Fecal specimens for parasites: options for collection[a] *(continued)*

Option	Pros	Cons
The laboratory will pool three specimens for examination; perform *one* concentration and examine *three* permanent stained smears.	Three specimens are collected by the patient (three separate collection vials) over 7–10 days; pooling by the laboratory for the concentration would probably be sufficient for the identification of helminth eggs. Examination of the three separate permanent stained smears (one from each vial) would maximize recovery of intestinal protozoa in areas of the country where these organisms are most common.	Might miss light helminth infection (eggs and larvae) due to the pooling of the three specimens for the concentration; however, with a permanent stain performed on each of the three specimens, this approach would probably be the next best option in lieu of the standard approach (concentration and permanent stained smear performed on every stool). Coding and billing would have to match the work performed; this may present some problems where work performed does not match existing codes.
The patient collects three stools but puts a sample of stool from all three specimens into a single vial (patient is given a single vial only).	Pooling of the specimens would require only a single vial.	This would complicate patient collection and very likely result in poorly preserved specimens, especially regarding the recommended ratio of stool to preservative and the lack of proper mixing of specimen and fixative.
Perform immunoassays on selected patients[b] using methods for *Giardia lamblia, Cryptosporidium* spp., and/or the *Entamoeba histolytica*/*E. dispar* group or *Entamoeba histolytica.*	Would be more cost-effective than performing immunoassay procedures on all specimens; however, information required to group patients into categories is often not received with the specimens. *This approach assumes that the physicians have guidance in terms of correct ordering options* (*see* table below).	Laboratories rarely receive information that would allow them to place a patient in a particular risk group: children <5 yr old, children from day care centers (may or may not be symptomatic), patients with immunodeficiencies, and patients from outbreaks. Performance of immunoassay procedures on every stool is not cost-effective, and the positivity rate will be low unless an outbreak situation is involved.
Perform immunoassays and O&P examinations on request[c]: *Giardia lamblia, Cryptosporidium* spp. and/or *Entamoeba histolytica/E. dispar* group, or *Entamoeba histolytica* A number of variables will determine the approach to immunoassay testing and the O&P examination (geography, parasites recovered, positivity rate, and physician requests). Immunoassays and/or O&P examinations should be separately ordered, reported, and billed.	This approach will limit the number of stools on which immunoassay procedures for parasites are performed. Immunoassay results do not have to be confirmed by any other testing (such as O&P examinations or modified acid-fast stains). If specific kit performance problems have been identified, individual laboratories may prefer to do additional testing. *However, the fecal immunoassays are more sensitive than the O&P examination and special stains (modified acid-fast stains).* Also, this may be considered duplicate testing and may not be approved for reimbursement unless specifically ordered by the physician.	This approach will require education of the physician clients regarding appropriate times and patients for whom fecal immunoassays should be ordered. Educational initiatives must also include information on the test report indicating the pathogenic parasites that will *not* be detected using these methods. It is critical to make sure clients know that if patients have become asymptomatic, further testing may not be required. *However*, if the patient remains symptomatic, then further testing (O&P exams) is required. Remember, a single O&P may not reveal all organisms present. Present plan to physicians for approval: immunoassays or O&P examinations, procedure discussion, report formats, clinical relevance, and limitations on each approach.

[a] O&P, ovum and parasite; FA, fluorescent-antibody immunoassay. See the following references:
Cartwright, C. P. 1999. Utility of multiple stool specimen ova and parasite examinations in a high-prevalence setting. *J. Clin. Microbiol.* **37:**2408–2411.
Hiatt, R. A., E. K. Markell, and E. Ng. 1995. How many stool examinations are necessary to detect pathogenic intestinal protozoa? *Am. J. Trop. Med. Hyg.* **53:**36–39.
Kehl, K. S. C. 1996. Screening stools for *Giardia* and *Cryptosporidium:* are antigen tests enough? *Clin. Microbiol. Newsl.* **18:**133–135.
Morris, A. J., M. L. Wilson, and L. B. Reller. 1992. Application of rejection criteria for stool ovum and parasite examinations. *J. Clin. Microbiol.* **30:**3213–3216.
Siegel, D. L., P. H. Edelstein, and I. Nachamkin. 1990. Inappropriate testing for diarrheal diseases in the hospital. *JAMA* **263:**979–982.

[b] See in particular the first two references listed above.

[c] See table below. Regarding ordering recommendations for routine O&P examinations or fecal immunoassays, it is difficult to know when you may be in an early outbreak situation where testing of all specimens for *Giardia lamblia*, *Cryptosporidium* spp., or both may be relevant. Extensive efforts are under way to encourage communication among laboratories, water companies, pharmacies, and public health officials regarding the identification of potential or actual outbreaks. If it appears that an outbreak is in the early stages, then performing the immunoassays on request can be changed to testing all stools.

Approaches to stool parasitology: test ordering[a]

Patient and/or situation	Test ordered[b]	Follow-up test ordered
Patient with diarrhea and AIDS or other cause of immunodeficiency *or* Patient with diarrhea involved in a potential waterborne outbreak (municipal water supply)	*Cryptosporidium* or *Giardia/Cryptosporidium* immunoassay	If immunoassays are negative and symptoms continue, special stains for microsporidia (modified trichrome stain) and other coccidia (modified acid-fast stain) and O&P exam should be performed.
Patient with diarrhea (person associated with a nursery school or day care center or a camper or backpacker) *or* Patient with diarrhea involved in a potential waterborne outbreak (resort setting) *or* Patient with diarrhea who lives within areas of the United States where *Giardia* is the most common organism seen	*Giardia* or *Giardia/Cryptosporidium* immunoassay	If immunoassays are negative and symptoms continue, special stains for microsporidia and other coccidia (see above) and O&P exam should be performed.
Patient with diarrhea and relevant history of travel outside of the United States *or* Patient with diarrhea who is a past or present resident of a developing country *or* Patient in an area of the United States where parasites other than *Giardia* are found (large metropolitan centers such as New York, NY; Los Angeles, CA; Washington, DC; and Miami, FL)	O&P exam, *Entamoeba histolytica/E. dispar* immunoassay; immunoassay for confirmation of *E. histolytica;* various tests for *Strongyloides* may be relevant (even in the absence of eosinophilia), particularly if there is any history of pneumonia (migrating larvae in lungs) or sepsis or meningitis (fecal bacteria carried by migrating larvae); agar plate culture is the most sensitive diagnostic approach for *Strongyloides*	The O&P exam is designed to detect and identify a broad range of parasites (amebae, flagellates, ciliates, *Isospora belli,* and helminths). If exams are negative and symptoms continue, special tests for coccidia (fecal immunoassays, modified acid-fast stains, or autofluorescence) and microsporidia (modified trichrome stains or calcofluor white stains) should be performed; fluorescent stains are also options.
Patient with unexplained eosinophilia	Although the O&P exam is recommended, agar plate culture for *Strongyloides stercoralis* (more sensitive than the O&P exam) is also recommended, particularly if there is any history of pneumonia (migrating larvae in lungs) or sepsis or meningitis (fecal bacteria carried by migrating larvae)	If tests are negative and symptoms continue, additional O&P exams and special tests for microsporidia (modified trichrome stains, calcofluor white stains, or fluorescent stains) and other coccidia (modified acid-fast stains, autofluorescence, or fluorescent stains) should be performed.
Patient with diarrhea (suspected food-borne outbreak)	Test for *Cyclospora cayetanensis* (modified acid-fast stain, autofluorescence, or fluorescent stains)	If tests are negative and symptoms continue, special procedures for microsporidia and other coccidia and O&P exam should be performed.

[a] For abbreviations, see footnote *a* above.

[b] Depending on the particular immunoassay kit used, various single or multiple organisms may be included. Selection of a particular kit depends on many variables: clinical relevance, cost, ease of performance, training, personnel availability, number of test orders, training of physician clients, sensitivity, specificity, equipment, time to result, etc. Very few laboratories handle this type of testing exactly the same. Many options are clinically relevant and acceptable for good patient care. It is critical that the laboratory report indicate specifically which organisms could be identified using the kit; a negative report should list the organisms relevant to that particular kit. *It is important to remember that sensitivity and specificity data for all of these fecal immunoassay kits (FA, EIA, and cartridge formats) are comparable.*

9.2.2 Preservation of Specimens

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Fecal specimens that cannot be processed and examined in the recommended time should be placed in an appropriate preservative or combination of preservatives for examination later (1, 2, 3, 4). Preservatives will prevent the deterioration of any parasites that are present. A number of fixatives for preserving protozoa and helminths are available. Each preservative has specific limitations, and no single solution enables all techniques to be performed with optimal results. The choice of preservative should give the laboratory the capability to perform a concentration technique and prepare a permanent stained smear for every specimen submitted for fecal examination (1, 2, 3, 4).

II. SPECIMEN

Observe standard precautions.

The fecal specimen should be collected as described previously (*see* procedures 9.1 and 9.2.1). A portion of the specimen should be placed in the preservative immediately after passage. Excellent directions are available with stool collection vials and/or kits, and this approach eliminates many of the problems encountered with fresh stool collection. See Appendix 9.2.2–1 for a summary table on fecal preservatives and specimen collection.

III. MATERIALS

A. Wooden sticks, applicator sticks, tongue depressors, or other appropriate implement for transferring and mixing the specimen

B. Reagents (*see* Appendix 9.2.2–1)

C. Equipment
Standard

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Obtain a fresh, anticoagulated blood specimen, and prepare a buffy coat sample.

B. Mix approximately 2 g of soft, fresh fecal specimen (normal stool, containing no parasites) with several drops of the buffy coat cells.

C. Prepare several fecal smears, and fix immediately in Schaudinn's fixative to be quality controlled.

D. Mix the remaining feces-buffy coat mixture in 10 ml of polyvinyl alcohol (PVA), modified PVA, or sodium acetate-acetic acid-formalin (SAF) to be quality controlled.

E. Allow 30 min for fixation, and then prepare several fecal smears. Allow to dry thoroughly (30 to 60 min) at room temperature.

F. Stain slides by using normal staining procedure.

G. After staining, if WBCs appear well fixed and display typical morphology, assume that any intestinal protozoa placed in the same lot number of preservative would also be well fixed, provided the fecal sample was fresh and fixed within the recommended time limits.

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. Add a portion of fecal material to the preservative vial to give a 3:1 or 5:1 ratio of preservative to fecal material (a grape-sized formed specimen or about 5 ml of liquid specimen).

C. Mix well by stirring with an applicator stick or the "Spork" insert that is attached to the fixative vial lid to give a homogeneous solution.

D. Allow to stand for 30 min at room temperature to allow adequate fixation.

E. If using commercial collection systems, follow the manufacturer's directions concerning shaking the vials, etc.

POSTANALYTICAL CONSIDERATIONS

VI. PROCEDURE NOTES

A. Most of the commercially available kits have a "fill to" line on the vial label to indicate how much fecal material to add to ensure adequate preservation of the fecal material.

B. Although the two-vial system (one vial of 10% buffered formalin [concentration] and one vial of PVA [permanent stained smear]) has always been the "gold standard," laboratories are beginning to use other options. Changes in the selection of fixatives are based on the following.

1. Problems with disposal of mercury-based fixatives

2. Cost of a two-vial system compared with that of a single collection vial

3. Selection of specific stains (trichrome, iron-hematoxylin) to use with specific fixatives

4. Immunoassay procedures (EIA, fluorescent-antibody assay [FA], or immunochromatographic cartridge) cannot be performed on specimens preserved in certain fixatives; check with the manufacturer.

C. Points to consider when selecting a stool fixative

1. Overall fixation efficacy for trophozoites, cysts, oocysts, microsporidial spores, eggs, and larvae

2. Ability to perform both the concentration and permanent stained smear from the preserved specimen

3. Selection of a one- or two-vial collection system

4. Preparation of reagents in-house or commercial purchase

5. Decision to perform immunoassay procedures (EIA, fluorescent-antibody assay, cartridge formats) from preserved specimen

6. Need to perform special stains from the preserved specimen (modified acid-fast stains for *Cryptosporidium*, *Cyclospora*, and *Cystoisospora* (*Isospora*) and modified trichrome stains for microsporidia)

VII. LIMITATIONS OF THE PROCEDURE

A. Adequate fixation still depends on the following.

1. Meeting recommended time limits for lag time between passage of the specimen and fixation

2. Use of the correct ratio of specimen to fixative

3. Thorough mixing of the preservative and specimen

B. Unless the appropriate stain is used with each fixative, the final permanent stained smear may be difficult to examine (organisms hard to see and/or identify).

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.

2. **Garcia, L. S.** 2009. *Practical Guide to Diagnostic Parasitology*, 2nd ed. ASM Press, Washington, DC.

REFERENCES *(continued)*

3. **Melvin, D. M., and M. M. Brooke.** 1985. *Laboratory Procedures for the Diagnosis of Intestinal Parasites*, p. 163–189. U.S. Department of Health, Education, and Welfare publication no. (CDC) 85-8282. U.S. Government Printing Office, Washington, DC.

4. **NCCLS.** 2005. *Procedures for the Recovery and Identification of Parasites from the Intestinal Tract*. Approved guideline M28-A2. NCCLS, Wayne, PA.

APPENDIX 9.2.2–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. Schaudinn's fixative

This preservative is used with fresh stool specimens or samples from the intestinal mucosal surface. Many laboratories that receive specimens from in-house patients (no problem with delivery times) may select this approach. Permanent stained smears are then prepared from fixed material.

1. Mercuric chloride, saturated aqueous solution

mercuric chloride ($HgCl_2$)	110 g
distilled water	1,000 ml

Dissolve the mercuric chloride in distilled water by heating (use a hood if available); allow the solution to cool until crystals form. Filter the solution into a glass-stoppered bottle and store until needed for stock solution preparation.

2. Schaudinn's fixative (stock solution)

mercuric chloride, saturated aqueous solution	600 ml
ethyl alcohol, 95%	300 ml

Immediately before use, add 5 ml of glacial acetic acid per 100 ml of stock solution.

3. Advantages
   a. Designed to be used for the fixation of slides prepared from fresh fecal specimens or samples from the intestinal mucosal surfaces
   b. Prepared slides can be stored in the fixative for up to a week without distortion of protozoan organisms.
   c. Easily prepared in the laboratory
   d. Available from a number of commercial suppliers
4. Disadvantages
   a. Not recommended for use in concentration techniques
   b. Has poor adhesive properties with liquid or mucoid specimens
   c. Contains mercury compounds (mercuric chloride), which may cause disposal problems. Additional information can be found in section 15.

B. PVA

PVA (1, 2, 7) is a plastic resin that is normally incorporated into Schaudinn's fixative. The PVA powder serves as an adhesive for the stool material; i.e., when the stool-PVA mixture is spread onto the glass slide, it adheres because of the PVA component. Fixation is still accomplished by the Schaudinn's fluid itself. Perhaps the greatest advantage in the use of PVA is the fact that a permanent stained smear can be prepared. PVA fixative solution is highly recommended as a means of preserving cysts and trophozoites for examination at a later time. The use of PVA also permits specimens to be shipped (by regular mail service) from any location in the world to a laboratory for subsequent examination. PVA is particularly useful for liquid specimens and should be used at a ratio of 3 parts PVA to 1 part fecal specimen.

1. PVA fixative

PVA	10.0 g
ethyl alcohol, 95%	62.5 ml
mercuric chloride, saturated aqueous solution	125.0 ml
acetic acid, glacial	10.0 ml
glycerin	3.0 ml

   a. Mix the liquid ingredients in a 500-ml beaker.
   b. Add the PVA powder (stirring is not recommended).

APPENDIX 9.2.2–1 *(continued)*

c. Cover the beaker with a large petri dish, heavy waxed paper, or foil, and allow PVA to soak overnight.
d. Heat the solution slowly to 75°C. When this temperature is reached, remove the beaker and swirl the mixture until a homogeneous, slightly milky solution is obtained (30 s).

2. Advantages
   a. Ability to prepare permanent stained smears and perform concentration techniques
   b. Good preservation of protozoan trophozoites and cyst stages
   c. Long shelf life (months to years) in tightly sealed containers at room temperature
   d. Commercially available from a number of sources
   e. Allows shipment of specimens
3. Disadvantages
   a. Some organisms (*Trichuris trichiura* eggs, *Giardia lamblia* cysts, and *Isospora belli* oocysts) are not concentrated as well from PVA as from formalin-based fixatives, and morphology of some ova and larvae may be distorted.
   b. Contains mercury compounds (Schaudinn's fixative), which may cause disposal problems
   c. May turn white and gelatinous when aliquotted into small amounts (begins to dehydrate) or if refrigerated
   d. Difficult to prepare in the laboratory

C. Modified PVA (copper base) (7)

Although there has been a great deal of interest in developing preservatives that do not use mercury compounds, substitute compounds may not provide good preservation of protozoan morphology on the permanent stained smear. Copper sulfate has been tried most frequently, but it does not provide results equal to those seen with mercuric chloride (3, 5).

1. Copper sulfate solution

   $CuSO_4 \cdot 5H_2O$ ..... 20.0 g
   distilled water .....1,000 ml

   Add the $CuSO_4 \cdot 5H_2O$ to 1,000 ml of distilled water heated to 100°C. Mix until dissolved.
2. Modified PVA fixative (stock solution)

   copper sulfate solution .....600 ml
   ethyl alcohol, 95% .....300 ml

   Immediately before use, add 5 ml of glacial acetic acid per 100 ml of stock solution.
3. Advantages
   a. Can be used for concentration techniques and stained smears
   b. Contains no mercury compounds (usually prepared with copper sulfate)
   c. Commercially available from a number of suppliers. These products (commercial formulas) apparently contain other "fixation agents" that may produce better overall fixation than the formula presented above.
4. Disadvantages
   a. Does not provide high quality of preservation of protozoan morphology on stained slides (3)
   b. Staining characteristics of protozoan organisms are variable; identification may be difficult, particularly when compared with staining characteristics seen with mercuric chloride-based fixatives.

D. Modified PVA (zinc base) (4, 6)

A number of these fixatives are now available commercially, although they do not provide the same overall quality of fixation seen with mercury-based fixatives and the specific formulas are proprietary. Morphological differences based on organism fixation can be placed in perspective as follows.

Mercury-based fixatives: How beautiful is the organism?
Zinc-based fixatives: Can you identify the organism?
(In terms of clinical relevance, the last is the critical question.)

Although some of the organisms do not present with "textbook-quality" morphological features, the majority of the time most organisms can be identified. In general, trophozoites tend to fix well, while cyst forms do not. The most difficult organisms to identify in zinc-preserved specimens are *Endolimax nana* cysts.

APPENDIX 9.2.2–1 *(continued)*

1. Advantages
   a. Can be used for concentration techniques and stained smears
   b. Contains no mercury compounds (usually prepared with zinc sulfate)
   c. Commercially available from a number of suppliers. These products (commercial formulas) contain other "fixation agents" that are proprietary.
2. Disadvantages
   a. Does not provide the same quality of preservation for good protozoan morphology on stained slides as seen in mercury-fixed solutions (4)
   b. Staining characteristics of protozoan organisms are variable; identification may be difficult, particularly when compared with staining characteristics seen from mercuric chloride-based fixatives.

E. SAF

SAF (9) lends itself to both the concentration technique and the permanent stained smear and has the advantage of not containing mercuric chloride, as is found in Schaudinn's fluid and PVA. It is a liquid fixative much like 10% formalin. The sediment is used to prepare the permanent smear, and it is recommended that the stool material be placed on an albumin-coated slide to improve adherence to the glass. SAF is considered a "softer" fixative than mercuric chloride. The morphology of organisms will not be quite as sharp after staining as that of organisms originally fixed in solutions containing mercuric chloride. Staining SAF-fixed material with iron-hematoxylin appears to reveal organism morphology more clearly than staining SAF-fixed material with trichrome.

1. SAF fixative

sodium acetate	1.5 g
acetic acid, glacial	2.0 ml
formaldehyde, 37 to 40% solution	4.0 ml
distilled water	92.0 ml

2. Mayer's albumin
   Equal parts egg white and glycerin. Place 1 drop on a microscope slide, and add 1 drop of SAF-preserved fecal sediment (from the concentration procedure). Allow the smear to dry at room temperature for 30 min prior to staining.
3. Advantages
   a. Can be used for concentration techniques and stained smears
   b. Contains no mercury compounds
   c. Long shelf life
   d. Easily prepared or commercially available from a number of suppliers
4. Disadvantages
   a. Has poor adhesive properties. Albumin-coated slides are recommended for stained smears.
   b. Protozoan morphology with trichrome stain not as clear as with PVA smears. Hematoxylin staining gives better results.
   c. More difficult for inexperienced workers to use

F. MIF

Merthiolate (thimerosal)-iodine-formalin (MIF) (1) is a good stain preservative for most kinds and stages of parasites found in feces and is useful for field surveys. It is used with all common types of stools and aspirates; protozoa, eggs, and larvae can be diagnosed without further staining in temporary wet mounts. Many laboratories using this fixative examine the material only as a wet preparation (direct smear and/or concentration sediment). MIF is prepared in two stock solutions that are stored separately and mixed immediately before use.

1. MIF fixative
   a. Solution I (stored in a brown bottle)

distilled water	50 ml
formaldehyde (USP)	5 ml
thimerosal (tincture of merthiolate, 1:1,000)	40 ml
glycerin	1 ml

APPENDIX 9.2.2–1 *(continued)*

b. Solution II (Lugol's solution: good for several weeks in a tightly stoppered brown bottle)

distilled water ..... 100 ml
potassium iodide (KI) crystals ..... 10 g
iodine crystals (add after KI dissolves) ..... 5 g

Combine 9.4 ml of solution I with 0.6 ml of solution II just before use.

2. Advantages
   a. Combination of preservative and stain (merthiolate), especially useful in field surveys
   b. Protozoan cysts and helminth eggs and larvae can be diagnosed from temporary wet-mount preparations.
3. Disadvantages
   a. Difficult to prepare permanent stained smears
   b. Iodine component unstable; needs to be added immediately prior to use
   c. Concentration techniques may give unsatisfactory results.
   d. Morphology of organisms becomes distorted after prolonged storage.

G. 5 or 10% Formalin (1, 2, 7, 8)

Formalin (1) is an all-purpose fixative that is appropriate for helminth eggs and larvae and protozoan cysts. Two concentrations are commonly used: 5% which is recommended for preservation of protozoan cysts, and 10%, which is recommended for helminth eggs and larvae. Most commercial manufacturers provide 10%, which is most likely to kill all helminth eggs. To help maintain organism morphology, formalin can be buffered with sodium phosphate buffers, i.e., neutral formalin.

1. 10% Formalin

formaldehyde (USP) ..... 100 or 50 ml (for 5%)

Formaldehyde is normally purchased as a 37 to 40% HCHO solution; however, for dilution, it should be considered to be 100%.

0.85% NaCl ..... 900 or 950 ml (for 5%)

Dilute 100 ml of formaldehyde with 900 ml of 0.85% NaCl solution. (Distilled water may be used instead of NaCl solution.)

If you want to use buffered formalin, the following approach is recommended.

Na_2HPO_4 ..... 6.10 g
NaH_2PO_4 ..... 0.15 g

Mix the two thoroughly, and store the dry mixture in a tightly closed bottle. Prepare 1 liter of 10 or 5% formalin, and add 0.8 g of the buffer salt mixture.

2. Advantages
   a. Good routine preservative for protozoan cysts and helminth eggs and larvae. Materials can be preserved for several years.
   b. Can be used for concentration techniques (sedimentation techniques)
   c. Long shelf life and commercially available
   d. Neutral formalin (buffered with sodium phosphate) helps maintain organism morphology with prolonged storage.
3. Disadvantage

Permanent stained smears cannot be prepared from formalin-preserved fecal specimens.

APPENDIX 9.2.2–1 *(continued)*

Preservatives used in diagnostic parasitology (stool specimens)[a]

Preservative	Concentration	Permanent stained smear	Immunoassays (*Giardia lamblia*, *Cryptosporidium* spp.)	Comments
5 or 10% formalin	Yes	No	Yes	EIA, FA, cartridge
5 or 10% buffered formalin	Yes	No	Yes	EIA, FA, cartridge
MIF	Yes	Polychrome IV stain	ND[f]	No published data
SAF	Yes	Iron-hematoxylin	Yes	EIA, FA, cartridge
PVA[b]	Yes, but rarely used	Trichrome or iron hematoxylin	No	PVA interferes with immunoassays
Modified PVA[c]	Yes, but rarely used	Trichrome or iron hematoxylin	No	PVA interferes with immunoassays
Modified PVA[d]	Yes	Trichrome or iron hematoxylin	Some but not all (rinse step may remove problematic compounds)	PVA interferes with immunoassays
Single-vial systems[e]	Yes	Trichrome or iron hematoxylin	Some are now real universal fixatives	Check with the manufacturer
Schaudinn's (without PVA)[b]	Yes, but rarely used	Trichrome or iron hematoxylin	No	Mercury interferes with immunoassays

[a] See references 1, 2, 7, and 8.

[b] This fixative uses the mercuric chloride base in the Schaudinn's fluid; this formulation is still considered the "gold standard," against which all other fixatives are evaluated (organism morphology after permanent staining). Additional fixatives prepared with non-mercuric chloride-based compounds are used; however, the overall organism morphology is not as good.

[c] This modification uses a copper sulfate base rather than mercuric chloride.

[d] This modification uses a zinc base rather than mercuric chloride and works well with both trichrome and iron hematoxylin stains.

[e] These modifications use a combination of ingredients (including zinc) but are prepared from proprietary formulas and may or may not contain PVA. The aim is to provide a fixative that can be used for the fecal concentration, permanent stained smear, and available immunoassays for *Giardia lamblia*, *Cryptosporidium* spp., and *Entamoeba histolytica* (or the *Entamoeba histolytica/E. dispar* group). Universal fixatives do not contain formalin, PVA, or mercury compounds (concentration, permanent stain, fecal immunoassays). However, fresh/frozen specimens are still required for testing *Entamoeba histolytica* or the *Entamoeba histolytica/E. dispar* group.

[f] ND, no data.

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
2. **Garcia, L. S.** 2009. *Practical Guide to Diagnostic Parasitology*, 2nd ed. ASM Press, Washington, DC.
3. **Garcia, L. S., R. Y. Shimizu, T. C. Brewer, and D. A. Bruckner.** 1983. Evaluation of intestinal parasite morphology in polyvinyl alcohol preservative: comparison of copper sulfate and mercuric chloride base for use in Schaudinn's fixative. *J. Clin. Microbiol.* **17:**1092–1095.
4. **Garcia, L. S., R. Y. Shimizu, A. Shum, and D. A. Bruckner.** 1993. Evaluation of intestinal protozoan morphology in polyvinyl alcohol preservative: comparison of zinc sulfate- and mercuric chloride-based compounds for use in Schaudinn's fixative. *J. Clin. Microbiol.* **31:**307–310.
5. **Horen, W. P.** 1981. Modification of Schaudinn's fixative. *J. Clin. Microbiol.* **13:**204–205.
6. **Isenberg, H. D. (ed.).** 1998. *Essential Procedures for Clinical Microbiology*. ASM Press, Washington, DC.
7. **Melvin, D. M., and M. M. Brooke.** 1985. *Laboratory Procedures for the Diagnosis of Intestinal Parasites*, p. 163–189. U.S. Department of Health, Education, and Welfare publication no. (CDC) 85-8282. U.S. Government Printing Office, Washington, DC.
8. **NCCLS.** 2005. *Procedures for the Recovery and Identification of Parasites from the Intestinal Tract.* Approved guideline M28-A2. NCCLS, Wayne, PA.
9. **Scholten, T. H., and J. Yang.** 1974. Evaluation of unpreserved and preserved stools for the detection and identification of intestinal parasites. *Am. J. Clin. Pathol.* **62:**563–567.

9.2.3 Shipment of Specimens

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

In outpatient situations, it may be necessary for a specimen to be shipped to the laboratory for examination. Only preserved fecal specimens should be shipped, as any delays in examination may result in deterioration of parasitic organisms (1–3). Prior fixation also reduces the risk of infection from any etiologic agents present in the specimen. The U.S. Postal Service regulates the shipment of clinical specimens through the mail. It is the responsibility of the sender to conform to these regulations (1–3).

II. SPECIMENS

Observe standard precautions.

A. Preserved fecal specimens in collection vials
B. Fecal smears for staining and examination for parasitic organisms
C. Blood smears for staining and examination for blood parasites (thin blood films should be fixed in methyl alcohol prior to shipment)

III. MATERIALS

A. Reagents
None

B. Supplies
1. Primary container (vial, test tube, or bottle) containing the clinical specimen (feces or blood)
2. Secondary container in which the primary container will be placed (sealed and water tight, durable) with absorbent material to contain leakage
3. Shock-absorbent material for protecting the contents (when shipping fecal or blood smears or tubes of blood)
4. Outer shipping container of fiberboard or other sturdy material in which the secondary container will be placed
5. Instructions for patient specimen collection and handling
6. Appropriate mailing labels

C. Equipment
None

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Familiarity with U.S. postal regulations is mandatory. Post up-to-date regulations.
B. Review relevant postal regulations at routine safety meetings for the staff, and document the review.

V. PROCEDURE

A. Place the primary container of preserved fecal material into the secondary container (metal sleeve or a sealable bag), and seal.
B. Place into the mailing container, and seal.
C. Label appropriately for shipment.
D. Wrap glass slides in shock-absorbent material to protect from breakage, or place them in a sturdy slide container.
E. Slides need not be placed in double containers for shipping.
F. Place padded slides in shipping container and label appropriately.

VI. PROCEDURE NOTES

A. Some commercially available mailing containers consist of a screw-cap inner metal sleeve that will hold a preservative vial that is placed inside a screw-cap cardboard mailing sleeve. Absorbent material can be placed between the two containers to absorb any leakage. This container can be used to mail a single specimen.
B. Padded envelopes for mailing sets of preservative vials are available in several sizes. The absorbent material is shredded newspaper inside the envelope layers.
C. Clinical specimens exceeding 50 ml per parcel must be packaged in a fiberboard box or shipping container of equivalent strength.

VII. LIMITATIONS OF THE PROCEDURE

A. The same limitations apply to all fixatives, regardless of whether they are delivered by mail.
B. Each vial or tube containing liquid should be as full as possible. If it is only partly full, some of the liquid may get splashed onto the walls of the container and dry out during transit. Also, the larger the air space, the more shaking will occur, and this may be detrimental to any delicate organisms in the specimen (even if they are preserved).

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
2. **McVicar, J. W., and J. Suen.** 1995. Packaging and shipping biological materials, p. 239. *In* D. O. Fleming, J. H. Richardson, J. J. Tulis, and D. Vesley (ed.), *Laboratory Safety: Principles and Practices*, 2nd ed. ASM Press, Washington, DC.
3. **World Health Organization.** 1997. Guidelines for the safe transport of infectious specimens and diagnostic specimens. World Health Organization, Geneva, Switzerland.

9.3.1 Macroscopic Examination of Fecal Specimens: Age and Physical Description

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The age of fresh fecal specimens is an important factor in the diagnosis of parasitic infections (1–4). The date and time of passage must be provided for each specimen submitted to the laboratory. The physical characteristics of a fresh fecal specimen may aid in determining what types of organisms may be present (1–4). Fecal specimens are described as formed, semiformed, soft, loose, or watery. Loose or watery specimens may contain trophozoites, whereas formed or semiformed specimens are more likely to contain cyst stages. Helminth eggs or larvae may be found in any type of specimen but are more difficult to find in liquid specimens because of the dilution factor. One can also see if blood and/or mucus is present, although if present, neither one necessarily indicates a parasitic infection. When the fresh specimen is examined visually in the collection container, adult pinworms *(Enterobius vermicularis)* and tapeworm proglottids may also be seen.

II. SPECIMENS

(See procedure 9.2.1.)

Observe standard precautions.

A. Fresh fecal specimen in waterproof container
B. Preserve or examine liquid or soft stools containing blood or mucus within 30 min of passage.
C. Preserve or examine semiformed specimens within 1 h of passage.
D. Preserve or examine formed specimens within the same day.
E. Fecal specimens that cannot be processed and examined in the recommended time period should be placed in an appropriate preservative or combination of preservatives for examination at a later time. Preservatives will prevent the deterioration of any parasites that are present. A number of fixatives are available (*see* Appendix 9.2.2–1) for preserving protozoan and helminth organisms. The choice of preservative should give the laboratory the capability to perform a concentration technique and prepare a permanent stained smear on every specimen submitted for fecal examination (2, 3).

ANALYTICAL CONSIDERATIONS

III. PROCEDURE

A. *Wear gloves when performing this procedure.*
B. Examine the specimen macroscopically to determine consistency.
C. Examine the surface of the fecal specimen for the presence of blood or mucus.
D. Sample areas of blood or mucus for examination for trophic amebae.
E. Examine the surface of the specimen and the area underneath the specimen for possible organisms (adult pinworms or tapeworm proglottids).
F. Check information for date and time of passage.

IV. RESULTS

A. Adult helminths or portions of helminths may be recovered and seen with the naked eye. Examples include *E. vermicularis* adult worms, *Ascaris lumbricoides* adult worms, and tapeworm proglottids.

B. Occasionally, other helminths may be recovered (hookworm, *Strongyloides stercoralis*), but identification requires the use of the microscope.

POSTANALYTICAL CONSIDERATIONS

V. REPORTING RESULTS

A. Report the presence of adult helminths or portions of helminths. Morphology and size are usually adequate for identification of pinworm and *Ascaris* adults and tapeworm proglottids. (Identification to the species level will require India ink injection [*see* procedure 9.5.6].)

Examples: *Ascaris lumbricoides* adult worm identified.
Enterobius vermicularis adult worm identified.
Taenia saginata gravid proglottid identified.

B. Report the presence of blood on or in the fecal specimen.

Example: Fresh blood seen on stool specimen.

VI. PROCEDURE NOTES

A. Trophic amebae or flagellates are found most frequently in liquid or soft specimens and tend to disintegrate rapidly at room temperature.

B. Trophozoites and cyst stages may be found in semiformed specimens.

C. Cyst stages are found most frequently in formed specimens and will not lose characteristic morphology at room temperature for approximately 1 day.

D. Eggs and larvae in fresh fecal specimens do not lose characteristic morphology at room temperature as rapidly as trophozoites or cysts. Some eggs (hookworm) may hatch if the specimen is kept unpreserved at room temperature for more than a day.

E. Refrigeration of the fresh fecal specimen will delay deterioration of the parasitic organisms.

F. Freezing of the fecal specimen is not recommended, as characteristic morphology of the parasitic organisms may be altered.

G. Never incubate fecal specimens.

H. Laboratories that receive the fecal specimen in preservative vials must rely on information that is submitted with the specimen as to the consistency of the specimen.

I. Many commercially available kits contain vials with labels which allow the patient to indicate the original consistency of the specimen.

J. A clean vial (containing no preservative) can be provided to patients for submitting a portion of the fresh specimen for determining consistency.

K. Dark or tarry fecal specimens usually indicate bleeding in the upper gastrointestinal tract.

L. Bright red blood indicates bleeding at a lower level or around the rectum.

M. Barium causes feces to be light tan to white. These specimens should be rejected.

N. Ingested iron and some antidiarrheal compounds may cause the specimen to be dark to black.

O. Yellowish specimens may be noted in cases of fat malabsorption, which is seen commonly in infection with *Giardia lamblia.*

P. Vegetable material is frequently seen in fecal specimens and must be differentiated from helminths. Size and gross morphology are used to differentiate vegetable material from helminth parasites (*see* Appendixes 9.10.1–1 and 9.10.3–1 at the end of this section).

VII. LIMITATIONS OF THE PROCEDURE

Although there are some benefits (organism motility) associated with the examination of fresh fecal specimens, many laboratories have switched to stool preservative collection kits. Many intestinal parasites tend to disintegrate soon after collection, particularly if there is a time lag between specimen collection and preservation. In order to eliminate time lag problems and ensure adequate organism morphology, stool collection kits are recommended. Also, remember that organisms other than parasites can cause diarrhea; you might want to check whether bacterial cultures or viral studies have been ordered.

REFERENCES

1. **Ash, L. R., and T. C. Orihel.** 1991. *Parasites: a Guide to Laboratory Procedures and Identification.* ASCP Press, Chicago, IL.
2. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
3. **Melvin, D. M., and M. M. Brooke.** 1985. *Laboratory Procedures for the Diagnosis of Intestinal Parasites*, p. 163–189. U.S. Department of Health, Education, and Welfare publication no. (CDC) 85-8282. U.S. Government Printing Office, Washington, DC.
4. **NCCLS.** 2005. *Protection of Laboratory Workers from Occupationally Acquired Infections.* Approved guideline M29-A3. NCCLS, Wayne, PA.

9.3.2 Calibration of Microscope with an Ocular Micrometer

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The identification of protozoa and other parasites depends on several factors, one of which is size. Any laboratory doing diagnostic work in parasitology should have a calibrated microscope available for precise measurements. Measurements are made with a micrometer disk that is placed in the ocular of the microscope; the disk is usually calibrated as a line divided into 50 units (U). Depending on the objective magnification used, the divisions in the disk represent different measurements. The ocular disk division must be compared with a known calibrated scale, usually a stage micrometer with a scale of 0.1- and 0.01-mm divisions (1) (Fig. 9.3.2–1).

II. MATERIALS

A. Supplies

1. Ocular micrometer disk (line divided into 50 U) (any laboratory supply distributor: Fisher, Baxter, Scientific Products, VWR, etc.)
2. Stage micrometer with a scale of 0.1- and 0.01-mm divisions (Fisher, Baxter, Scientific Products, VWR, etc.)
3. Immersion oil
4. Lens paper

B. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives. Other objective magnifications (50× oil or 60× oil immersion lenses) may also be used.
2. Oculars should be 10×. Some may prefer 5×; however, smaller magnification may make final identifications more difficult.
3. Single 10× ocular to be used to calibrate all laboratory microscopes (to be used when any organism is being measured)

ANALYTICAL CONSIDERATIONS

III. QUALITY CONTROL

A. Recalibrate the microscope periodically. If the scope receives heavy use, once a year is recommended, or when any parts are replaced or changed on the microscope.

B. Often the measurement of RBCs (approximately 7.5 μm) is used to check the calibrations of the three magnifications (×100, ×400, ×1,000).

C. Latex or polystyrene beads of a standardized diameter can be used to check the calculations and measurements (Sigma, J. T. Baker, etc.). Beads of 10 and 90 μm are recommended.

D. Record all measurements in QC records.

IV. PROCEDURE

A. Unscrew the eye lens of a 10× ocular, and place the micrometer disk (engraved side down) within the ocular. Use lens paper to handle the disk; keep all surfaces free of dust or lint.

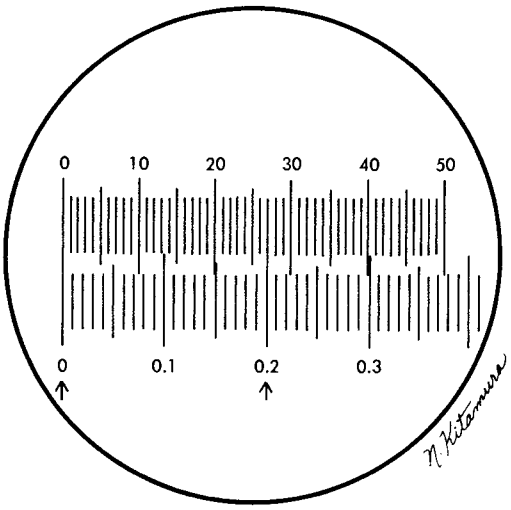


Figure 9.3.2–1 Ocular micrometer, top scale; stage micrometer, bottom scale (from **L. S. Garcia,** *Diagnostic Medical Parasitology,* 5th ed., 2007, ASM Press, Washington, DC).

IV. PROCEDURE *(continued)*

B. Place the calibrated micrometer on the stage, and focus on the scale. You should be able to distinguish the difference between the 0.1- and 0.01-mm divisions. Make sure you understand the divisions on the scale before proceeding.

C. Adjust the stage micrometer so that the "0" line on the ocular micrometer is exactly lined up on top of the 0 line on the stage micrometer.

D. When these two 0 lines are lined up, do not move the stage micrometer any farther. Look to the right of the 0 lines for another set of lines superimposed on each other. The second set of lines should be as far to the right of the 0 lines as possible; however, the distance varies with the objectives being used (Fig. 9.3.2–1).

E. Count the number of ocular divisions between the 0 lines and the point where the second set of lines is superimposed. Then, on the stage micrometer, count the number of 0.1-mm divisions between the 0 lines and the second set of superimposed lines.

F. Calculate the portion of a millimeter that is measured by a single small ocular unit.

G. When the high dry and oil immersion objectives are used, the 0 line of the stage micrometer will increase in size, whereas the ocular 0 line will remain the same size. The thin ocular 0 line should be lined up in the center or at one edge of the broad stage micrometer 0 line. Thus, when the second set of superimposed lines is found, the thin ocular line should be lined up in the center or at the corresponding edge of the broad stage micrometer line.

H. Calculate the factors as follows.

Examples:

$$\frac{\text{stage reading (mm)}}{\text{ocular reading}} \times \frac{1{,}000\ \mu\text{m}}{1\ \text{mm}} = \text{ocular units } (\mu\text{m})$$

$$\text{Low power } (10\times)\text{: } \frac{0.8\ \text{mm}}{100\ \text{U}} \times \frac{1{,}000\ \mu\text{m}}{1\ \text{mm}} = 8.0\ \mu\text{m (factor)}$$

$$\text{High dry power } (40\times)\text{: } \frac{0.1\ \text{mm}}{50\ \text{U}} \times \frac{1{,}000\ \mu\text{m}}{1\ \text{mm}} = 2.0\ \mu\text{m (factor)}$$

$$\text{Oil immersion } (100\times)\text{: } \frac{0.05\ \text{mm}}{62\ \text{U}} \times \frac{1{,}000\ \mu\text{m}}{1\ \text{mm}} = 0.8\ \mu\text{m (factor)}$$

IV. PROCEDURE *(continued)*

Examples: If a helminth egg measures 15 ocular units by 7 ocular units with the high dry objective, then multiply the measurements by the factor 2.0 μm (for that objective). The egg then measures 30 by 14 μm and is probably *Clonorchis sinensis.*

If a protozoan cyst measures 27 ocular units with the oil immersion objective, then multiply the measurement by the factor 0.8 μm (for that objective). The cyst then measures 21.6 μm.

V. RESULTS

A. For each objective magnification, a factor will be generated (1 ocular unit = certain number of micrometers).

B. If standardized latex or polystyrene beads or an RBC is measured with various objectives, the size for the object measured should be the same (or very close), regardless of the objective magnification.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Post the multiplication factor for each objective either on the base of the microscope or on a nearby wall or bulletin board for easy reference.

B. Once the number of ocular lines per width and length of the organism is measured, then, depending on the objective magnification, the factor (1 ocular unit = certain number of micrometers) can be applied to the number of lines to obtain the width and length of the organism.

C. Comparison of these measurements with reference measurements in various books and manuals should confirm the organism identification.

VII. PROCEDURE NOTES

A. The final multiplication factors will be only as good as your visual comparison of the ocular 0 and stage micrometer 0 lines.

B. As a rule of thumb, the high dry objective (40×) factor should be approximately 2.5 times more than the factor obtained from the oil immersion objective (100×). The low-power objective (10×) factor should be approximately 10 times that of the oil immersion objective (100×).

VIII. LIMITATIONS OF THE PROCEDURE

A. After each objective has been calibrated, the oculars containing the disk and/or these objectives cannot be interchanged with corresponding objectives or oculars on another microscope.

B. Each microscope used to measure organisms must be calibrated as a unit. The original oculars and objectives that were used to calibrate the microscope must also be used when an organism is measured.

C. The objective containing the ocular micrometer can be stored until needed. This single ocular can be inserted when measurements are taken. However, this particular ocular containing the ocular micrometer disk must also have been used as the ocular during microscope calibration.

REFERENCE

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology,* 5th ed. ASM Press, Washington, DC.

9.3.3 Microscopic Examination of Fecal Specimens: Direct Smears

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The microscopic examination of a direct smear has several purposes: to assess the worm burden of a patient, to provide a quick diagnosis of a heavily infected specimen, to check organism motility, and to diagnose parasites that may be lost in concentration techniques (1–5).

II. SPECIMEN

Observe standard precautions.

Any fresh stool specimen that has not been refrigerated is acceptable. Since trophozoites within preserved specimens would exhibit no motility on a direct smear, it is not necessary to perform this procedure on specimens submitted in preservatives (10% formalin, sodium acetate-acetic acid-formalin, and various types of polyvinyl alcohol, single-vial preservative collection systems).

III. MATERIALS

A. Reagents (*see* Appendix 9.3.3–1)
The reagents indicated below are available commercially.
1. 0.85% NaCl
2. D'Antoni's iodine or Lugol's iodine
   Aliquot some of the iodine into a brown dropper bottle. The working solution should resemble a strong tea color and should be discarded when it lightens in color (usually within 10 to 14 days).

B. Supplies
1. Microscope slides (1 by 3 in. or larger)
2. Coverslips (no. 1, 22 by 22 mm or larger)
3. Pasteur pipettes and bulbs
4. Screw-cap test tubes (16 by 125 mm)
5. Beaker (500 ml)
6. Brown glass-stoppered bottle (250 ml)
7. Two brown glass dropper bottles

C. Equipment
1. Binocular microscope with 10×, 40×, and 100× objectives; phase-contrast and/or differential interference contrast optics preferred
2. Oculars should be 10×. Some may prefer 5×; however, smaller magnification may make final identifications more difficult.
3. Magnetic stirrer and stir bar

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check the working iodine solution each time it is used or periodically (once a week).
1. The iodine should be free of any signs of bacterial and/or fungal contamination.

IV. QUALITY CONTROL *(continued)*

2. The color should be that of strong tea (discard if too light).
3. Protozoan cysts should contain yellow-gold cytoplasm, brown glycogen material, and paler refractile nuclei. Human WBCs mixed with negative stool can be used as a QC specimen. The human cells will stain with the same color as that seen in the protozoa.

B. The microscope should be calibrated, and the original optics used for the calibration should be in place on the microscope. Post the calibration factors for all objectives on the microscope for easy access (*see* procedure 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Record all QC results.

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. Place 1 drop of 0.85% NaCl on the left side of the slide and 1 drop of iodine (working solution) on the right side of the slide.

C. Take a very small amount of fecal specimen (about the amount picked up on the end of an applicator stick when introduced into the specimen), and thoroughly emulsify the stool in the saline and iodine preparations (use separate sticks for each).

D. Place a coverslip (22 by 22 mm) on each suspension.

E. Systematically scan both suspensions with the 10× objective. The entire coverslip area should be examined.

F. If you see something suspicious, use the 40× objective for more-detailed study. At least one-third of the coverslip should be examined with the 40× objective, even if nothing suspicious has been seen.

VI. RESULTS

Protozoan trophozoites and/or cysts and helminth eggs and larvae may be seen and identified.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. You may (saline) or may not (iodine) be able to identify motile trophozoites to the species level (depending on the clarity of the morphology).
Example (positive report): *Giardia lamblia* trophozoites present.

B. You may or may not be able to identify protozoan cysts to the species level (depending on the clarity of the morphology).
Example (positive report): *Entamoeba coli* cysts present.

C. You may identify helminth eggs and/or larvae.
Examples: *Ascaris lumbricoides* eggs present.
Strongyloides stercoralis larvae present.

D. You may see artifacts and/or other structures. Report them.
Examples: Moderate Charcot-Leyden crystals present.
Few RBCs present.

VIII. PROCEDURE NOTES

A. In preserved specimens, formalin replaces the saline and can be used directly; however, you will not be able to see any organism motility (organisms are killed by 10% formalin).

VIII. PROCEDURE NOTES *(continued)*

B. Some workers prefer to make the saline and iodine mounts on separate slides. There is less chance of getting fluids on the microscope stage if separate slides are used (less total fluid on the slide and under the coverslip).

C. The microscope light should be reduced for low-power observations, since most organisms will be overlooked with bright light. Illumination should be regulated so that some of the cellular elements in the feces show refraction. Most protozoan cysts will refract under these light conditions.

IX. LIMITATIONS OF THE PROCEDURE

A. Results obtained with wet smears should usually be confirmed by permanent stained smears. Some protozoa are very small and difficult to identify to the species level by direct wet smears alone.

B. Confirmation is particularly important in the case of *Entamoeba histolytica/E. dispar* versus *Entamoeba coli.*

REFERENCES

1. **Garcia, L. S., J. W. Smith, and T. R. Fritsche.** 2003. *Cumitech 30A, Selection and Use of Laboratory Procedures for Diagnosis of Parasitic Infections of the Gastrointestinal Tract.* Coordinating ed., L. S. Garcia. ASM Press, Washington, DC.
2. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology,* 5th ed. ASM Press, Washington, DC.
3. **Markell, E. K., D. T. John, and W. A. Krotoski.** 1999. *Markell and Voge's Medical Parasitology,* 8th ed. W. B. Saunders Co., Philadelphia, PA.
4. **Melvin, D. M., and M. M. Brooke.** 1985. *Laboratory Procedures for the Diagnosis of Intestinal Parasites,* p. 163–189. U.S. Department of Health, Education, and Welfare publication no. (CDC) 85-8282. U.S. Government Printing Office, Washington, DC.
5. **NCCLS.** 2005. *Procedures for the Recovery and Identification of Parasites from the Intestinal Tract.* Approved guideline M28-A2. NCCLS, Wayne, PA.

APPENDIX 9.3.3–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. 0.85% NaCl

NaCl ..... 0.85 g
distilled water .....100.0 ml

1. Dissolve the sodium chloride in distilled water in a flask or bottle by using a magnetic stirrer.
2. Distribute 10 ml of the solution into each of 10 screw-cap tubes.
3. Label as 0.85% NaCl with an expiration date of 1 year.
4. Sterilize by autoclaving at 121°C for 15 min.
5. When cool, store at 4°C.

B. D'Antoni's iodine

potassium iodide (KI) .....1.0 g
powdered iodine crystals .....1.5 g
distilled water .....100.0 ml

1. Dissolve the potassium iodide and iodine crystals in distilled water in a flask or bottle by using a magnetic stirrer.
2. Some excess crystals of iodine should remain on the bottom of the bottle.
3. Store in a brown, glass-stoppered bottle at room temperature.
4. The solution is ready for immediate use. Label as D'Antoni's iodine with an expiration date of 1 year (the stock solution remains good as long as an excess of iodine crystals remains on the bottom of the bottle).
5. Aliquot some of the iodine into a brown dropper bottle. The working solution should resemble strong tea and should be discarded when it lightens in color (usually within 10 to 14 days).

APPENDIX 9.3.3–1 *(continued)*

C. Lugol's iodine

potassium iodide ..... 10.0 g
powdered iodine crystals .....5.0 g
distilled water .....100.0 ml

1. Follow the directions given above for D'Antoni's iodine, including the expiration date of 1 year.
2. Dilute a portion 1:5 with distilled water for routine use (working solution).
3. Place this working solution into a brown dropper bottle. The working solution should resemble strong tea and should be discarded when it lightens in color (usually within 10 to 14 days).

Diagnostic characteristics of organisms in wet mounts

Specimen	Protozoa	Helminths
Stool, other specimens from the gastrointestinal tract or urogenital system	Size, shape, stage (trophozoite, precyst, cyst, oocyst), motility (fresh specimens only), refractility, cytoplasm inclusions (chromatoidal bars, glycogen vacuoles, axonemes, axostyles, median bodies, sporozoites)	Egg, larvae, or adult; size; internal structure, as follows: for the egg, embryonated, opercular shoulders, abopercular thickenings or projections, hooklets, polar filaments, and spines; for larvae, head and tail morphology and digestive tract; and for the adult, nematode, cestode, or trematode

9.3.4

Microscopic Examination of Fecal Specimens: Concentration by Formalin-Ethyl Acetate Sedimentation

Fecal concentration has become a routine procedure as a part of the complete ova and parasite examination for parasites and allows the detection of small numbers of organisms that may be missed by using only a direct wet smear. There are two types of concentration procedures: flotation and sedimentation, both of which are designed to separate protozoan organisms and helminth eggs and larvae from fecal debris by centrifugation and/or differences in specific gravity.

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

By centrifugation, this concentration procedure leads to the recovery of all protozoa, eggs, and larvae present; however, the preparation contains more debris than the flotation procedure. Ethyl acetate is used as an extractor of debris and fat from the feces and leaves the parasites at the bottom of the suspension. Concentration by formalin-ethyl acetate sedimentation is recommended because it is the easiest to perform, allows recovery of the broadest range of organisms, and is least subject to technical error (1–5). It is also important to remember that concentrated fecal sediment is recommended for the modified acid-fast and modified trichrome stains used for the coccidia and microsporidia, respectively.

II. SPECIMEN

Observe standard precautions.

A. The specimen must be fresh or formalinized stool (5 or 10% buffered or nonbuffered formalin or sodium acetate-acetic acid-formalin [SAF]) (*see* procedures 9.1, 9.2.1, and 9.2.2).

B. Polyvinyl alcohol (PVA)-preserved specimens can be used (*see* item VIII below), as can specimens preserved in the single-vial collection systems (zinc-based, proprietary formulas).

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

1. Ethyl acetate
2. Formalin (5 or 10% buffered or nonbuffered or SAF)
3. 0.85% NaCl
4. D'Antoni's or Lugol's iodine

B. Supplies

1. Funnel
2. Gauze
3. Centrifuge tubes (15 ml)
4. Applicator sticks
5. Glass slides (1 by 3 in. or larger)
6. Coverslips (22 by 22 mm; no. 1 or larger)
7. Disposable glass or plastic pipettes

C. Equipment

1. Centrifuge (tabletop or floor model)
2. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
3. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check the reagents each time they are used. The formalin and saline should appear clear, without any visible contamination.

B. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements done with the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedure 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Concentrate known positive specimens and verify organism recovery at least quarterly and particularly after the centrifuge has been recalibrated.

D. Known positive microscope slides, Kodachrome (2- by 2-in.) projection slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

E. Record all QC results.

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. Transfer a half-teaspoon (about 4 g) of fresh stool into 10 ml of 10% formalin in a shell vial, unwaxed paper cup, or round-bottom tube (container may be modified to suit individual laboratory preferences). Mix the stool and formalin thoroughly. Let the mixture stand a minimum of 30 min for fixation. If the specimen is already in 5 or 10% formalin (or SAF or one of the other available single-vial collection system fixatives), restir the stool-formalin mixture.

C. Depending on the amount and viscosity of the specimen, strain a sufficient quantity through wet gauze into a conical 15-ml centrifuge tube to give the desired amount of sediment (0.5 to 1 ml) in step V.D below. Usually, 8 ml of the stool-formalin mixture prepared in step V.B will be sufficient. If the specimen is received in vials of preservative, then approximately 3 to 4 ml will be sufficient unless there is very little stool in the vial.

D. Add 0.85% NaCl (*see* item VIII below) almost to the top of the tube, and centrifuge for 10 min at 500 $\times$ *g*. The amount of sediment obtained should be approximately 0.5 to 1 ml.

E. Decant the supernatant fluid, and resuspend the sediment in saline. Add saline almost to the top of the tube, and centrifuge again for 10 min at 500 $\times$ *g*. This second wash may be eliminated if the supernatant fluid after the first wash is light tan or clear.

F. Decant the supernatant fluid, and resuspend the sediment on the bottom of the tube in 10% formalin. Fill the tube half full only. If the amount of sediment left in the bottom of the tube is very small or if the original specimen contained a lot of mucus, *do not add ethyl acetate* in step V.G; merely add the formalin, spin, decant, and examine the remaining sediment.

G. Add 4 to 5 ml of ethyl acetate. Stopper the tube, and shake it vigorously for at least 30 s, exerting pressure on the stopper throughout. Hold the tube so the stopper is directed away from your face.

H. After a 15- to 30-s wait, carefully remove the stopper.

I. Centrifuge for 10 min at 500 $\times$ *g*. Four layers should result: a small amount of sediment (containing the parasites) in the bottom of the tube, a layer of formalin, a plug of fecal debris on top of the formalin layer, and a layer of ethyl acetate at the top (Fig. 9.3.4–1).

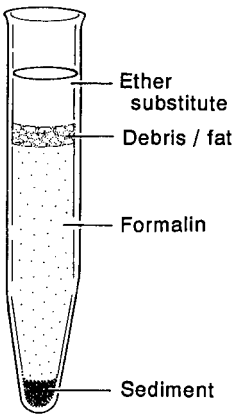


Figure 9.3.4–1 Diagram of specimen after centrifugation (sedimentation procedure).

V. PROCEDURE *(continued)*

J. Free the plug of debris by ringing the plug with an applicator stick; decant all of the supernatant fluid. After proper decanting, a drop or two of fluid remaining on the side of the tube may run down into the sediment. Mix this fluid with the sediment.

K. If the sediment is still somewhat solid, add a drop or two of saline to the sediment, mix, and add a small amount of material to a slide, add a coverslip, and examine.

L. Systematically scan with the 10× objective. The entire coverslip area should be examined.

M. If you see something suspicious, use the 40× objective for more-detailed study. At least one-third of the coverslip should be examined with the 40× objective, even if nothing suspicious has been seen. As in the direct wet smear, iodine can be added to enhance morphological detail.

VI. RESULTS

Protozoan trophozoites and/or cysts and helminth eggs and larvae may be seen and identified. Protozoan trophozoites are less likely to be seen.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. You may or may not be able to identify protozoan cysts to the species level (depending on the clarity of the morphology).
Example (positive report): *Giardia lamblia* cysts present.

B. You may identify helminth eggs and/or larvae.
Example: *Trichuris trichiura* eggs present.

C. You may also see artifacts and/or other structures. Report them.
Examples: Few Charcot-Leyden crystals present.
Moderate PMNs present.

VIII. PROCEDURE NOTES

A. Tap water may be substituted for 0.85% NaCl throughout this procedure, although the addition of water to fresh stool will cause *Blastocystis hominis* cyst forms to rupture. In addition to the original 10% formalin fixation, some workers prefer to use 10% formalin for all the rinses throughout the procedure.

B. Ethyl acetate is widely recommended as a substitute for ether. It can be used the same way in the procedure and is much safer. Hemo-De can also be used (6).

1. After the plug of debris is rimmed and excess fluid is decanted, while the tube is still upside down, swab the sides of the tube with a cotton-tipped applicator stick to remove excess ethyl acetate. This is particularly important if you are working with plastic centrifuge tubes. If the sediment is too dry after the tube has been swabbed, add several drops of saline before preparing your wet smear for examination.

2. If you have excess ethyl acetate in the smear of the sediment prepared for examination, bubbles will obscure the material you are trying to see.

C. If specimens are received in SAF, then begin at step V.B.

D. If specimens are received in PVA or one of the other available single vial collection system fixatives, modify the first two steps of the procedure (steps V.A and B) as follows:

1. Immediately after stirring the stool-fixative mixture with applicator sticks, pour approximately one-half of the mixture into a tube (container optional) and add 0.85% saline almost to the top of the tube.

VIII. PROCEDURE NOTES *(continued)*

2. Filter the stool-fixative-saline mixture through wet gauze into a 15-ml centrifuge tube. Follow the standard procedure from here to completion, beginning with step V.C.

E. Too much or too little sediment will result in an ineffective concentration.

F. Let the centrifuge reach the recommended speed before you begin to monitor centrifugation time. If the centrifugation time at the proper speed is reduced, some organisms (*Cryptosporidium* oocysts or microsporidial spores) may not be recovered in the sediment.

IX. LIMITATIONS OF THE PROCEDURE

A. Results obtained with wet smears should usually be confirmed by permanent stained smears. Some protozoa are very small and difficult to identify to the species level by direct wet smears alone.

B. Confirmation is particularly important in the case of *Entamoeba histolytica*/*E. dispar* versus *Entamoeba coli.*

C. Certain organisms, such as *Giardia* cysts, hookworm eggs, and occasionally *Trichuris* eggs, may not concentrate as well from PVA-preserved specimens as they do from those preserved in formalin. However, if there are enough *Giardia* cysts present to concentrate from formalin, then PVA should contain enough for detection on the permanent stained smear. In clinically important infections, the number of helminth eggs present would ensure detection regardless of the type of preservative used. Also, the morphology of *Strongyloides stercoralis* larvae is not as clear from PVA as from specimens fixed in formalin.

D. For unknown reasons, *Cystoisospora* (*Isospora*) oocysts concentrated from PVA-preserved specimens are routinely missed in the concentrate sediment.

E. At the centrifugation speed and time recommended in this procedure, there is anecdotal evidence to strongly indicate that *Cryptosporidium* oocysts and microsporidial spores should be recovered if present in the specimen. *The current recommendation is centrifugation at 500 × g for a minimum of 10 min for the recovery of coccidia and microsporidia.*

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
2. **Garcia, L. S.** 2009. *Practical Guide to Diagnostic Parasitology*, 2nd ed. ASM Press, Washington, DC.
3. **Markell, E. K., D. T. John, and W. A. Krotoski.** 1999. *Markell and Voge's Medical Parasitology*, 8th ed. W. B. Saunders Co., Philadelphia, PA.
4. **Melvin, D. M., and M. M. Brooke.** 1985. *Laboratory Procedures for the Diagnosis of Intestinal Parasites*, p. 163–189. U.S. Department of Health, Education, and Welfare publication no. (CDC) 85-8282. U.S. Government Printing Office, Washington, DC.
5. **NCCLS.** 2005. *Procedures for the Recovery and Identification of Parasites from the Intestinal Tract.* Approved guideline M28-A2. NCCLS, Wayne, PA.
6. **Neimeister, R., A. L. Logan, B. Gerber, J. H. Egleton, and B. Kieger.** 1987. Hemo-De as a substitute for ethyl acetate in formalin-ethyl acetate concentration technique. *J. Clin. Microbiol.* **25:**425–426.

APPENDIX 9.3.4–1

Automated Workstation for the Microscopic Analysis of Fecal Concentrates

The FE-2 (DiaSys Corporation, Waterbury, CT) is a countertop workstation that automates the microscopic analysis of fecal concentrates (Fig. 9.3.4–A1). The system automates the aspiration, resuspension, staining or dilution (based on user preference), transfer, presentation, and disposal of fecal concentrates. When the sample button is pressed, within 5 s two samples of fecal concentrate are automatically and simultaneously aspirated from the concentrate tube and transported to the glass dual-flow cells of the Optical Slide Assembly (Fig. 9.3.4–A2). Based on user preference, the FE-2 will also simultaneously stain or dilute one of the two samples to be examined. After the microscopic examination of the fecal suspension

APPENDIX 9.3.4–1 *(continued)*

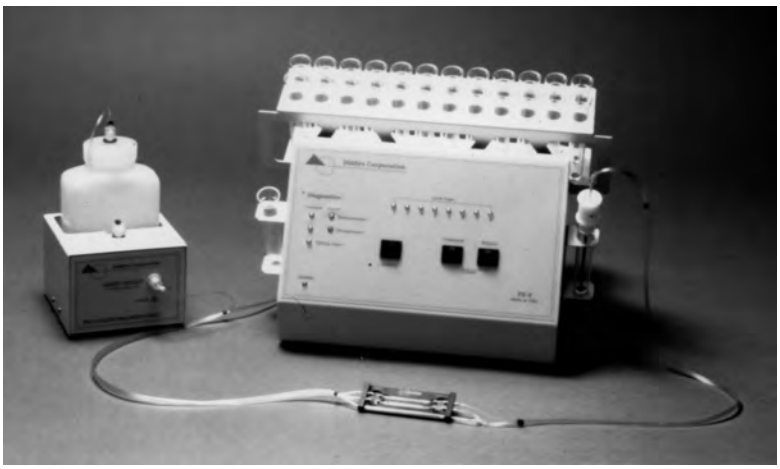


Figure 9.3.4–A1 Countertop workstation that automates the microscopic analysis of fecal concentrates (DiaSys Corp.) (from **L. S. Garcia,** *Diagnostic Medical Parasitology,* 5th ed., 2007, ASM Press, Washington, DC).

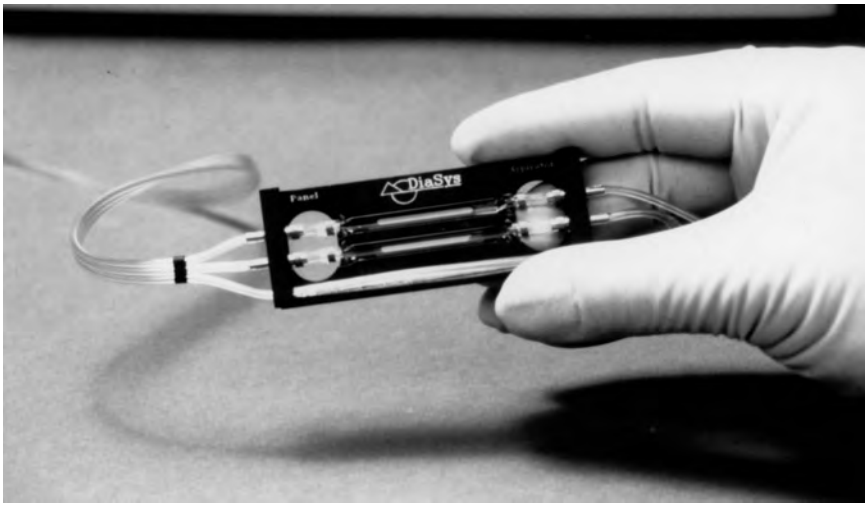


Figure 9.3.4–A2 Dual-flow-cell Optical Slide Assembly (DiaSys Corp.) that fits into the stage clips of any standard upright microscope (from **L. S. Garcia,** *Diagnostic Medical Parasitology,* 5th ed., 2007, ASM Press, Washington, DC).

within the glass viewing chambers, the flow chambers can be purged and cleaned so that they are ready for the next specimen. The dual-flow-cell Optical Slide Assembly is designed to fit within the stage clips of any standard, upright microscope. The Optical Slide Assembly accommodates bright-field, phase-contrast, polarized-light, and other common forms of microscopy. The system can be moved from one microscope to another or can be set up as a semipermanent station for fecal concentrate microscopy. Removal to another microscope just involves removing the Optical Slide Assembly from the microscope stage.

9.3.5 Microscopic Examination of Fecal Specimens: Concentration by Zinc Sulfate Flotation

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The flotation procedure permits the separation of protozoan cysts and certain helminth eggs from excess debris through the use of a liquid with a high specific gravity. The parasitic elements are recovered in the surface film, and the debris remains in the bottom of the tube. This technique yields a cleaner preparation than the sedimentation procedure; however, some helminth eggs (operculated eggs and/or very dense eggs such as unfertilized *Ascaris* eggs) do not concentrate well with the flotation method (1–6). The specific gravity of the zinc sulfate may be increased, although this usually causes more distortion in the organisms present and is not recommended for routine clinical use. To ensure detection of all possible organisms, examine both the surface film and the sediment. For most laboratories, this is not a practical approach.

II. SPECIMEN

Observe standard precautions.

The specimen must be fresh or formalinized stool (5 or 10% buffered or nonbuffered formalin or sodium acetate-acetic acid-formalin [SAF]).

III. MATERIALS

A. Reagents (*see* Appendix 9.3.5–1)
- **1.** Formalin (5 or 10% buffered or nonbuffered) or SAF
- **2.** 0.85% NaCl
- **3.** Zinc sulfate (33% aqueous solution)

B. Supplies
- **1.** Funnel
- **2.** Gauze
- **3.** Centrifuge tubes (15 ml)
- **4.** Applicator sticks
- **5.** Glass slides (1 by 3 in. or larger)
- **6.** Coverslips (22 by 22 mm; no. 1 or larger)
- **7.** Disposable glass or plastic pipettes
- **8.** Wire loop (bacteriology)
- **9.** Graduated cylinder

C. Equipment
- **1.** Centrifuge (tabletop or floor model)
  Swinging buckets are preferred to a fixed-head model.
- **2.** Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
- **3.** Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
- **4.** Hydrometer (with a range that includes 1.18 to 1.20)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check the reagents each time they are used.
The formalin, saline, and zinc sulfate should appear clear, without any visible contamination.

B. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the micro-

IV. QUALITY CONTROL *(continued)*

scope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedure 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Concentrate known positive specimens, and verify organism recovery at least quarterly and particularly after the centrifuge has been recalibrated.

D. Known positive microscope slides, Kodachrome (2- by 2-in.) projection slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

E. Record all QC results.

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. Transfer a half-teaspoon (about 4 g) of fresh stool into 10 ml of 10% formalin in a shell vial, unwaxed paper cup, or round-bottom tube (container may be modified to suit individual laboratory preferences). Mix the stool and formalin thoroughly. Let the mixture stand a minimum of 30 min for fixation. If the specimen is already in 5 or 10% formalin (or SAF), restir the stool-formalin mixture.

C. Depending on the size and density of the specimen, strain a sufficient quantity through wet gauze into a conical 15-ml centrifuge tube to give the desired amount of sediment (0.5 to 1 ml) in step V.D below. Usually, 8 ml of the stool-formalin mixture prepared in step V.B will be sufficient. If the specimen is received in vials of preservative (10% formalin or SAF), then approximately 3 to 4 ml will be sufficient unless the specimen has very little stool in the vial.

D. Add 0.85% NaCl (*see* item VIII below) almost to the top of the tube, and centrifuge for 10 min at 500 $\times$ *g*. The amount of sediment obtained should be approximately 0.5 to 1 ml. Too much or too little sediment will result in an ineffective concentration.

E. Decant the supernatant fluid, resuspend the sediment in 0.85% NaCl almost to the top of the tube, and centrifuge for 10 min at 500 $\times$ *g*. This second wash may be eliminated if the supernatant fluid after the first wash is light tan or clear.

F. Decant the supernatant fluid, and resuspend the sediment on the bottom of the tube in 1 to 2 ml of zinc sulfate. Fill the tube to within 2 to 3 mm of the rim with additional zinc sulfate.

G. Centrifuge for 1 min at 500 $\times$ *g*. Allow the centrifuge to come to a stop without interference or vibration. Two layers should result: a small amount of sediment in the bottom of the tube and a layer of zinc sulfate. The protozoan cysts and some helminth eggs will be in the surface film; some operculated and/or heavy eggs will be in the sediment (Fig. 9.3.5–1).

H. Without removing the tube from the centrifuge, remove 1 or 2 drops of the surface film with a Pasteur pipette or a freshly flamed (and allowed to cool) wire loop and place them on a slide. Do not use the loop as a "dipper"; simply touch the surface (bend the loop portion of the wire 90° so the loop is parallel with the surface of the fluid). Make sure the pipette tip or wire loop is not below the surface film.

I. Add a coverslip to the preparation. Iodine may be added to the preparation.

J. Systematically scan with the 10$\times$ objective. The entire coverslip area should be examined.

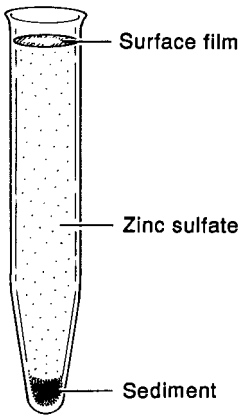


Figure 9.3.5–1 Diagram of specimen after centrifugation (flotation procedure).

V. PROCEDURE *(continued)*

K. If you see something suspicious, use the 40× objective for more-detailed study. At least one-third of the coverslip should be examined with the 40× objective, even if nothing suspicious has been seen. As in the direct wet smear, iodine can be added to enhance morphological detail.

VI. RESULTS

Protozoan trophozoites and/or cysts and helminth eggs and larvae may be seen and identified.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. You may or may not be able to identify protozoan cysts to the species level (depending on the clarity of the morphology).
Example: *Giardia lamblia* cysts present.

B. You may identify helminth eggs and/or larvae.
Example: *Trichuris trichiura* eggs present.

C. You may see and report artifacts and/or other structures.
Examples: Few Charcot-Leyden crystals present.
Moderate PMNs present.

VIII. PROCEDURE NOTES

A. Tap water (*see* step V.D below) may be substituted for 0.85% NaCl throughout this procedure; some workers prefer to use 10% formalin for all the rinses throughout the procedure.

B. If fresh stool is used (nonformalin preservatives), then the zinc sulfate should be prepared with a specific gravity of 1.18.

C. If specimens are received in SAF, then begin at step V.B.

D. If fresh specimens are received, the standardized procedure requires the stool to be rinsed in distilled water prior to the addition of zinc sulfate in step V.F. *However, the addition of fresh stool to distilled water will destroy any* Blastocystis hominis *organisms present and is not a recommended approach.*

E. Some workers prefer to remove the tubes from the centrifuge prior to sampling the surface film. This is acceptable; however, there is more chance that the surface film will be disturbed prior to sampling.

F. Some workers prefer to add a small amount of zinc sulfate to the tube so that the fluid forms a slightly convex meniscus. A coverslip is then placed on top of the tube so that the undersurface touches the meniscus. Leave undisturbed for 5 min. Carefully remove the coverslip, and place it on a slide for examination.

G. When using the hydrometer (with the solution at room temperature), mix the solution well. Float the hydrometer in the solution, giving it a slight twist to see that it is completely free from the sides of the container. Read the bottom meniscus, and correct for temperature if necessary. Most hydrometers are calibrated at 20°C. A difference of 3°C between the solution temperature (room temperature) and the hydrometer calibration temperature requires a correction of 0.001, to be added if above 20°C and subtracted if below 20°C.

H. The longer centrifugation times for the wash steps (V.D and E) are necessary for the possible recovery of *Cryptosporidium* oocysts or microsporidial spores.

IX. LIMITATIONS OF THE PROCEDURE

A. Results obtained with wet smears should usually be confirmed by permanent stained smears. Some protozoa are very small and difficult to identify to the species level by direct wet smears alone.

B. Confirmation is particularly important in the case of *Entamoeba histolytica*/*E. dispar* versus *Entamoeba coli.*

IX. LIMITATIONS OF THE PROCEDURE *(continued)*

C. Protozoan cysts and thin-shelled helminth eggs are subject to collapse and distortion when left in contact with high-specific-gravity zinc sulfate for more than a few minutes. Remove the surface film for examination within 5 min of the time the centrifuge comes to a stop. The longer the organisms are in contact with the zinc sulfate, the more distortion you will see on microscopic examination of the surface film.

D. If zinc sulfate is the only concentration method used, examine both the surface film and the sediment to ensure detection of all possible organisms.

REFERENCES

1. **Garcia, L. S., J. W. Smith, and T. R. Fritsche.** 2003. *Cumitech 30A, Selection and Use of Laboratory Procedures for Diagnosis of Parasitic Infections of the Gastrointestinal Tract.* Coordinating ed., L. S. Garcia. ASM Press, Washington, DC.
2. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology,* 5th ed. ASM Press, Washington, DC.
3. **Garcia, L. S.** 2009. *Practical Guide to Diagnostic Parasitology,* 2nd ed. ASM Press, Washington, DC.
4. **Markell, E. K., D. T. John, and W. A. Krotoski.** 1999. *Markell and Voge's Medical Parasitology,* 8th ed. W. B. Saunders Co., Philadelphia, PA.
5. **Melvin, D. M., and M. M. Brooke.** 1985. *Laboratory Procedures for the Diagnosis of Intestinal Parasites,* p. 163–189. U.S. Department of Health, Education, and Welfare publication no. (CDC) 85-8282. U.S. Government Printing Office, Washington, DC.
6. **NCCLS.** 2005. *Procedures for the Recovery and Identification of Parasites from the Intestinal Tract.* Approved guideline M28-A2. NCCLS, Wayne, PA.

APPENDIX 9.3.5–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. Formalin (5 or 10% buffered or nonbuffered) or SAF (*see* procedure 9.2.2)
B. 0.85% NaCl
C. Zinc sulfate (33% aqueous solution)

zinc sulfate	330 g
distilled water	670 ml

1. Dissolve the zinc sulfate in distilled water in an appropriate flask or beaker with a magnetic stirrer.
2. Adjust the specific gravity to 1.20 by adding more zinc sulfate or distilled water. Use a specific gravity of 1.18 when using fresh stool (nonformalinized).

D. Store in a glass-stoppered bottle with an expiration date of 24 months.

◩ **NOTE:** There are no specific guidelines for how often the specific gravity of the zinc sulfate solution should be checked. However, due to the possibility of evaporation, the specific gravity of the solution should probably be checked twice each year. If the zinc sulfate flotation concentration method is used routinely, the specific gravity of the solution will be checked after preparation and then twice each year. Remember to store the solution in a tightly stoppered bottle.

9.3.6 Microscopic Examination of Fecal Specimens: Permanent Stained Smear (Trichrome)

It is generally recognized that stained fecal films are the single most productive means of stool examination for intestinal protozoa. The permanent stained smear facilitates detection and identification of cysts and trophozoites and affords a permanent record of the protozoa encountered. Small protozoa missed by direct smear and concentration techniques are often seen on the stained smear. It also allows laboratories to refer the slide to a specialist for help when they have encountered an organism with an unusual morphology or have difficulty with the identification. *For these reasons, the permanent stained smear is recommended for use with every stool specimen submitted for a routine parasite examination (1–6).*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The trichrome technique of Wheatley (8) for fecal specimens is a modification of Gomori's original staining procedure for tissue (3). It is a rapid, simple procedure that produces uniformly well-stained smears of intestinal protozoa, human cells, yeast cells, and artifact material.

II. SPECIMEN

Observe standard precautions.

A. The specimen usually consists either of unconcentrated fresh stool smeared on a microscope slide and immediately fixed in Schaudinn's fixative or of polyvinyl alcohol (PVA)-preserved stool smeared on a slide and allowed to air dry (*see* procedure 9.2.2).

B. Stool preserved in sodium acetate-acetic acid-formalin (SAF) or any of the single-vial fixatives for parasitology can also be used (*see* procedure 9.2.2).

III. MATERIALS

A. Reagents (*see* Appendix 9.3.6–1)
Trichrome stain

B. Supplies
1. Disposable glass or plastic pipettes
2. Glass slides, frosted at one end (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. 12 covered Coplin jars or staining dishes (with slide rack)

C. Equipment
1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Fume hood to contain staining setup (optional)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. For QC of Schaudinn's, SAF, or PVA fixative, see procedure 9.2.2.

B. Stool samples used for QC can be either fixed stool specimens known to contain protozoa or PVA-preserved negative stools to which buffy coat cells have been added. Use a QC smear prepared from a positive PVA specimen or PVA containing buffy coat cells when a new stain is prepared or at least once each month. Cultured protozoa can also be used.

C. Include a QC slide when you use a new lot number of reagents, when you add new reagents after cleaning the dishes, and at least monthly.

D. If the xylene becomes cloudy or has an accumulation of water in the bottom of the staining dish, use fresh 100% ethanol and xylene.

E. Cover all staining dishes to prevent evaporation of reagents.

F. Depending on the volume of slides stained, change staining solutions on an as-needed basis.

G. When the smear is thoroughly fixed and the stain is performed correctly, the cytoplasm of protozoan trophozoites will be blue-green, with sometimes a tinge of purple. Cysts tend to be slightly more purple. Nuclei and inclusions (chromatoid bodies, RBCs, bacteria, and Charcot-Leyden crystals) are red, sometimes tinged with purple. The background material usually stains green, providing a nice color contrast with the protozoa. This contrast is more distinct than that obtained with the hematoxylin stain.

H. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

I. Known positive microscope slides, Kodachrome 2- by 2-in. projection slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

J. Record all QC results, including a description of QC specimens tested.

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. Slide preparation

1. Fresh fecal specimens

a. When the specimen arrives, prepare two slides with applicator sticks and *immediately* (without drying) place them in Schaudinn's fixative (*see* procedure 9.2.2). Allow the specimen to fix for a minimum of 30 min; overnight fixation is acceptable. The stool smeared on the slide should be thin enough that newsprint can be read through the smear. Proceed with the trichrome staining procedure (*see* item V.C.1 below).

b. If the fresh specimen is liquid, place 3 or 4 drops of PVA (*see* procedure 9.2.2) on the slide, mix several drops of fecal material with the PVA, spread the mixture, and allow it to dry for several hours in a 37°C incubator or overnight at room temperature (25°C). Proceed with the trichrome staining procedure by placing the slides in iodine-alcohol (*see* item V.B.2 below).

V. PROCEDURE *(continued)*

2. PVA-preserved fecal specimens (mercuric chloride base) (*see* procedure 9.2.2)
- **a.** Allow the stool specimens that are preserved in PVA to fix for at least 30 min. Thoroughly mix the contents of the PVA bottle with two applicator sticks.
- **b.** Pour some of the PVA-stool mixture onto a paper towel, and allow it to stand for 3 min to absorb the PVA. *Do not eliminate this step.*
- **c.** With an applicator stick, apply some of the stool material from the paper towel to two slides, and allow them to dry for several hours in a 37°C incubator or overnight at room temperature (25°C).
- **d.** Place the dry slides into iodine-alcohol (*see* item V.C.2 below).
- **e.** If the stool was not thoroughly mixed with PVA by the patient, apply some stool material to two slides and immediately immerse in Schaudinn's fixative for a minimum of 30 min; then proceed with the trichrome method (*see* item V.C.1 below).

3. Modified PVA-preserved fecal specimens (copper or zinc base, single-vial systems)
- **a.** Allow the stool specimens that are preserved in PVA or another fixative to fix for at least 30 min. Thoroughly mix the contents of the fixative vial with two applicator sticks.
- **b.** Pour some of the fixative-stool mixture onto a paper towel, and allow it to stand for 3 min to absorb the PVA. *Do not eliminate this step if the fixative contains PVA.*
- **c.** With an applicator stick, apply some of the stool material from the paper towel to two slides, and allow them to dry for several hours in a 37°C incubator or overnight at room temperature (25°C).
- **d.** Begin the trichrome staining process at step V.C.4 or V.C.5.

4. SAF-preserved fecal specimens (*see* procedure 9.2.2)
- **a.** Thoroughly mix the SAF-stool mixture, and strain through gauze into a 15-ml centrifuge tube.
- **b.** After centrifugation (10 min at 500 $\times$ *g*), decant the supernatant fluid. The final sediment should be about 0.5 to 1.0 ml. If necessary, adjust by repeating step V.B.1 or by suspending the sediment in saline (0.85% NaCl) and removing part of the suspension.
- **c.** Prepare a smear from the sediment for later staining.
- **d.** After drying, place the smear in 70% alcohol (*see* item V.C.3 below) (iodine-alcohol step can be eliminated).

C. Staining smears

1. Remove slide from Schaudinn's fixative, and place slide in 70% ethanol for 5 min.

2. Place slide in 70% ethanol plus iodine for 1 min for fresh specimens or 5 to 10 min for PVA-preserved air-dried smears. All slides exposed to mercuric-chloride-based fixatives must be placed in the iodine dish to remove the mercury. The subsequent rinses in ethanol remove the iodine. At the point the slide is placed into trichrome stain, both the mercury and iodine have been removed from the fecal smear.

3. Place slide in 70% ethanol for 5 min.*

4. Place in 70% ethanol again for 3 min.* Fecal smears prepared from SAF-, non-mercury-based-PVA-, or single-vial-preserved stool material do not require the iodine step (V.C.2 above) and can be placed in this alcohol dish before trichrome staining.

5. Place in trichrome stain for 10 min. Fecal smears prepared from modified PVA-fixed material (copper or zinc base) do not require the iodine step (V.C.2 above) or subsequent alcohol rinses (V.C.3 and 4 above) but can be

V. PROCEDURE *(continued)*

placed directly into the trichrome stain (this step). One alcohol rinse may be used (*see* item V.C.4) before this trichrome step; some labs prefer this approach.

6. Place in 90% ethanol plus acetic acid for 1 to 3 s. Immediately drain the rack (*see* item VIII below), and proceed to the next step. Do not allow slides to remain in this solution.
7. Dip several times in 100% ethanol. Use this step as a rinse.
8. Place in two changes of 100% ethanol for 3 min each.*
9. Place in xylene for 5 to 10 min.*
10. Place in xylene again for 5 to 10 min.*
11. Mount with coverslip (no. 1 thickness) by using mounting medium (e.g., Permount).
12. Allow the smear to dry overnight or for 1 h at 37°C.
13. Examine the smear microscopically with the 100× objective. Examine at least 200 to 300 oil immersion fields.

*Slides may be held for up to 24 h in these solutions without harming the quality of the smear or the stainability of organisms.

VI. RESULTS

A. Protozoan trophozoites and cysts will be readily seen.
B. Helminth eggs and larvae may not be easily identified; therefore, examine wet mounts of concentrates.
C. Yeast and human cells can be identified. Human cells include macrophages, PMNs, and RBCs. Yeasts include single and budding cells and pseudohyphae.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism and stage (do not use abbreviations).
Example: *Entamoeba histolytica/E. dispar* trophozoites
B. Quantitate the number of *Blastocystis hominis* seen (rare, few, moderate, many). Do not quantitate other protozoa.
C. Note and quantitate the presence of human cells.
Example: Moderate WBCs, many RBCs, few macrophages, rare Charcot-Leyden crystals
D. Report and quantitate yeast cells.
Example: Moderate budding yeast cells and few pseudohyphae
E. Save positive slides for future reference. Record information prior to storage (name, patient number, organisms present).
F. Quantitation of parasites, cells, yeast cells, and artifacts
Few = ≤2 per 10 oil immersion fields (×1,000)
Moderate = 3 to 9 per 10 oil immersion fields (×1,000)
Many = ≥10 per 10 oil immersion fields (×1,000)

VIII. PROCEDURE NOTES

A. Fixation of specimens is important. Improperly fixed specimens will result in protozoan forms that are nonstaining or predominantly red.
B. Spread the PVA-stool mixture to the edges of the glass slide; this will cause the film to adhere to the slide during staining. It is also important to thoroughly dry the slides to prevent the material from washing off during staining.
C. Always drain slides between solutions. Touch the end of the slide to a paper towel for 2 s to remove excess fluid before proceeding to the next step.
D. Incomplete removal of mercuric chloride (Schaudinn's fixative and PVA) may cause the smear to contain highly refractive granules that may prevent finding

VIII. PROCEDURE NOTES *(continued)*

or identifying any organisms present (1). Since the 70% ethanol–iodine solution removes the mercury complex, it should be changed at least weekly to maintain the strong-tea color.

E. To restore weakened trichrome stain, remove cap and allow the ethanol to evaporate (ethanol carried over on staining rack from previous dish). After a few hours, add fresh stain to restore lost volume. Older, more concentrated stain produces more intense colors and may require slightly longer destaining times (an extra dip).

F. Smears that are predominantly green may be due to the inadequate removal of iodine by the 70% ethanol (*see* items VIII.D and E above). Lengthening the time of these steps or changing the 70% ethanol more frequently will help.

G. In the final stages of dehydration (steps V.C.8, 9, and 10), keep the 100% ethanol and the xylenes as free from water as possible. Coplin jars must have tight-fitting caps to prevent both evaporation of reagents and absorption of moisture. If the xylene becomes cloudy after addition of slides from the 100% ethanol, return the slides to fresh 100% ethanol, and replace the xylene.

H. If the smears peel or flake off, the specimen might have been inadequately dried on the slide (in the case of PVA-fixed specimens) or the slides might have been greasy. Slides do not have to be cleaned with alcohol prior to use.

I. If the stain appears unsatisfactory and it is not possible to obtain another slide to stain, restain the unsatisfactory slide. Place the slide in xylene to remove the coverslip, and reverse the dehydration steps by adding 50% ethanol as the last step. Destain the slide in 10% acetic acid for several hours, and then wash it thoroughly first in water, then in 50% ethanol, and then in 70% ethanol. Place the slide in the trichrome stain for 8 min, and complete the staining procedure (7).

IX. LIMITATIONS OF THE PROCEDURE

A. The permanent stained smear is not recommended for staining helminth eggs or larvae. However, occasionally they may be recognized and identified.

B. Examine the smear under the oil immersion lens (100×) for the identification of protozoa, human cells, Charcot-Leyden crystals, yeast cells, and artifact material.

C. This high-magnification examination is recommended for protozoa.

D. Screening the smear under the low-magnification lens (10×) might reveal eggs or larvae, but this is not recommended as a routine approach.

E. Helminth eggs and larvae and *Cystoisospora* (*Isospora*) *belli* oocysts are best seen in wet preparations.

F. *Cryptosporidium* spp. and *Cyclospora cayetanensis* are generally not seen on a trichrome-stained smear (modified acid-fast stains or immunoassays are recommended).

G. Microsporidial spores will not be seen on a trichrome-stained smear (modified trichrome stains are recommended).

REFERENCES

1. **Garcia, L. S., J. W. Smith, and T. R. Fritsche.** 2003. *Cumitech 30A, Selection and Use of Laboratory Procedures for Diagnosis of Parasitic Infections of the Gastrointestinal Tract.* Coordinating ed., L. S. Garcia. ASM Press, Washington, DC.
2. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
3. **Garcia, L. S.** 2009. *Practical Guide to Diagnostic Parasitology*, 2nd ed. ASM Press, Washington, DC.
4. **Gomori, G.** 1950. A rapid one-step trichrome stain. *Am. J. Clin. Pathol.* **20:**661–663.
5. **Markell, E. K., D. T. John, and W. A. Krotoski.** 1999. *Markell and Voge's Medical Parasitology*, 8th ed. W. B. Saunders Co., Philadelphia, PA.

REFERENCES *(continued)*

6. **Melvin, D. M., and M. M. Brooke.** 1985. *Laboratory Procedures for the Diagnosis of Intestinal Parasites*, p. 163–189. U.S. Department of Health, Education, and Welfare publication no. (CDC) 85-8282. U.S. Government Printing Office, Washington, DC.
7. **NCCLS.** 2005. *Procedures for the Recovery and Identification of Parasites from the Intestinal Tract.* Approved guideline M28-A2. NCCLS, Wayne, PA.
8. **Smith, J. W., and M. S. Bartlett.** 1991. Diagnostic parasitology: introduction and methods, p. 701–716. *In* A. Balows, W. J. Hausler, Jr., K. L. Hermann, H. D. Isenberg, and H. J. Shadomy (ed.), *Manual of Clinical Microbiology*, 5th ed. American Society for Microbiology, Washington, DC.
9. **Wheatley, W.** 1951. A rapid staining procedure for intestinal amoebae and flagellates. *Am. J. Clin. Pathol.* **21:**990–991.

APPENDIX 9.3.6–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. Trichrome stain

chromotrope 2R	0.6 g
light green SF	0.3 g
phosphotungstic acid	0.7 g
acetic acid (glacial)	1.0 ml
distilled water	100 ml

Prepare the stain by adding 1.0 ml of acetic acid to the dry components. Allow the mixture to stand (ripen) for 15 to 30 min at room temperature. Add 100 ml of distilled water. Properly prepared stain will be purple. Store in a glass or plastic bottle at room temperature. The shelf life is 24 months.

B. 70% Ethanol

C. 70% Ethanol plus iodine

Prepare a stock solution by adding iodine crystals to 70% alcohol until you obtain a dark solution (1 to 2 g/100 ml). To use, dilute the stock solution with 70% alcohol until a dark reddish brown (strong-tea color) is obtained. As long as the color is acceptable, new working solution does not have to be replaced. Replacement time will depend on the number of smears stained and the size of the container (one to several weeks).

D. 90% Ethanol, acidified

90% ethanol	99.5 ml
acetic acid (glacial)	0.5 ml

Prepare by combining.

E. 100% Ethanol

F. Xylene (or xylene substitute)

APPENDIX 9.3.6–2

As an alternative to using mounting fluid on every slide, the following method can be used. This approach saves time (drying the slides after they are mounted) and eliminates the need for routine use of mounting fluids.

A. Remove the stained slides from the last dehydrating dish (step V.C.10 above).
B. Allow the slide to air dry (minimum of 30 min, especially if using xylene substitutes).
C. Place a drop of immersion oil directly onto the dry stool smear.
D. Allow the oil to "sink in" for a minimum of ~15 min.
E. Place a no. 1 coverslip onto the oil-covered stool smear.
F. Add 1 drop of immersion oil onto the coverslip and proceed to examine the smear using the 100× oil immersion objective. A 50× or 60× oil immersion objective can be used for screening.
G. *Do not use this approach unless you add the coverslip before examination of the smear. The dry stool material may be quite hard; the objective lens could accidentally be scratched if the stool smear is not covered before reading.*

9.3.7 Microscopic Examination of Fecal Specimens: Iron Hematoxylin Stain (Modified Spencer-Monroe Method)

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The iron hematoxylin stain is one of a number of stains used to make a permanent stained slide for detecting and quantitating parasitic organisms. Iron hematoxylin was the stain used for most of the original morphological descriptions of intestinal protozoa found in humans (1–4). Under oil immersion power (×1,000), one can examine the diagnostic features used to identify the protozoan parasite.

II. SPECIMEN

Observe standard precautions.

A. The specimen usually consists either of unconcentrated fresh stool smeared on a microscope slide and immediately fixed in Schaudinn's fixative or of polyvinyl alcohol (PVA)-preserved stool smeared on a slide and allowed to air dry (*see* procedure 9.2.2).

B. Stool preserved in sodium acetate-acetic acid-formalin (SAF) can also be used (*see* procedure 9.2.2).

III. MATERIALS

A. Reagents (*see* Appendix 9.3.7–1)

1. Iron hematoxylin stain
2. D'Antoni's iodine solution

B. Supplies

1. Glass slides, frosted at one end (1 by 3 in.)
2. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
3. Coplin jars or other suitable staining containers
4. Hematoxylin, crystals or powder
5. Ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$]
6. Ferric ammonium sulfate [$FeNH_4(SO_4)_2 \cdot 12H_2O$]
7. Hydrochloric acid, concentrated (HCl)
8. Potassium iodide (KI)
9. Iodine crystals, powdered (I_2)
10. Permount or other suitable mounting medium

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Fume hood to contain staining setup (optional)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. For QC of Schaudinn's SAF or PVA fixatives, see procedure 9.2.2.

B. Stool samples used for QC can be either fixed stool specimens known to contain protozoa or PVA-preserved negative stools to which buffy coat cells have been added. A QC smear prepared from a positive PVA specimen or PVA containing buffy coat cells should be used when a new stain is prepared or at least once each month. Cultured protozoa can also be used (2).

C. Include a QC slide when you use a new lot number of reagents, when you add new reagents after cleaning the dishes, and at least monthly.

D. If the xylene becomes cloudy or has an accumulation of water in the bottom of the staining dish, use fresh 100% ethanol and xylene.

E. Cover all staining dishes to prevent evaporation of reagents.

F. Depending on the volume of slides stained, change staining solutions on an as-needed basis.

G. Background material will stain blue-gray. Cells and organisms will stain various intensities of blue-gray. Inclusions, chromatoidal bodies, and nuclear structures will stain darker than the surrounding cytoplasm.

H. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

I. Known positive microscope slides, Kodachrome 2- by 2-in. projection slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

J. Record all QC results, including a description of QC specimens tested.

V. PROCEDURE

A. ***Wear gloves when performing this procedure.***

B. Slide preparation

1. Fresh fecal specimens

a. When the specimen arrives, prepare two slides with applicator sticks and *immediately* (without drying) place them in Schaudinn's fixative (*see* procedure 9.1). Allow the slides to fix for a minimum of 30 min; overnight fixation is acceptable. The amount of stool smeared on the slide should be thin enough that newsprint can be read through the smear.

b. If the fresh specimen is liquid, place 3 or 4 drops of PVA (*see* procedure 9.1) on the slide, mix several drops of fecal material with the PVA, spread the mixture, and allow it to dry for several hours in a 37°C incubator or overnight at room temperature (25°C).

c. Proceed with the hematoxylin staining procedure by placing the dry slides in iodine-alcohol (*see* item V.B.2 below).

2. PVA-preserved fecal specimens (*see* procedure 9.1)

a. Allow the stool specimens that are preserved in PVA to fix at least 30 min. Thoroughly mix the contents of the PVA bottle with two applicator sticks.

b. Pour some of the PVA-stool mixture onto a paper towel, and allow it to stand for 3 min to absorb the PVA. *Do not eliminate this step.*

V. PROCEDURE *(continued)*

c. With an applicator stick, apply some of the stool material from the paper towel to two slides, and allow them to dry for several hours in a 37°C incubator or overnight at room temperature.

d. Place the dry slides in iodine-alcohol (*see* item V.C.2 below).

e. If the stool was not thoroughly mixed with PVA by the patient, apply some stool material to two slides, and immediately immerse in Schaudinn's fixative for a minimum of 30 min; then proceed with the hematoxylin method.

3. SAF-preserved fecal specimens (*see* procedure 9.1)

a. Thoroughly mix the SAF-stool mixture, and strain through gauze into a 15-ml centrifuge tube.

b. After centrifugation (10 min at 500 × *g*), decant the supernatant fluid. The final sediment should be about 0.5 to 1.0 ml. If necessary, adjust by repeating step 1 or by suspending the sediment in saline (0.85% NaCl) and removing part of the suspension.

c. Prepare a smear from the sediment for later staining.

d. After drying, place the smear into 70% alcohol (*see* item V.C.3 below) (iodine-alcohol step can be eliminated).

C. Staining smears

1. Place slide in 70% ethanol for 5 min.

2. Place slide in iodine–70% ethanol (70% alcohol to which is added enough D'Antoni's iodine to obtain a strong tea color) solution for 2 to 5 min.

3. Place in 70% ethanol for 5 min. Begin procedure for SAF-fixed slides at this point.*

4. Wash slide in running tap water (constant stream of water into the container) for 10 min.

5. Place slide in iron hematoxylin working solution for 4 to 5 min.

6. Wash slide in running tap water (constant stream of water into the container) for 10 min.

7. Place slide in 70% ethanol for 5 min.*

8. Place slide in 95% ethanol for 5 min.*

9. Place slide in two changes of 100% ethanol for 5 min each.*

10. Place slide in two changes of xylene for 5 min each.*

11. Add Permount to the stained area of the slide, and cover with a coverslip.

12. Examine the smear microscopically with the 100× objective. Examine at least 200 to 300 oil immersion fields.

*Slides may be held for up to 24 h in these solutions without harming the quality of the smear or the stainability of organisms.

VI. RESULTS

A. Protozoan trophozoites and cysts will be readily seen.

B. Helminth eggs and larvae may not be easily identified; therefore, examine wet-mount concentrates.

C. Yeast and human cells can be identified. Human cells include macrophages, PMNs, and RBCs. Yeast cells include single and budding cells and pseudohyphae.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the complete scientific name (genus and species) of the organism and the stage seen.
Example: *Entamoeba histolytica/E. dispar* trophozoites

B. Quantitate the number of *Blastocystis hominis* seen (rare, few, moderate, many). Do not quantitate other protozoa.

C. Note and quantitate the presence of human cells.
Examples: Moderate WBCs, many RBCs, few macrophages, rare Charcot-Leyden crystals

D. Report and quantitate yeast cells.
Example: Moderate budding yeast cells and few pseudohyphae

E. Save positive slides for future reference. Record information prior to storage (name, patient number, organisms present).

F. Quantitation of parasites, cells, yeast cells, and artifacts
Few = ≤ per 10 oil immersion fields (×1,000)
Moderate = 3 to 9 per 10 oil immersion fields (×1,000)
Many = ≥10 per 10 oil immersion fields (×1,000)

VIII. PROCEDURE NOTES

A. Once the staining process has begun, do not allow the slides to dry until they have been placed in xylene.

B. Always drain slides between solutions. Touch the end of the slide to a paper towel for 2 s to remove excess fluid before proceeding to the next step.

C. Incomplete removal of mercuric chloride (Schaudinn's fixative and PVA) may cause the smear to contain highly refractive granules that may prevent finding or identifying any organisms present (4). Since the 70% ethanol–iodine solution removes the mercury complex, it should be changed at least weekly to maintain the port wine or strong tea color.

D. When large numbers of slides are stained, the working hematoxylin solution may be diluted and affect the quality of the stain. If dilution occurs, discard the working solution, and prepare a fresh working solution.

E. The shelf life of the stock hematoxylin solutions may be extended by keeping the solutions in the refrigerator at 4°C. Because of crystal formation in the working solutions, it may be necessary to filter them before preparing a new working solution.

F. In the final stages of dehydration (*see* items V.C.9 and 10 above), keep the 100% ethanol and the xylenes as free from water as possible. Coplin jars must have tight-fitting caps to prevent both evaporation of reagents and absorption of moisture. If the xylene becomes cloudy after addition of slides from the 100% ethanol, return the slides to fresh 100% ethanol, and replace the xylene.

G. If the smears peel or flake off, the specimen might have been inadequately dried on the slide (in the case of PVA-fixed specimens) or the slides might have been greasy. Slides do not have to be cleaned with alcohol prior to use.

H. Other iron hematoxylin formulations may call for 10 g of stain per 100 ml of 95 or 100% ethanol. These are also acceptable.

IX. LIMITATIONS OF THE PROCEDURE

A. The permanent stained smear is not recommended for staining helminth eggs or larvae. However, occasionally they may be recognized and identified.

B. The smear should be examined with the oil immersion lens (100×) for the identification of protozoa, human cells, Charcot-Leyden crystals, yeast cells, and artifact material.

C. This high-magnification examination is recommended for protozoa.

IX. LIMITATIONS OF THE PROCEDURE *(continued)*

D. Screening the smear under low magnification (10×) might reveal eggs or larvae, but this is not recommended as a routine approach.

E. Helminth eggs and larvae and *Isospora belli* oocysts are best seen in wet preparations.

F. *Cryptosporidium*, *Cystoisospora* (*Isospora*), and *Cyclospora* oocysts will not be seen on an iron hematoxylin-stained smear (acid-fast stains or immunoassays are recommended).

G. Microsporidial spores will not be seen on an iron hematoxylin-stained smear (modified trichrome stains are recommended).

REFERENCES

1. **Garcia, L. S., J. W. Smith, and T. R. Fritsche.** 2003. *Cumitech 30A, Selection and Use of Laboratory Procedures for Diagnosis of Parasitic Infections of the Gastrointestinal Tract.* Coordinating ed., L. S. Garcia. ASM Press, Washington, DC.
2. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
3. **NCCLS.** 2005. *Procedures for the Recovery and Identification of Parasites from the Intestinal Tract.* Approved guideline M28-A2. NCCLS, Wayne, PA.
4. **Spencer, F. M., and L. S. Monroe.** 1976. *The Color Atlas of Intestinal Parasites*, 2nd ed. Charles C Thomas, Publisher, Springfield, IL.

APPENDIX 9.3.7–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. Iron hematoxylin stain

1. Solution 1

hematoxylin (crystal or powder) ……10 g
ethanol (absolute) ……1,000 ml

Place solution in a stoppered clear flask or bottle, and allow to ripen in a lighted room for at least 1 week at room temperature.

2. Solution 2

ferrous ammonium sulfate
$[Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O]$ ……10 g
ferric ammonium sulfate
$[FeNH_4(SO_4)_2 \cdot 12H_2O]$ ……10 g
hydrochloric acid (HCl) (concentrated) …..10 ml
distilled water ……1,000 ml

3. Working solution
Mix equal volumes of solutions 1 and 2. The working solution should be made fresh every week.

B. D'Antoni's iodine solution

potassium iodide (KI) …… 1 g
distilled water …… 100 ml

Add 1.5 g of powered iodine crystals to the KI solution to saturate the solution. Undissolved iodine crystals should be present. Store the solution in the dark in a brown bottle (stock solution). Working solutions should be made fresh every 2 weeks and can be made by filtering the stock solution to remove undissolved crystals.

C. Ethanol: 70, 95, and 100%

D. Xylene (or xylene substitute)

APPENDIX 9.3.7–2

Modified Iron Hematoxylin Stain (Incorporating Carbol Fuchsin Step)

The following combination staining method for SAF-preserved fecal specimens was developed to allow the microscopist to screen for acid-fast organisms in addition to other intestinal parasites. For those laboratories using iron hematoxylin stains in combination with SAF-fixed material and modified acid-fast stains for *C. parvum, Cyclospora cayetanensis*, and *C. belli*, this modification represents an improved approach to current staining methods. This combination stain provides savings in both time and personnel use. Any fecal specimen submitted in SAF fixative can be used. Fresh fecal specimens after fixation in SAF for 30 min can also be used. This combination stain approach is not recommended for specimens preserved in Schaudinn's fixative or PVA.

I. REAGENTS

A. Mayer's albumin

Add an equal quantity of glycerin to a fresh egg white. Mix gently and thoroughly. Store at 4°C and indicate an expiration date of 3 months. Mayer's albumin from commercial suppliers can normally be stored at 25°C for 1 year [e.g., product 756; E. M. Diagnostic Systems Inc., 480 Democrat Rd., Gibbstown, NJ 08027; (800) 443-3637].

B. Stock solution of hematoxylin stain

hematoxylin powder ……10 g
ethanol (95 or 100%) ……1,000 ml

1. Mix well until dissolved.
2. Store in a clear glass bottle in a light area. Allow to ripen for 14 days before use.
3. Store at room temperature with an expiration date of 1 year.

C. Mordant

ferrous ammonium sulfate
$[Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O]$ ……10 g
ferric ammonium sulfate
$[FeNH_4(SO_4)_2 \cdot 12H_2O]$ ……10 g
hydrochloric acid (concentrated) ……10 ml
distilled water to ……1,000 ml

D. Working solution of hematoxylin stain

1. Mix equal quantities of stock solution of stain and mordant.
2. Allow mixture to cool thoroughly before use (prepare at least 2 h prior to use). The working solution should be made fresh every week.

E. Picric acid

Mix equal quantities of distilled water and an aqueous saturated solution of picric acid to make a 50% saturated solution.

F. Acid-alcohol decolorizer

hydrochloric acid (concentrated) ……30 ml
alcohol to ……1,000 ml

G. 70% alcohol and ammonia

70% alcohol ……50 ml
ammonia ……0.5 to 1.0 ml

Add enough ammonia to bring the pH to approximately 8.0.

H. Carbol fuchsin

1. To make basic fuchsin (solution A), dissolve 0.3 g of basic fuchsin in 10 ml of 95% ethanol.
2. To make phenol (solution B), dissolve 5 g of phenol crystals in 100 ml of distilled water. (Gentle heat may be needed.)
3. Mix solution A with solution B.
4. Store at room temperature. Solution is stable for 1 year.

APPENDIX 9.3.7–2 *(continued)*

II. PROCEDURE

A. Prepare slide.
   1. Place 1 drop of Mayer's albumin on a labeled slide.
   2. Mix the sediment from the SAF concentration well with an applicator stick.
   3. Add approximately 1 drop of the fecal concentrate to the albumin and spread the mixture over the slide.

B. Allow slide to air dry at room temperature (smear will appear opaque when dry).
C. Place slide in 70% alcohol for 5 min.
D. Wash in container (not running water) of tap water for 2 min.
E. Place slide in Kinyoun's stain for 5 min.
F. Wash slide in running tap water (constant stream of water into container) for 1 min.
G. Place slide in acid-alcohol decolorizer for 4 min.

◩ **NOTE:** This step can also be performed as follows.

1. Place slide in acid-alcohol decolorizer for 2 min.
2. Wash slide in running tap water (constant stream of water into container) for 1 min.
3. Place slide in acid-alcohol decolorizer for 2 min.
4. Wash slide in running tap water (constant stream of water into container) for 1 min.
5. Continue staining sequence with step II.I below (iron hematoxylin working solution).

H. Wash slide in running tap water (constant stream of water into container) for 1 min.
I. Place slide in iron hematoxylin working solution for 8 min.
J. Wash slide in distilled water (in container) for 1 min.
K. Place slide in picric acid solution for 3 to 5 min.
L. Wash slide in running tap water (constant stream of water into container) for 10 min.
M. Place slide in 70% alcohol plus ammonia for 3 min.
N. Place slide in 95% alcohol for 5 min.
O. Place slide in 100% alcohol for 5 min.
P. Place slide in two changes of xylene for 5 min.

III. PROCEDURE NOTES

A. The first 70% alcohol step acts with the Mayer's albumin to "glue" the specimen to the glass slide. The specimen may wash off if insufficient albumin is used or if the slides are not completely dry prior to staining.
B. The working hematoxylin stain should be checked each day of use by adding a drop of stain to alkaline tap water. If a blue color does not develop, prepare fresh working stain solution.
C. The picric acid differentiates the hematoxylin stain by removing more stain from fecal debris than from the protozoa and removing more stain from the organism cytoplasm than from the nucleus. When properly stained, the background should be various shades of gray-blue and protozoa should be easily seen with medium blue cytoplasm and dark blue-black nuclei.

Supplemental Reading

Palmer, J. 1991. Modified iron hematoxylin/kinyoun stain. *Clin. Microbiol. Newsl.* **13:**39–40.

9.3.8 Calcofluor White for Detection of Microsporidial Spores and *Acanthamoeba* Cysts

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Calcofluor white (CFW) can be used for the direct detection of *Acanthamoeba* cysts and microsporidial spores from clinical specimens (1, 4). The active ingredient is 4,4′-bis-(4-anilino-bis-diethylamino-5-triazin-2-ylamino)-2,2′-stilbene disulfonic acid, which is a nonspecific fluorescent dye binding to the polysaccharide polymer of amebic cysts and microsporidial spores (3). It is very simple to use and provides a rapid and reliable means of demonstrating these organisms.

Acanthamoeba, a free-living ameba found in soil, sewage, freshwater, salt water, or brackish water, can cause painful keratitis that may lead to eye removal or loss of eye function and has also been implicated in granulomatous amebic encephalitis (1) (see also procedure 9.9.2). A key to successful treatment of *Acanthamoeba*-caused infection is the rapid detection of the organism in patient samples. Culture may require several days, delaying diagnosis and treatment.

The diagnosis of intestinal microsporidiosis *(Enterocytozoon bieneusi* and *Encephalitozoon intestinalis)* has depended on the use of invasive procedures and subsequent examination of biopsy specimens, often by electron microscopy methods. CFW is a useful technique performed on fresh or formalin-fixed stool specimens to demonstrate microsporidial spores. However, the stain is nonspecific, and other structures within the specimen may also stain (yeasts, etc.).

II. SPECIMEN

Observe standard precautions.

With the exception of stool, collect all specimens aseptically, and hold them at room temperature (24 to 28°C). Do not freeze or refrigerate specimens. Use sterile containers and solutions where indicated; any remaining specimen may be used to inoculate culture media.

A. Stool

1. The specimen can be fresh stool or stool that has been preserved in 5 or 10% formalin, sodium acetate-acetic acid-formalin (SAF), or some of the newer single-vial system fixatives.
2. Any specimen other than tissue thought to contain microsporidia could be stained by these methods.
3. Polyvinyl alcohol-preserved fecal material is not recommended.

B. CSF

1. Centrifuge at 250 $\times$ *g* for 10 min.
2. Remove and place into another sterile tube all except 0.5 ml of the supernatant fluid.
3. Suspend the sediment with remaining supernatant fluid for examination.

C. Tissue

Triturate a small portion in sterile water or saline.

1. Brain
2. Corneal scrapings or biopsy specimen
3. Lung
4. Skin lesion

II. SPECIMEN *(continued)*

D. Swab of conjunctiva or corneal ulcer

1. Place swab in 2 ml of sterile water or saline in a tube.
2. Vigorously shake the cotton-tipped portion of the swab in the liquid to suspend specimen.
3. Remove swab from tube, and return to original holder.
4. The sterile water can be examined directly or concentrated by centrifugation if a larger volume is used.

E. Contact lens paraphernalia

1. Contact lens solutions
Solutions must be from opened containers already used by patient. If volume of solution is greater than 2 ml, then centrifuge prior to examination (250 $\times$ *g* for 10 min).
2. Contact lenses
Submit in 2 ml of sterile water or saline. Examine a small portion of lens and the fluid containing the lens.

F. Water samples

1. Collect at least 100 ml of water in a sterile container.
2. Concentrate specimen by filtration or centrifugation prior to examination (2).

G. Slide prepared by physician

Submit at least two slides.
1. Place specimen in the center of the slide, covering no more than a dime-sized area.
2. Circle the material with a wax pencil or magic marker to denote location of the specimen.
3. Air dry slides thoroughly.
4. Place the slide in a slide holder or envelope for staining in the laboratory.

III. MATERIALS

A. Reagents (*see* Appendix 9.3.8–1)

B. Supplies

1. Microscope slides (1 by 3 in.), one ring (15 mm), frosted
2. Microscope slides (1 by 3 in.), two rings (12 mm), frosted
3. Coverslip (24 by 40 mm), no. 1 thickness
4. Sterile polystyrene, round-bottom tubes (with cap), 12 by 75 mm
5. Sterile transfer pipettes
6. Sterile wooden applicator sticks
7. Sterile swabs
8. Disposable latex gloves
9. Biohazard container for disposal of contaminated supplies and patient specimens.
10. Positive fecal specimen (microsporidial spores)
11. *Acanthamoeba castellanii* ATCC 30010 stock culture
12. *Escherichia coli* ATCC 25922 stock culture
13. Wax pencil or magic marker

C. Equipment

1. Epifluorescence microscope equipped with an exciter filter that transmits the 250- to 400-nm group of intense mercury spectral emission lines (Zeiss UGI or G365). View through a barrier filter (Zeiss 41 or LP420), which removes UV while transmitting visible blue light and longer wavelengths (1, 3).
2. Biological safety cabinet

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. A QC slide must be run with each batch of specimens stained with CFW.

B. Unfortunately, the only way to perform acceptable QC procedures for this method is to use actual microsporidial spores as the control organisms (Medical Chemical Corporation; http://www.med-chem.com). Obtaining these positive controls may be somewhat difficult. It is particularly important to use the actual

IV. QUALITY CONTROL *(continued)*

organisms because the spores are difficult to stain and the size is very small (1 to 2.5 μm). Prepare control slides for microsporidia as follows:

1. Using a 10-μl aliquot of *concentrated* (formalin-ethyl acetate sedimentation concentration; centrifugation at 500 × *g* for 10 min), preserved liquid stool (5 or 10% formalin or SAF), prepare the smear by spreading the material over an area of 45 by 25 mm.
2. Allow the smear to air dry.

C. Prepare control slides for amebae as follows.

1. Make a suspension of *A. castellanii* from stock culture with sterile water or Page ameba saline.
2. Make a suspension of *E. coli* from stock culture with sterile water or Page ameba saline.
3. Add 1 drop of the *A. castellanii* suspension to one of the rings of a two-ring slide and 1 drop of the *E. coli* suspension to the other ring.
4. Allow the smear to air dry.
5. Fix the smear in absolute methanol for 3 to 5 min.
6. Store at room temperature. The smears are stable for 1 year.

D. Positive control

1. *Acanthamoeba* cysts are doubled walled (10 to 25 μm), and outer wall is wrinkled. The cysts will fluoresce.
2. The spores will be ovoid and refractile, and the spore wall will fluoresce. Occasionally, the polar tube can be seen either as a stripe or as a diagonal line across the spore; however, the internal spore contents will normally not be visible.

E. Negative control

1. *E. coli* will not fluoresce.
2. Most of the bacteria and other debris will not fluoresce. However, there will still be some yeast and debris that may also fluoresce.

F. Perform all scheduled maintenance on all equipment.

G. Record all QC results, including a description of QC specimens tested.

H. Known positive microscope slides, projection slides (2 by 2 in.), photographs, and reference books should be available at the workstation. Images are also available online on various Web sites.

V. PROCEDURE

A. Slide preparation of clinical specimens

1. Using a sterile swab, stick, or pipette, thinly spread the specimen evenly over the area circumscribed by the ring.

   ◩ **NOTE:** Do not apply excessive specimen on the slide, because the smear may be too thick to visualize any organisms present.
2. Allow the smear to air dry.
3. Fix the smear in absolute methanol for 3 to 5 min.
4. Air dry.

B. Stain procedure

1. Add 3 or 4 drops of CFW and 3 or 4 drops of Evan's blue into a tube (12 by 75 mm) and mix well.
2. Add several drops of this mixture to the specimen and allow to stand for 5 min.
3. Turn slide on its side and allow excess stain to run off.
4. Add coverslip, blot excess stain from slide, and examine immediately.

VI. RESULTS

A. Microsporidial spores will fluoresce. The outer wall (1 to 3 μm) will fluoresce, with the interior being clear, or perhaps the horizontal or diagonal stripe will fluoresce.

B. *Acanthamoeba* cysts will fluoresce. Cysts are double walled (10 to 25 μm), and the outer cyst wall is wrinkled (hexacanth cyst). Although more rare than *Acanthamoeba*, the cysts of *Balamuthia mandrillaris* are usually spherical, appear to have two walls (outer irregular wall and inner round wall), and measure 6 to 30 μm in diameter. *Naegleria* cysts can be confirmed from culture plates but are not seen in clinical specimens. They tend to measure from 7 to 15 μm and have a thick double wall.

C. Yeast cells, pseudohyphae, hyphae, and other fungal elements will stain with CFW (2).

D. *Pneumocystis jirovecii* can be detected with CFW (3).

E. Bacteria will not fluoresce.

F. Epithelial cells and blood cells will stain red by Evan's blue counterstain.

G. Cotton fibers will fluoresce strongly and can be distinguished as artifacts.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Positive

Report as microsporidial spores or *Acanthamoeba* cysts seen. Notify physician immediately.

B. Negative

No organisms seen.

VIII. PROCEDURE NOTES

A. Collagen, elastin, and keratin also fluoresce.

B. Other microorganisms (e.g., yeast cells, fungi) will fluoresce.

C. Although bacteria will not fluoresce, microsporidial spores (approximately the same size as some small yeasts and bacteria [1 to 2 μm]) will fluoresce. These organisms have been implicated as a cause of eye disease and have been found in other body tissues. The use of modified trichrome stains would be helpful in differentiating microsporidial spores from other organisms.

D. One drop of 10% KOH can be added to the CFW reagent, and a wet mount can be made of the specimens which require clearing or teasing (e.g., skin scrapings, hair, or viscous specimens).

E. Various concentrations of CFW and Evan's blue are commercially available. Some work better than others.

F. Confirm findings (positive or negative) with culture.

G. Depending on what filter combination is used, the cysts will fluoresce either blue-white or apple green.

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
2. **Green, L. K., and D. G. Moore.** 1987. Fluorescent compounds that nonspecifically stain fungi. *Lab. Med.* **18:**456–458.
3. **Polysciences, Inc.** March 2003. Data sheet 316.
4. **Wilhelmus, K. R., M. S. Osato, R. L. Font, N. M. Robinson, and D. B. Jones.** 1986. Rapid diagnosis of *Acanthamoeba* keratitis using calcofluor white. *Arch. Ophthalmol.* **104:**1309–1312.

SUPPLEMENTAL READING

Baselski, V. S., and M. K. Robinson. 1989. A staining kit for detection of opportunistic pathogens in bronchoalveolar lavage specimens. *Am. Clin. Lab.* **8:**36–37.

Polysciences, Inc. 2003. Material safety data sheet for Fungi-Fluor kit, catalog no. 17442. Polysciences, Inc., Warrington, PA.

Ryan, N. J., G. Sutherland, K. Coughlan, M. Globan, J. Doultree, J. Marshall, R. W. Baird, J. Pedersen, and B. Dwyer. 1993. A new trichrome-blue stain for detection of microsporidial species in urine, stool, and nasopharyngeal specimens, *J. Clin. Microbiol.* **31:**3264–3269.

Weber, R., R. T. Bryan, R. L. Owen, C. M. Wilcox, L. Gorelkin, and G. S. Visvesvara. 1992. Improved light-microscopical detection of microsporidia spores in stool and duodenal aspirates. *N. Engl. J. Med.* **326:**161–166.

APPENDIX 9.3.8–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. Commercially available solution of CFW with an Evan's blue counterstain (such as Fungi-Fluor kit, catalog no. 17442; Polysciences, Inc., Warrington, PA) or solution prepared as follows:

1. 0.1% CFW

CFW M2R, purified	0.1 g
distilled water	99.9 ml

Mix. Filter, and store in dark container. The mixture is stable at room temperature for 1 year.

2. 0.5% Evan's blue

Evan's blue	0.5 g
distilled water	99.5 ml

Mix. The mixture is stable at room temperature for 1 year.

B. Page's ameba saline (1×)

NaCl	6 mg
$MgSO_4 \cdot 7H_2O$	0.2 mg
$CaCl_2 \cdot 2H_2O$	0.2 mg
Na_2HPO_4	7.1 mg
KH_2PO_4	6.8 mg
distilled water	500 ml

Autoclave at 121°C for 15 min. Store refrigerated in a glass bottle. The mixture is stable for 3 months.

C. Absolute methanol

D. Sterile distilled water

9.4.1 Special Stains for Coccidia: Modified Kinyoun's Acid-Fast Stain (Cold)

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Cryptosporidium and *Cystoisospora* (*Isospora*) species have been recognized as causes of severe diarrhea in immunocompromised hosts, but they can also cause diarrhea in immunocompetent hosts. Oocysts in clinical specimens may be difficult to detect without special staining. *Cyclospora cayetanensis* has also been reported to be acid fast. Modified acid-fast stains are recommended for demonstrating these organisms. Unlike the Ziehl-Neelsen modified acid-fast stain, the modified Kinyoun acid-fast stain does not require heating the reagents used for staining and uses a mild decolorizer (1–3).

II. SPECIMEN

Concentrated sediment of fresh or formalin-preserved stool may be used. Other types of clinical specimens such as duodenal fluid, bile, or pulmonary (induced sputum, bronchial washings, biopsy specimens) may also be stained after centrifugation.

III. MATERIALS

Observe standard precautions.

A. Reagents (*see* Appendix 9.4.1–1)
1. Absolute methanol
2. 50% Ethanol
3. Kinyoun carbol fuchsin
4. 1% Sulfuric acid
5. Methylene blue

B. Supplies
1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)

C. Equipment
1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Tabletop centrifuge
4. Staining rack

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. A control slide of *Cryptosporidium* spp. from a 10% formalin-preserved specimen is included with each staining batch run. If the cryptosporidia stain well, any *Cystoisospora* (*Isospora*) oocysts present will also take up the stain, as will *C. cayetanensis.*

IV. QUALITY CONTROL *(continued)*

B. *Cryptosporidium* stains pink-red. Oocysts are 4 to 6 μm in diameter, and four sporozoites may be present internally. The background should stain uniformly blue.
C. Check the specimen (macroscopically) for adherence to the slide.
D. Record all QC results.
E. Known positive microscope slides, projection slides (2 by 2 in.), photographs, and reference books should be available at the workstation. Images are also available online on various Web sites.

V. PROCEDURE

A. Smear 1 or 2 drops of specimen on the slide, and allow it to air dry. Do not make the smears too thick (you should be able to see through the wet material before it dries). Prepare two smears.
B. Fix with absolute methanol for 1 min. Allow to air dry.
C. Flood slide with Kinyoun's carbol fuchsin, and stain for 5 min.
D. Rinse slide briefly (3 to 5 s) with 50% ethanol.
E. Rinse thoroughly with water.
F. Decolorize with 1% sulfuric acid for 2 min or until no more color runs from the slide.
G. Rinse slide with water. Drain.
H. Counterstain with methylene blue for 1 min.
I. Rinse slide with water. Air dry.
J. Examine using low-power or high dry power objectives. To see internal morphology, use oil immersion objective (100×).

VI. RESULTS

A. With this cold Kinyoun acid-fast method, *Cyclospora* and the oocysts of *Cryptosporidium* and *Cystoisospora* (*Isospora*) will stain pink to red to deep purple. Some of the four sporozoites may be visible in the *Cryptosporidium* oocysts. Some of the *Cystoisospora* (*Isospora*) immature oocysts (entire oocyst) will stain, while in oocysts that are mature, the two sporocysts within the oocyst wall will usually stain pink to purple and there will be a clear area between the stained sporocysts and the oocyst wall. The background will stain blue. If *Cyclospora* oocysts are present (uncommon), they tend to be approximately 8 to 10 μm, they resemble *C. parvum* but are larger, and they have no definite internal morphology; the acid-fast staining will tend to be more variable than that seen with *Cryptosporidium* or *Cystoisospora* (*Isospora*). Modified acid-fast stains stain the *Cyclospora* oocysts from light pink to deep red, and some of the oocysts will contain granules or have a bubbly appearance, often being described as looking like "wrinkled cellophane." Even with the 1% acid decolorizer, some oocysts of *Cyclospora* may appear clear or very pale. If the patient has a heavy infection with microsporidia (immunocompromised patient), small (1- to 2-μm) spores may be seen but may not be recognized as anything other than bacteria or small yeast cells.
B. There is usually a range of color intensity in the organisms present.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism and stage. Do not use abbreviations.
Examples: *Cryptosporidium* spp. oocysts or *Cystoisospora* (*Isospora*) *belli* oocysts or *Cyclospora cayetanensis* oocysts
B. Call the physician when these organisms are identified.

VIII. PROCEDURE NOTES

A. Routine stool examination stains are not recommended; however, the sedimentation concentration is acceptable (500 × *g* for 10 min) for the recovery and identification of *Cryptosporidium* and *Cyclospora.* Routine concentration (formalin-ethyl acetate) can be used to recover *Cystoisospora* (*Isospora*) oocysts, but routine permanent stains are not reliable for this purpose.

B. Polyvinyl alcohol-preserved specimens are not acceptable for staining with the modified acid-fast stain. However, specimens preserved in SAF are perfectly acceptable.

C. Avoid the use of wet gauze filtration (an old, standardized method of filtering stool prior to centrifugation) with too many layers of gauze that may trap organisms and prevent them from flowing into the fluid to be concentrated. It is recommended that no more than two layers of gauze be used; another option is to use the commercially available concentrators that use no gauze but instead use plastic or metal screens.

D. Other organisms, such as acid-fast bacteria and some *Nocardia* spp., stain positive.

E. It is very important that smears not be too thick. Thick smears may not adequately destain.

F. Concentration of the specimen is essential for demonstrating organisms (500 × *g* for 10 min). The number of organisms seen in the specimens may vary from numerous to very few.

G. Because of their mucoid consistency, some specimens require treatment with 10% KOH. Add 10 drops of 10% KOH to the sediment, and vortex until homogeneous. Rinse with 10% formalin, and centrifuge (500 × *g* for 10 min). Without decanting the supernatant, take 1 drop of the sediment and smear it thinly on a slide.

H. Commercial concentrators and reagents are available (*see* Appendix 9.10.6–1 at the end of this section).

I. Weak concentrations of sulfuric acid (1.0 to 3.0%) are normally used. Stronger concentrations will remove too much stain.

J. There is some debate about whether organisms lose their abilities to take up the acid-fast stain after long-term storage in 10% formalin. Use of the hot modified acid-fast method might eliminate this problem (1).

K. Centrifuge specimens in capped tubes, and *wear gloves during all phases of specimen processing.*

L. Currently, no commercial immunoassays are available for *Cyclospora.* However, several reagents are in the research phase.

IX. LIMITATIONS OF THE PROCEDURE

A. Light infections (low number of oocysts) may be missed. Immunoassay methods for *Cryptosporidium* are more sensitive.

B. Multiple specimens must be examined, since the numbers of oocysts in the stool will vary from day to day. A series of three specimens submitted on alternate days is recommended.

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology,* 5th ed. ASM Press, Washington, DC.
2. **Ma, P., and R. Soave.** 1983. Three step stool examination for cryptosporidiosis in 10 homosexual men with protracted diarrhea. *J. Infect. Dis.* **147:**824–828.
3. **Miller, J. M.** 1991. Quality control of media, reagents, and stains, p. 1203–1225. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), *Manual of Clinical Microbiology,* 5th ed. American Society for Microbiology, Washington, DC.

APPENDIX 9.4.1–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

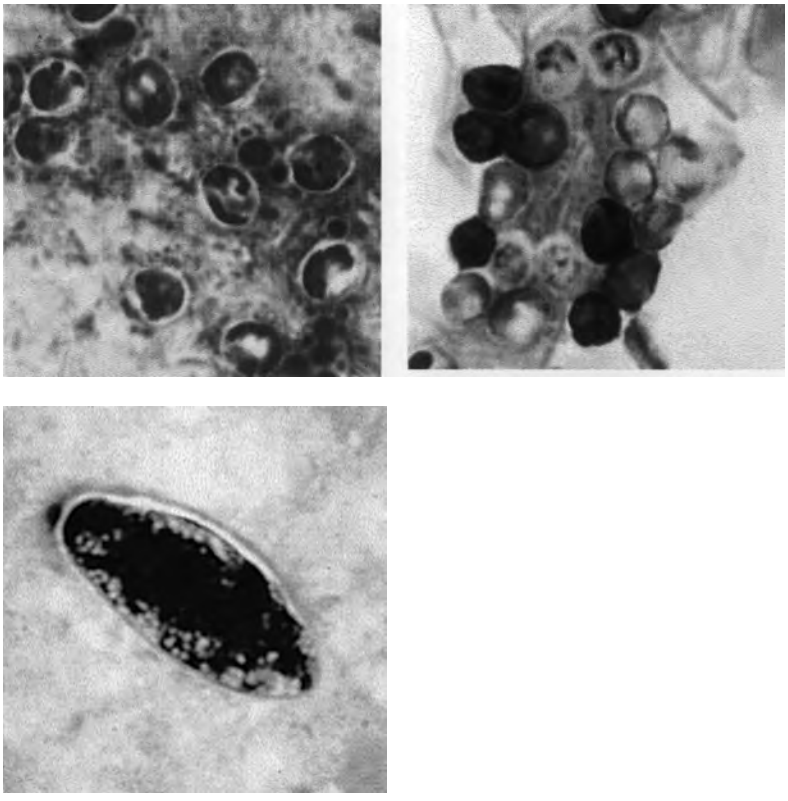
A. 50% Ethanol
1. Add 50 ml of absolute ethanol to 50 ml of distilled water.
2. Store at room temperature. Stable for 1 year.

B. Kinyoun carbol fuchsin
1. Dissolve 4 g of basic fuchsin in 20 ml of 95% ethanol (solution A).
2. Dissolve 8 g of phenol crystals in 100 ml of distilled water (solution B).
3. Mix solutions A and B together.
4. Store at room temperature. Stable for 1 year.

C. 1% Sulfuric acid
1. Add 1 ml of concentrated sulfuric acid to 99 ml of distilled water.
2. Store at room temperature. Stable for 1 year.

D. Methylene blue
1. Dissolve 0.3 g of methylene blue in 100 ml of 95% ethanol.
2. Store at room temperature. Stable for 1 year.

APPENDIX 9.4.1–2



(Top) *Cryptosporidium* oocysts; sporozoites are visible within some oocysts. (Bottom) *Cystoisospora* (*Isospora*) immature oocyst; note that the entire oocyst stains with modified acid-fast stain.

9.4.2 Special Stains for Coccidia: Modified Ziehl-Neelsen Acid-Fast Stain (Hot)

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Cryptosporidium and *Cystoisospora* (*Isospora*) species have been recognized as causes of severe diarrhea in immunocompromised hosts, but they can also cause diarrhea in immunocompetent hosts. Oocysts in clinical specimens may be difficult to detect without special staining. *Cyclospora cayetanensis* has also been reported to be acid fast. Modified acid-fast stains are recommended for demonstrating these organisms. Application of heat to carbol fuchsin assists in the staining, and the use of a mild decolorizer allows the organisms to retain their pink-red color (1–5).

II. SPECIMENS

Observe standard precautions.

Concentrated sediment of fresh or formalin-preserved stool may be used. Other types of clinical specimens such as duodenal fluid, bile, or pulmonary (induced sputum, bronchial washings, biopsy specimens) may also be stained, after centrifugation.

III. MATERIALS

A. Reagents (*see* Appendix 9.4.2–1)
- **1.** Carbol fuchsin
- **2.** 5% Sulfuric acid
- **3.** Methylene blue

B. Supplies
- **1.** Disposable glass or plastic pipettes
- **2.** Glass slides (1 by 3 in., or larger if you prefer)
- **3.** Coverslips (22 by 22 mm; no. 1, or larger if you prefer)

C. Equipment
- **1.** Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
- **2.** Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
- **3.** Tabletop centrifuge
- **4.** Staining rack
- **5.** 70°C heating block
- **6.** Alcohol lamp or Bunsen burner

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. A control slide of *Cryptosporidium* spp. from a 10% formalin-preserved specimen is included with each staining batch run. If the cryptosporidia stain well, any *Cystoisospora* (*Isospora*) oocysts present will also take up the stain, as will *C. cayetanensis.*

B. *Cryptosporidium* stains pink-red. Oocysts are 4 to 6 μm in diameter, and four sporozoites may be present internally. The background should stain uniformly blue.

C. Check the specimen (macroscopically) for adherence to the slide.

IV. QUALITY CONTROL *(continued)*

D. Record all QC results, including a description of QC specimens tested.

E. Known positive microscope slides, projection slides (2 by 2 in.), photographs, and reference books should be available at the workstation. Images are also available online on various Web sites.

V. PROCEDURE

A. Smear 1 or 2 drops of specimen on the slide, and allow it to air dry. Do not make the smears too thick (you should be able to see through the wet material before it dries). Prepare two smears.

B. Dry on a heating block (70°C) for 5 min, or air dry.

C. Place slide on staining rack, and flood with carbol fuchsin.

D. With alcohol lamp or Bunsen burner, gently heat slide to steaming by passing flame under slide. Discontinue heating once the stain begins to steam. Do not boil.

E. Allow to stain 5 min. If slide dries, add more stain without additional heating.

F. Rinse thoroughly with water. Drain.

G. Decolorize with 5% sulfuric acid for 30 s. (Thicker smears may require longer to destain.)

H. Rinse with water. Drain.

I. Flood slide with methylene blue for 1 min.

J. Rinse with water, drain, and air dry.

K. Examine with low-power or high dry power objectives. To see internal morphology, use oil immersion objective (100×).

VI. RESULTS

A. With this modified acid-fast method, the oocysts of *Cyclospora, Cryptosporidium,* and *Cystoisospora* (*Isospora*) will stain pink to red to deep purple. Some of the four sporozoites may be visible in the *Cryptosporidium* oocysts. Some of the *Cystoisospora* (*Isospora*) immature oocysts (entire oocyst) will stain, while in oocysts that are mature, the two sporocysts within the oocyst wall will stain pink to purple and there will be a clear area between the stained sporocysts and the oocyst wall. The background will stain blue. If *Cyclospora* oocysts are present (uncommon), they tend to be approximately 8 to 10 μm, they resemble *Cryptosporidium parvum* but are larger, and they have no definite internal morphology; the acid-fast staining will tend to be more variable than that seen with *Cryptosporidium* or *Cystoisospora* (*Isospora*). Modified acid-fast stains stain the *Cyclospora* oocysts from light pink to deep red, and some of the oocysts will contain granules or have a bubbly appearance, often being described as looking like "wrinkled cellophane." With the 5% acid decolorizer, some oocysts of *Cyclospora* may appear clear or very pale. If the patient has a heavy infection with microsporidia (immunocompromised patient), small (1- to 2-μm) spores may be seen but may not be recognized as anything other than bacteria or small yeast cells.

B. There is usually a range of color intensity in the organisms present.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism and stage. Do not use abbreviations.
Examples: *Cryptosporidium* sp. oocysts or *Cystoisospora* (*Isospora*) *belli* oocysts or *Cyclospora cayetanensis* oocysts

B. Call the physician when these organisms are identified.

VIII. PROCEDURE NOTES

A. Routine stool examination stains are not recommended for the recovery and identification of *Cryptosporidium* and *Cyclospora*; however, the sedimentation concentration is acceptable (500 × *g* for 10 min). Routine concentration (formalin-ethyl acetate) can be used to recover *Cystoisospora* (*Isospora*) oocysts, but routine permanent stains are not reliable for this purpose.

B. Polyvinyl alcohol-preserved specimens are not acceptable for staining with the modified acid-fast stain. However, specimens preserved in sodium acetate-acetic acid-formalin are perfectly acceptable.

C. Avoid the use of wet gauze filtration (an old, standardized method of filtering stool prior to centrifugation) with too many layers of gauze that may trap organisms and prevent them from flowing into the fluid to be concentrated. It is recommended that no more than two layers of gauze be used; another option is to use the commercially available concentrators that use no gauze but instead use plastic or metal screens.

D. Other organisms, such as acid-fast bacteria and some *Nocardia* spp., stain positive.

E. It is very important that smears not be too thick. Thick smears may not adequately destain.

F. Concentration of specimen is essential for demonstrating organisms. The number of organisms seen in the specimen may vary from numerous to very few.

G. Because of their mucoid consistency, some specimens require treatment with 10% KOH. Add 10 drops of 10% KOH to the sediment, and vortex until homogeneous. Rinse with 10% formalin, and centrifuge (500 × *g* for 10 min). Without decanting the supernatant, take 1 drop of the sediment and smear it thinly on a slide.

H. Commercial concentrators and reagents are available (*see* Appendix 9.10.6–1 at the end of this section).

I. Do not boil stain. Gently heat until steam rises from the slide. Do not allow the stain to dry on the slide.

J. Various concentrations of sulfuric acid (0.25 to 10%) may be used, but destaining time will vary according to the concentration used. Generally, a 1 or 5% solution is used.

K. There is some debate about whether organisms lose their abilities to take up the acid-fast stain after long-term storage in 10% formalin. Use of this hot modified acid-fast method might eliminate the problem (1).

L. Centrifuge specimens in capped tubes, and *wear gloves during all phases of specimen processing.*

M. Currently, no commercial immunoassays are available for *Cyclospora*. However, several reagents are in the research phase.

IX. LIMITATIONS OF THE PROCEDURE

A. Light infections (low number of oocysts) may be missed. Immunoassay methods for *Cryptosporidium* are more sensitive.

B. Multiple specimens must be examined, since the numbers of oocysts in the stool will vary from day to day. A series of three specimens submitted on alternate days is recommended.

REFERENCES

1. **Current, W. L., and L. S. Garcia.** 1991. Cryptosporidiosis. *Clin. Microbiol. Rev.* **3:**325–324.
2. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
3. **Garcia, L. S., D. A. Bruckner, T. C. Brewer, and R. Y. Shimizu.** 1983. Techniques for the recovery and identification of *Cryptosporidium* oocysts from stool specimens. *J. Clin. Microbiol.* **18:**185–190.
4. **Henriksen, S. A., and J. F. L. Pohlenz.** 1981. Staining cryptosporidia by a modified Ziehl-Neelsen technique. *Acta Vet. Scand.* **22:** 594–596.
5. **Miller, J. M.** 1991. Quality control of media, reagents, and stains, p. 1203–1225. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), *Manual of Clinical Microbiology*, 5th ed. American Society for Microbiology, Washington, DC.

APPENDIX 9.4.2–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. Carbol fuchsin

1. Basic fuchsin (solution A)
   Dissolve 0.3 g of basic fuchsin in 10 ml of 95% ethanol.
2. Phenol (solution B)
   Dissolve 5 g of phenol crystals in 100 ml of distilled water. (Gentle heat may be needed.)
3. Mix solution A with solution B.
4. Store at room temperature. Stable for 1 year.

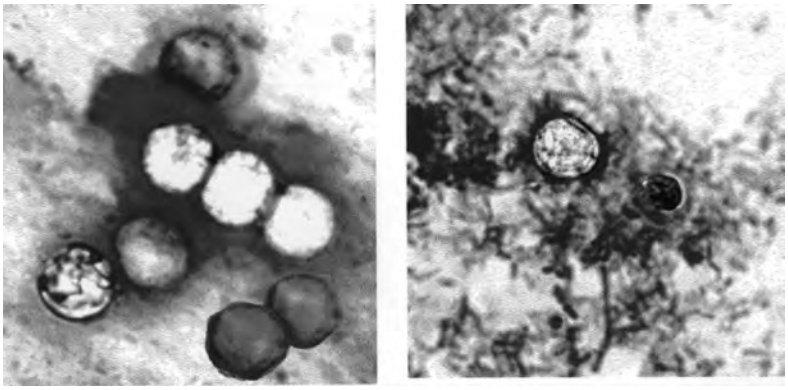
B. 5% Sulfuric acid

1. Add 5 ml of concentrated sulfuric acid to 95 ml of distilled water.
2. Store at room temperature. Stable for 1 year.

C. Methylene blue

1. Dissolve 0.3 g of methylene blue chloride in 100 ml of distilled water.
2. Store at room temperature. Stable for 1 year.

APPENDIX 9.4.2–2



(Left) *Cyclospora* oocysts (note variation in staining). (Right) The large, clear object is a *Cyclospora* oocyst (8 to 10 μm); the darker object to the right is a *Cryptosporidium* oocyst (4 to 6 μm). Note that *Cyclospora* tends to look like wrinkled cellophane when it does not stain well.

9.4.3 Special Stains for Microsporidia: Modified Trichrome-Weber Green

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The diagnosis of intestinal microsporidiosis *(Enterocytozoon bieneusi* and *Encephalitozoon intestinalis)* has depended on the use of invasive procedures and subsequent examination of biopsy specimens, often by electron microscopy methods. However, the need for a practical method for the routine clinical laboratory has stimulated some work in the development of additional methods. Slides prepared from fresh or formalin-fixed stool specimens can be stained by a new chromotrope-based technique and can be examined with light microscopy. This staining method is based on the fact that stain penetration of the microsporidial spore is very difficult; thus, the dye content in the chromotrope 2R is higher than that routinely used to prepare Wheatley's modification of Gomori's trichrome method, and the staining time is much longer (90 min) (1–3). At least several of these stains are available commercially from a number of suppliers.

II. SPECIMEN

Observe standard precautions.

The specimen can be fresh stool or stool that has been preserved in 5 or 10% formalin, sodium acetate-acetic acid-formalin (SAF), or some of the newer single-vial system fixatives. Actually, urine or any specimen other than tissue thought to contain microsporidia could be stained by these methods. Polyvinyl alcohol-preserved fecal material is not recommended.

III. MATERIALS

A. Reagents (*see* Appendix 9.4.3–1)

B. Supplies

1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. Glass or plastic centrifuge tubes (15 ml)
5. Coplin jars or other suitable staining containers
6. Chromotrope 2R
7. Aniline blue
8. Phosphotungstic acid
9. Acetic acid (glacial)
10. Distilled water

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Tabletop centrifuge

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Unfortunately, the only way to perform acceptable QC procedures for this method is to use actual microsporidial spores as the control organisms. Obtaining these positive controls may be somewhat difficult. It is particularly important to use the actual organisms because the spores are difficult to stain and the size is very small (1 to 2.5 μm).

B. A QC slide should be included with each run of stained slides, particularly if the staining setup is used infrequently.

C. All staining dishes should be covered to prevent evaporation of reagents (screw-cap Coplin jars or glass lids).

D. Depending on the volume of slides stained, staining solutions will have to be changed on an as-needed basis.

E. When the smear is thoroughly fixed and the stain is performed correctly, the spores will be ovoid and refractile, with the spore wall being bright pinkish red. Occasionally, the polar tube can be seen either as a stripe or as a diagonal line across the spore. The majority of the bacteria and other debris will tend to stain green. However, there will still be some bacteria and debris that will stain red.

F. The specimen is also checked for adherence to the slide (macroscopically).

G. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted on the body of the microscope). Although recalibration every 12 months may not be necessary, this will vary from laboratory to laboratory, depending on equipment care and use. Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

H. Known positive microscope slides, Kodachrome 2- by 2-in. projection slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

I. Record all QC results; the laboratory should also have an action plan for "out-of-control" results.

V. PROCEDURE

A. Using a 10-μl aliquot of *concentrated* (formalin-ethyl acetate sedimentation concentration; centrifugation at 500 × *g* for 10 min), preserved liquid stool (5 or 10% formalin or SAF), prepare the smear by spreading the material thinly over an area of 45 by 25 mm.

B. Allow the smear to air dry.

C. Place the smear in absolute methanol for 5 min.

D. Allow the smear to air dry.

E. Place in modified trichrome stain for 90 min.

F. Rinse in acid-alcohol for no more than 10 s.

G. Dip slides several times in 95% alcohol. Use this step as a rinse.

H. Place in 95% alcohol for 5 min.

I. Place in 100% alcohol for 10 min.

J. Place in xylene substitute for 10 min.

K. Mount with coverslip (no. 1 thickness), using mounting medium (this step is optional).

L. Examine smears under oil immersion (1,000×) and read at least 100 fields; the examination time will probably be at least 10 min per slide.

VI. RESULTS

A. The microsporidial spore wall should stain pinkish to red (1 to 2 μm), with the interior of the spore being clear or perhaps showing a horizontal or diagonal stripe that represents the polar tube. The background will appear green (Weber stain).

B. Other bacteria, some yeast cells, and some debris will stain pink to red; the shapes and sizes of the various components may be helpful in differentiating the spores from other structures.

C. The results from this staining procedure should be reported only if the positive control smears are acceptable.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

Report the organism and stage. Do not use abbreviations.

Examples (stool specimens): Microsporidial spores seen.
Enterocytozoon bieneusi or *Encephalitozoon intestinalis* present (if from fecal specimen); the two organisms cannot be differentiated on the basis of size or morphology.

Example (from urine): *Encephalitozoon intestinalis* seen (identification to the species level highly likely); generally this organism is involved in disseminated cases from the gastrointestinal tract to kidneys and will be found in urine.

VIII. PROCEDURE NOTES

A. It is mandatory that positive control smears be stained and examined each time patient specimens are stained and examined.

B. Because of the difficulty in getting stain penetration through the spore wall, prepare thin smears and do not reduce the staining time in trichrome. Also, make sure that the slides are not left too long in the decolorizing agent (acid-alcohol). If the control organisms are too light, leave them in the trichrome longer and shorten the time to two dips in the acid-alcohol solution. Also, remember that the 95% alcohol rinse after the acid-alcohol should be performed quickly to prevent additional destaining from the acid alcohol reagent.

C. When you purchase the chromotrope 2R, obtain the highest dye content available. Two sources are Harleco (Gibbstown, NJ) and Sigma Chemical Co. (St. Louis, MO) (dye content among the highest [85%]).

D. In the final stages of dehydration, the 100% ethanol and the xylenes (or xylene substitutes) should be kept as free from water as possible. Coplin jars must have tight-fitting caps to prevent both evaporation of reagents and absorption of moisture. If the xylene becomes cloudy after addition of slides from 100% alcohol, return the slides to 100% alcohol and replace the xylene with fresh stock.

IX. LIMITATIONS OF THE PROCEDURE

A. Although this staining method will stain the microsporidia, the range of stain intensity and the small size of the spores will cause some difficulty in identifying these organisms. Since this procedure will result in many other organisms or objects staining in stool specimens, differentiation of the microsporidia from surrounding material will still be very difficult. There also tends to be some slight size variation among the spores. The production of immunoassay reagents should provide a more specific and sensitive approach to the identification of microsporidia in patient samples.

IX. LIMITATIONS OF THE PROCEDURE *(continued)*

B. If the patient has severe watery diarrhea, there will be less artifact material in the stool to confuse with the microsporidial spores. However, if the stool is semiformed or formed, the amount of artifact material will be much greater; thus, the spores will be much harder to detect and identify. Also, remember that the number of spores will vary according to the stool consistency (the more diarrhetic, the more spores that will be present).

C. Those who developed some of these procedures feel that concentration procedures result in an actual loss of microsporidial spores; thus, there is a strong recommendation to use unconcentrated, formalinized stool. However, there are no data indicating what centrifugation speeds, etc., were used in the study.

D. *In the UCLA Clinical Microbiology Laboratory, data (unpublished) have been generated to indicate that centrifugation at 500 × g for 10 min increases dramatically the number of microsporidial spores available for staining (from the concentrate sediment). This is the same protocol used in that laboratory for centrifugation of all stool specimens, regardless of the suspected organism.*

E. Avoid the use of wet gauze filtration (an old, standardized method of filtering stool prior to centrifugation) with too many layers of gauze that may trap organisms and prevent them from flowing into the fluid to be concentrated. It is recommended that no more than two layers of gauze be used. Another option is to use the commercially available concentration systems that use metal or plastic screens for filtration.

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology,* 5th ed. ASM Press, Washington, DC.
2. **NCCLS.** 2005. *Procedures for the Recovery and Identification of Parasites from the Intestinal Tract.* Approved guideline M28-A2. NCCLS, Wayne, PA.
3. **Weber, R., R. T. Bryan, R. L. Owen, C. M. Wilcox, L. Gorelkin, G. S. Visvesvara, and The Enteric Opportunistic Infections Working Group.** 1992. Improved light-microscopical detection of microsporidia spores in stool and duodenal aspirates. *N. Engl. J. Med.* **326:**161–166.

APPENDIX 9.4.3–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. Trichrome stain (modified for microsporidia) (Weber green)

chromotrope 2R	6.0 g*
fast green	0.15 g
phosphotungstic acid	0.7 g
acetic acid (glacial)	3.0 ml
distilled water	100.0 ml

*10 times the normal trichrome stain formula

1. Prepare the stain by adding 3.0 ml of acetic acid to the dry ingredients. Allow the mixture to stand (ripen) for 30 min at room temperature.
2. Add 100 ml of distilled water. Properly prepared stain will be dark purple.
3. Store in a glass or plastic bottle at room temperature. The shelf life is at least 24 months.

B. Acid-alcohol

90% ethyl alcohol	995.5 ml
Acetic acid (glacial)	4.5 ml

Prepare by combining the two solutions.

9.4.4 Special Stains for Microsporidia: Modified Trichrome-Ryan Blue

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The diagnosis of intestinal microsporidiosis *(Enterocytozoon bieneusi* and *Encephalitozoon intestinalis)* has depended on the use of invasive procedures and subsequent examination of biopsy specimens, often by electron microscopy methods. However, the need for a practical method for the routine clinical laboratory has stimulated some work in the development of additional methods. Slides prepared from fresh or formalin-fixed stool specimens can be stained by a new chromotrope-based technique and can be examined with light microscopy. This staining method is based on the fact that stain penetration of the microsporidial spore is very difficult; thus, the dye content in the chromotrope 2R is higher than that routinely used to prepare Wheatley's modification of Gomori's trichrome method, and the staining time is much longer (90 min) (1–3).

A number of variations to the modified trichrome (Weber green) were tried in an attempt to improve the contrast between the color of the spores and the background staining. Optimal staining was achieved by modifying the composition of the trichrome solution. This stain is also available commercially from a number of suppliers.

II. SPECIMEN

Observe standard precautions.

The specimen can be fresh stool or stool that has been preserved in 5 or 10% formalin, sodium acetate-acetic acid-formalin (SAF), or some of the newer single-vial system fixatives. Actually, any specimen other than tissue thought to contain microsporidia could be stained by these methods. Polyvinyl alcohol-preserved fecal material is not recommended.

III. MATERIALS

A. Reagents (*see* Appendix 9.4.4–1)

B. Supplies

1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. Glass or plastic centrifuge tubes (15 ml)
5. Coplin jars or other suitable staining containers
6. Chromotrope 2R
7. Aniline blue
8. Phosphotungstic acid
9. Acetic acid (glacial)
10. Distilled water

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Tabletop centrifuge

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Unfortunately, the only way to perform acceptable QC procedures for this method is to use actual microsporidial spores as the control organisms. Obtaining these positive controls may be somewhat difficult. It is particularly important to use the actual organisms because the spores are difficult to stain and the size is very small (1 to 2.5 μm).

B. A QC slide should be included with each run of stained slides, particularly if the staining setup is used infrequently.

C. All staining dishes should be covered to prevent evaporation of reagents (screw-cap Coplin jars or glass lids).

D. Depending on the volume of slides stained, staining solutions will have to be changed on an as-needed basis.

E. When the smear is thoroughly fixed and the stain is performed correctly, the spores will be ovoid and refractile, with the spore wall being bright pinkish red. Occasionally, the polar tube can be seen either as a stripe or as a diagonal line across the spore. The majority of the bacteria and other debris will tend to stain blue. However, there will still be some bacteria and debris that will stain red.

F. The specimen is also checked for adherence to the slide (macroscopically).

G. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted on the body of the microscope). Although recalibration every 12 months may not be necessary, this will vary from laboratory to laboratory, depending on equipment care and use. Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

H. Known positive microscope slides, Kodachrome 2- by 2-in. projection slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

I. Record all QC results; the laboratory should also have an action plan for "out-of-control" results.

V. PROCEDURE

A. Using a 10-μl aliquot of *concentrated* (formalin-ethyl acetate sedimentation concentration; centrifugation at 500 × *g* for 10 min), preserved liquid stool (5 or 10% formalin or SAF), prepare the smear by spreading the material thinly over an area of 45 by 25 mm.

B. Allow the smear to air dry.

C. Place the smear in absolute methanol for 5 or 10 min.

D. Allow the smear to air dry.

E. Place in modified trichrome stain for 90 min.

F. Rinse in acid-alcohol for no more than 10 s.

G. Dip slides several times in 95% alcohol. Use this step as a rinse (no more than 10 s).

H. Place in 95% alcohol for 5 min.

I. Place in 95% alcohol for 5 min.

J. Place in 100% alcohol for 10 min.

K. Place in xylene substitute for 10 min.

L. Mount with coverslip (no. 1 thickness), using mounting medium (this step is optional).

V. PROCEDURE *(continued)*

M. Examine smears under oil immersion (1,000×) and read at least 100 fields; the examination time will probably be at least 10 min per slide.

VI. RESULTS

A. The microsporidial spore wall should stain pinkish to red (1 to 2.5 μm), with the interior of the spore being clear or perhaps showing a horizontal or diagonal stripe that represents the polar tube. The background will appear blue (Ryan stain).

B. Other bacteria, some yeast cells, and some debris will stain pink to red; the shapes and sizes of the various components may be helpful in differentiating the spores from other structures.

C. The results from this staining procedure should be reported only if the positive control smears are acceptable.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

Report the organism and stage. Do not use abbreviations.

Examples (stool specimens): Microsporidial spores seen.
Enterocytozoon bieneusi or *Encephalitozoon intestinalis* present (if from fecal specimen); the two organisms cannot be differentiated on the basis of size or morphology.

Example (from urine): *Encephalitozoon intestinalis* seen (identification to species highly likely); generally this organism is involved in disseminated cases from the gastrointestinal tract to kidneys and will be found in urine.

VIII. PROCEDURE NOTES

A. It is mandatory that positive control smears be stained and examined each time patient specimens are stained and examined.

B. Because of the difficulty in getting stain penetration through the spore wall, prepare thin smears and do not reduce the staining time in trichrome. Also, make sure that the slides are not left too long in the decolorizing agent (acid-alcohol). If the control organisms are too light, leave them in the trichrome longer and shorten the time to two dips in the acid-alcohol solution. Also, remember that the 95% alcohol rinse after the acid-alcohol should be performed quickly to prevent additional destaining from the acid-alcohol reagent.

C. When you purchase the chromotrope 2R, obtain the highest dye content available. Two sources are Harleco (Gibbstown, NJ) and Sigma Chemical Co. (St. Louis, MO) (dye content among the highest [85%]).

D. In the final stages of dehydration, the 100% ethanol and the xylenes (or xylene substitutes) should be kept as free from water as possible. Coplin jars must have tight-fitting caps to prevent both evaporation of reagents and absorption of moisture. If the xylene becomes cloudy after addition of slides from 100% alcohol, return the slides to 100% alcohol and replace the xylene with fresh stock.

IX. LIMITATIONS OF THE PROCEDURE

A. Although this staining method will stain the microsporidia, the range of stain intensity and the small size of the spores will cause some difficulty in identifying these organisms. Since this procedure will result in many other organisms or objects staining in stool specimens, differentiation of the microsporidia from surrounding material will still be very difficult. There also tends to be some slight size variation among the spores. The production of immunoassay reagents should provide a more specific and sensitive approach to the identification of microsporidia in patient samples.

B. If the patient has severe watery diarrhea, there will be less artifact material in the stool to confuse with the microsporidial spores. However, if the stool is semiformed or formed, the amount of artifact material will be much greater; thus, the spores will be much harder to detect and identify. Also, remember that the number of spores will vary according to the stool consistency (the more diarrhetic, the more spores that will be present).

C. Those who developed some of these procedures feel that concentration procedures result in an actual loss of microsporidial spores; thus, there is a strong recommendation to use unconcentrated, formalinized stool. However, there are no data indicating what centrifugation speeds, etc., were used in the study.

D. *In the UCLA Clinical Microbiology Laboratory, data (unpublished) have been generated to indicate that centrifugation at 500× g for 10 min increases dramatically the number of microsporidial spores available for staining (from the concentrate sediment). This is the same protocol used in that laboratory for centrifugation of all stool specimens, regardless of the suspected organism.*

E. Avoid the use of wet gauze filtration (an old, standardized method of filtering stool prior to centrifugation) with too many layers of gauze that may trap organisms and prevent them from flowing into the fluid to be concentrated. It is recommended that no more than two layers of gauze be used. Another option is to use the commercially available concentration systems that use metal or plastic screens for filtration.

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology,* 5th ed. ASM Press, Washington, DC.
2. **NCCLS.** 2005. *Procedures for the Recovery and Identification of Parasites from the Intestinal Tract.* Approved guideline M28-A2. NCCLS, Wayne, PA.
3. **Ryan, N. J., G. Sutherland, K. Coughlan, M. Globan, J. Doultree, J. Marshall, R. W. Baird, J. Pedersen, and B. Dwyer.** 1993. A new trichrome-blue stain for detection of microsporidial species in urine, stool, and nasopharyngeal specimens. *J. Clin. Microbiol.* **31:**3264–3269.

APPENDIX 9.4.4–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. Trichrome stain (modified for microsporidia) (Ryan blue)

chromotrope 2R ……6.0 g*
aniline blue ……0.5 g
phosphotungstic acid …… 0.25 g
acetic acid (glacial) ……3.0 ml
distilled water ……100.0 ml

*10 times the normal trichrome stain formula

1. Prepare the stain by adding 3.0 ml of acetic acid to the dry ingredients. Allow the mixture to stand (ripen) for 30 min at room temperature.

APPENDIX 9.4.4–1 *(continued)*

2. Add 100 ml of distilled water and adjust the pH to 2.5 with 1.0 M HCl. Properly prepared stain will be dark purple. The staining solution should be protected from light.
3. Store in a glass or plastic bottle at room temperature. The shelf life is at least 24 months.

B. Acid-alcohol

90% ethyl alcohol	995.5 ml
acetic acid (glacial)	4.5 ml

Prepare by combining the two solutions.

APPENDIX 9.4.4–2

Modified Trichrome Stain for the Microsporidia (1) (Kokoskin Hot Method)

Changes in temperature from room temperature to 50°C and the staining time from 90 to 10 min have been recommended as improvements for the modified trichrome staining methods. The procedure is as follows.

1. Using a 10-μl aliquot of unconcentrated, preserved liquid stool (5 or 10% formalin or SAF), prepare the smear by spreading the material over an area of 45 by 25 mm.
2. Allow the smear to air dry.
3. Place the smear in absolute methanol for 5 min.
4. Allow the smear to air dry.
5. Place in trichrome stain for 10 min at a temperature of 50°C.
6. Rinse in acid-alcohol for no more than 10 s.
7. Dip slides several times in 95% alcohol. Use this step as a rinse (no more than 10 s).
8. Place in 95% alcohol for 5 min.
9. Place in 100% alcohol for 10 min.
10. Place in xylene substitute for 10 min.
11. Mount with coverslip (no. 1 thickness), using mounting medium.
12. Examine smears under oil immersion (1,000×) and read at least 100 fields; the examination time will probably be at least 10 min per slide.

Reference

1. **Kokoskin, E., T. W. Gyorkos, A. Camus, L. Cedilotte, T. Purtill, and B. Ward.** 1994. Modified technique for efficient detection of microsporidia. *J. Clin. Microbiol.* **32:**1074–1075.

9.4.5

Special Stains for Microsporidia: Acid-Fast Modified Trichrome Stain for Coccidia and the Microsporidia

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The detection of coccidia and the microsporidia from stool specimens has depended on two separate stains. However, a method is now available that will stain both organisms, an important improvement since dual infections have been demonstrated in AIDS patients. This acid-fast modified trichrome stain yields results comparable to those obtained by the Kinyoun and modified trichrome methods and considerably reduces the time necessary for microscopic examination. Also, it appears that modified trichrome stains and staining with fluorochromes are equally useful in the diagnosis of microsporidiosis; however, a combination of both methods may be more sensitive in cases where the number of spores is very low (1, 2).

II. SPECIMEN

Observe standard precautions.

The specimen can be fresh stool or stool that has been preserved in 5 or 10% formalin, sodium acetate-acetic acid-formalin (SAF), or some of the newer single-vial system fixatives. Actually, urine or any specimen other than tissue thought to contain microsporidia could be stained by these methods. Polyvinyl alcohol-preserved fecal material is not recommended.

III. MATERIALS

A. Reagents (*see* Appendix 9.4.5–1)

B. Supplies

1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. Glass or plastic centrifuge tubes (15 ml)
5. Coplin jars or other suitable staining containers
6. Chromotrope 2R
7. Aniline blue
8. Phosphotungstic acid
9. Carbol fuchsin
10. Phenol
11. Acetic acid (glacial)
12. Distilled water

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult
3. Tabletop centrifuge

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Unfortunately, the only way to perform acceptable QC procedures for this method is to use actual microsporidial spores and coccidian oocysts as the control organisms. Obtaining these positive controls may be somewhat difficult. It is particularly important to use the actual organisms because the spores are difficult to stain and the size is very small (1 to 2.5 μm).

IV. QUALITY CONTROL *(continued)*

B. A QC slide should be included with each run of stained slides, particularly if the staining setup is used infrequently.

C. All staining dishes should be covered to prevent evaporation of reagents (screw-cap Coplin jars or glass lids).

D. Depending on the volume of slides stained, staining solutions will have to be changed on an as-needed basis.

E. When the smear is thoroughly fixed and the stain is performed correctly, the spores will be ovoid and refractile, with the spore wall being bright pinkish red. Occasionally, the polar tube can be seen either as a stripe or as a diagonal line across the spore. The majority of the bacteria and other debris will tend to stain blue. However, there will still be some bacteria and debris that will stain red. The coccidia *(Cryptosporidium*, *Cyclospora*, and *Cystoisospora* [*Isospora*]*)* will stain as with any modified acid-fast stain: from pink to violet.

F. The specimen is also checked for adherence to the slide (macroscopically).

G. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted on the body of the microscope). Although recalibration every 12 months may not be necessary, this will vary from laboratory to laboratory, depending on equipment care and use. Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

H. Known positive microscope slides, Kodachrome 2- by 2-in. projection slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

I. Record all QC results; the laboratory should also have an action plan for "out-of-control" results.

V. PROCEDURE

A. Using a 10-μl aliquot of *concentrated* (formalin-ethyl acetate sedimentation concentration; centrifugation at 500 × *g* for 10 min), preserved liquid stool (5 or 10% formalin or SAF), prepare the smear by spreading the material thinly over an area of 45 by 25 mm.

B. Allow the smear to air dry.

C. Place the smear in absolute methanol for 5 or 10 min.

D. Allow the smear to air dry.

E. Place in carbol fuchsin solution for 10 min (no heat required).

F. Briefly rinse with tap water.

G. Decolorize with 0.5% acid-alcohol.

H. Briefly rinse with tap water.

I. Place in modified trichrome stain for 30 min at 37°C.

J. Rinse in acid-alcohol for no more than 10 s.

K. Dip slides several times in 95% alcohol. Use this step as a rinse (no more than 10 s).

L. Place in 95% alcohol for 30 s.

M. Allow slides to air dry.

N. Examine smears under oil immersion (1,000×) and read at least 100 fields; the examination time will probably be at least 10 min per slide.

VI. RESULTS

A. The microsporidial spore wall should stain pink (1 to 2.5 μm), with the interior of the spore being clear or perhaps showing a horizontal or diagonal stripe that represents the polar tube. A vacuole may also be visible in some spores. The *Cryptosporidium* oocysts will stain bright pink or violet (4 to 6 μm). The background will appear blue.

B. Other bacteria, some yeast cells, and some debris will stain pink to red; the shapes and sizes of the various components may be helpful in differentiating the spores from other structures.

C. The results from this staining procedure should be reported only if the positive control smears are acceptable.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

Report the organism and stage. Do not use abbreviations.

Examples (stool specimens): 1. Microsporidial spores seen.
Enterocytozoon bieneusi or *Encephalitozoon intestinalis* present (if from fecal specimen); the two organisms cannot be differentiated on the basis of size or morphology.
2. *Cryptosporidium* species seen.

Example (from urine): *Encephalitozoon intestinalis* present (identification to species level highly likely); generally this organism is involved in disseminated cases from the gastrointestinal tract to kidneys and will be found in urine.

VIII. PROCEDURE NOTES

A. It is mandatory that positive control smears be stained and examined each time patient specimens are stained and examined.

B. Because of the difficulty in getting stain penetration through the spore wall, prepare thin smears and do not reduce the staining time in trichrome. Also, make sure that the slides are not left too long in the decolorizing agent (acid-alcohol). If the control organisms are too light, leave them in the trichrome longer and shorten the time to two dips in the acid-alcohol solution. Also, remember that the 95% alcohol rinse after the acid-alcohol should be performed quickly to prevent additional destaining from the acid-alcohol reagent.

C. When you purchase the chromotrope 2R, obtain the highest dye content available. Two sources are Harleco (Gibbstown, NJ) and Sigma Chemical Co. (St. Louis, MO) (dye content among the highest [85%]).

D. In the final stages of dehydration, the 100% ethanol and the xylenes (or xylene substitutes) should be kept as free from water as possible. Coplin jars must have tight-fitting caps to prevent both evaporation of reagents and absorption of moisture. If the xylene becomes cloudy after addition of slides from 100% alcohol, return the slides to 100% alcohol and replace the xylene with fresh stock.

IX. LIMITATIONS OF THE PROCEDURE

A. Although this staining method will stain the microsporidia, the range of stain intensity and the small size of the spores will cause some difficulty in identifying these organisms. Since this procedure will result in many other organisms or objects staining in stool specimens, differentiation of the microsporidia from surrounding material will still be very difficult. There also tends to be some slight size variation among the spores. The production of immunoassay reagents should provide a more specific and sensitive approach to the identification of microsporidia in patient samples.

IX. LIMITATIONS OF THE PROCEDURE *(continued)*

B. If the patient has severe watery diarrhea, there will be less artifact material in the stool to confuse with the microsporidial spores. However, if the stool is semiformed or formed, the amount of artifact material will be much greater; thus, the spores will be much harder to detect and identify. Also, remember that the number of spores will vary according to the stool consistency (the more diarrhetic, the more spores that will be present).

C. Those who developed some of these procedures feel that concentration procedures result in an actual loss of microsporidial spores; thus, there is a strong recommendation to use unconcentrated, formalinized stool. However, there are no data indicating what centrifugation speeds, etc., were used in the study.

D. *In the UCLA Clinical Microbiology Laboratory, data (unpublished) have been generated to indicate that centrifugation at 500 × g for 10 min increases dramatically the number of microsporidial spores available for staining (from the concentrate sediment). This is the same protocol used in that laboratory for centrifugation of all stool specimens, regardless of the suspected organism.*

E. Avoid the use of wet gauze filtration (an old, standardized method of filtering stool prior to centrifugation) with too many layers of gauze that may trap organisms and allow them to flow into the fluid to be concentrated. It is recommended that no more than two layers of gauze be used. Another option is to use the commercially available concentration systems that use metal or plastic screens for filtration.

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology,* 5th ed. ASM Press, Washington, DC.
2. **Ignatius, R., M. Lehmann, K. Miksits, T. Regnath, M. Arvand, E. Engelmann, U. Futh, H. Hahn, and J. Wagner.** 1997. A new acid-fast trichrome stain for simultaneous detection of *Cryptosporidium parvum* and microsporidial species in stool specimens. *J. Clin. Microbiol.* **35:** 446–449.

SUPPLEMENTAL READING

Moura, H., J. L. Nunes Da Silva, F. C. Sodre, P. Brasil, K. Wallmo, S. Wahlquist, S. Wallace, G. P. Croppo, and G. S. Visvesvara. 1996. Gram-chromatrope: a new technique that enhances detection of microsporidial spores in clinical samples. *J. Eukaryot. Microbiol.* **43:** 94S–95S.

APPENDIX 9.4.5–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. Trichrome stain (modified for microsporidia)

chromotrope 2R .....6.0 g*
aniline blue .....0.5 g
phosphotungstic acid .....0.7 g
acetic acid (glacial) .....3.0 ml
distilled water .....100.0 ml

*10 times the normal trichrome stain formula

1. Prepare the stain by adding 3.0 ml of acetic acid to the dry ingredients. Allow the mixture to stand (ripen) for 30 min at room temperature.
2. Add 100 ml of distilled water and adjust the pH to 2.5 with 1.0 M HCl. Properly prepared stain will be dark purple. The staining solution should be protected from light.
3. Store in a glass or plastic bottle at room temperature. The shelf life is at least 24 months.

B. Carbol fuchsin solution

Phenol solution
phenol ..... 25.0 g
distilled water .....500.0 ml

Saturated alcoholic fuchsin solution
basic fuchsin .....2.0 g
96% ethanol ..... 25.0 ml

Add the mixture of phenol and water to 25.0 ml of the saturated alcoholic fuchsin solution.

C. Acid-alcohol

90% ethyl alcohol .....995.5 ml
acetic acid (glacial) .....4.5 ml

Prepare by combining the two solutions.

9.4.6 Special Stain for *Cyclospora* Oocysts: Modified Safranin Technique with Microwave Heating

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Cyclospora is an emerging parasite causing intestinal disease both in immunocompetent persons with or without travel history and in immunocompromised patients (1). Oocysts stain modified acid-fast variable, and the variability may lead to misidentification of this parasite. An alternative to the modified acid-fast stain is the modified safranin stain with microwave heating, which may be easier to interpret and perform. This safranin-based stain uniformly stains the *Cyclospora* oocysts, unlike the variable staining with the modified acid-fast stain (2).

II. SPECIMEN

A. Concentrated sediment of fresh, 5 or 10% formalin or sodium acetate-acetic acid-formalin preserved stool may be used.
B. Polyvinyl alcohol-preserved fecal material is not recommended.
C. Specimens in potassium dichromate are not acceptable.

III. MATERIALS

A. Reagents (see Appendix 9.4.6–1)
1. 1% Safranin
2. 1% Methylene blue
3. Cytoseal 60

B. Supplies
1. Disposable glass or plastic pipettes
2. Glass microscope slides, 1 by 3 in.
3. Coverslips, 22 by 22 mm, no. 1
4. Staining jars

C. Equipment
1. Binocular microscope with 10×, 40×, and 100× objectives. Oculars should be 10×.
2. 60°C slide warmer
3. Microwave oven

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. The only way to perform acceptable QC procedures for this method is to use actual *Cyclospora* oocysts as the control organism. Obtaining these positive controls may be somewhat difficult.
B. A QC slide should be run with each batch of stained slides, particularly if the stain is used infrequently.
C. Depending on the volume of slides stained, staining solutions will have to be changed on an as-needed basis or every 10 uses.
D. When the smear is thoroughly fixed and the stain is performed correctly, the oocysts will appear uniformly reddish orange.
E. The specimen is also checked for adherence to the slide macroscopically.
F. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted on the body of the microscope). Although recalibration every 12 months may not be necessary, this will vary from laboratory to laboratory, depending on equipment care and use.

IV. QUALITY CONTROL *(continued)*

Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

G. Known positive microscope slides, Kodachrome 2- by 2-in. projection slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

H. Record all QC results; the laboratory should also have an action plan for "out-of-control" results.

V. PROCEDURE

A. Using a 10-μl aliquot of concentrated stool, prepare the smear by spreading the material thinly across the slide.

B. Allow the smear to dry on a 60°C slide warmer.

C. Cool slide to room temperature before staining.

D. Place the slide in a Coplin jar containing acidic alcohol (3% [vol/vol] HCl in methanol) and let stand for 5 min.

E. Wash off excess acidic alcohol with cold tap water.

F. Place the slide(s) into the Coplin jar containing the safranin solution in acidified water (pH 6.5) and microwave on full power (650 W) for 1 min. (Place staining jar in another container to catch overflow of stain because of boiling.)

G. Wash off excess stain with cold tap water.

H. Place the slide(s) in a Coplin jar containing 1% methylene blue for 1 min.

I. Rinse gently with cold tap water.

J. Air dry.

K. Coverslip the slide using Cytoseal 60.

L. Examine the smear with low-power or high dry power objectives. To see additional morphology, use the oil immersion objective (100×).

VI. RESULTS

Cyclospora oocysts will stain reddish-orange. The crinkled or wrinkled appearance of the oocyst wall will be evident. The oocysts will measure 8 to 10 μm.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism and the stage. Do not use abbreviations.
Example: *Cyclospora cayetanensis* oocysts

B. Notify the physician when this organism is identified.

VIII. PROCEDURE NOTES

A. It is mandatory that positive control smears be stained and examined each time patient specimens are stained and examined.

B. For best results, safranin stain should be changed after 10 microwave heatings.

C. The *Cyclospora* oocyst wall in the safranin-stained smears will exhibit weak to moderate autofluorescence.

D. The *Cyclospora* oocyst wall may be seen more clearly using Nomarski differential interference contrast microscopy.

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.

2. **Visvesvara, G. S., H. Moura, E. Kovacs-Nace, S. Wallace and M. L. Eberhard.** 1997. Uniform staining of *Cyclospora* oocysts in fecal smears by a modified safranin technique with microwave heating. *J. Clin. Microbiol.* **35:**730–733.

APPENDIX 9.4.6–1

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

1. Acid alcohol (3% HCl/methanol)

3 ml of hydrochloric acid
97 ml of absolute methanol

Slowly add 3 ml of hydrochloric acid to 97 ml of absolute methanol. Store at room temperature in an air-tight container.

2. Safranin (1%)

10 g of safranin
1,000 ml of distilled water

3. Methylene blue (1%)

1 g of methylene blue
100 ml of distilled water

9.5.1 "Culture" of Larval-Stage Nematodes: Baermann Technique

Strongyloides stercoralis larvae are usually the only larvae found in stool specimens. Depending on bowel transit time and the condition of the patient, rhabditiform and, rarely, filariform larvae may be present. If there is delay in examination of the stool, then embryonated ova and larvae of hookworm may be present. Culture of feces for larvae is useful to (i) reveal the presence of larvae when they are too scanty to be detected by concentration methods, (ii) distinguish whether the infection is due to *Strongyloides* or hookworm on the basis of rhabditiform larval morphology by allowing hookworm eggs to hatch and release first-stage larvae, and (iii) allow development of larvae into the filariform stage for further differentiation.

The use of certain fecal-culture methods (sometimes referred to as coproculture) is especially helpful in detecting light infections of hookworm, *Strongyloides* spp., and *Trichostrongylus* spp. and for specifically identifying parasites. Additionally, such techniques are useful for obtaining a large number of infective-stage larvae for research purposes. Three culture techniques and one enhanced-recovery method are described in this section.

Occasionally, it is necessary to examine stool specimens for scolices and proglottids of cestodes and for adult nematodes and trematodes to confirm the diagnosis and/or to identify a species. A method for the recovery of these stages is also described in procedure 9.5.6.

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The Baermann technique uses a special apparatus and relies on the principle that active larvae will migrate out of a fecal specimen that has been placed on a wire mesh covered with several layers of gauze (1, 2). Larvae migrate through the gauze into the water and settle to the bottom of the funnel, where they can be collected and examined. Modifications to simplify the procedure have been reported elsewhere (5). Besides being useful for diagnosis from stool specimens directly or after enhancement by culture, this technique can be used by epidemiologists to examine soil specimens for larvae.

II. SPECIMEN

Observe standard precautions.

The specimen must be fresh stool that has not been refrigerated.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagent

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

Bleach (full strength)

B. Supplies

1. Glass funnel (6 in. across at the mouth)
2. Rubber tubing to fit end of funnel
3. Clamp
4. Wire gauze screen or nylon filter
5. Centrifuge tubes (15-ml capacity)
6. Glass slides (1 by 3 in. or larger)
7. Disposable glass or plastic pipettes
8. Wooden applicator sticks (nonsterile)
9. Gauze
10. Ring stand and ring for holding funnel
11. Glass beaker (500 ml)

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Centrifuge, tabletop

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. To ensure reliable results, follow routine procedures for optimal collection and handling of specimens for parasitological examination.

B. If available, examine known positive and negative samples of stools (from laboratory animals) to make sure that the procedure is precise.

C. Review larval diagrams (any parasitology text) for confirmation of larval identification.

D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

E. Record all QC results.

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. If possible, use a fresh fecal specimen that has been obtained after administration of a mild saline cathartic, not a stool softener. Soft stool is recommended, but any fresh fecal specimen is acceptable.

C. Set up a clamp supporting a 6-in.-mouth glass funnel. Attach rubber tubing and a pinch clamp to the bottom of the funnel. Place a collection beaker underneath (*see* Fig. 9.5.1–1).

D. Place wire gauze or a nylon filter over the top of the funnel (or resting within the funnel), and then place a pad consisting of two layers of gauze over that.

E. Close the pinch clamp at the bottom of the tubing, and fill the funnel with tap water until it just soaks the gauze padding.

F. Spread a large amount of fecal material on the gauze padding so the specimen is in contact with water. If the fecal material is very firm, emulsify in water.

G. Allow the apparatus to stand for 2 or more hours, draw off 10 ml of fluid into the beaker by releasing the pinch clamp, centrifuge for 2 min at 500 $\times$ *g*, and examine the sediment under the microscope (100$\times$ and 400$\times$) for the presence of motile larvae. *Make sure that the end of the tubing is well inside the beaker before slowly releasing the pinch clamp. Infective larvae may be present; wear gloves when performing this procedure.*

H. If there are no larvae seen, allow the apparatus to stand at room temperature for 8 to 12 h and examine additional fluid (after centrifugation). Discard after 12 h.

VI. RESULTS

A. Larval nematodes (hookworm, *Strongyloides* spp., or *Trichostrongylus* spp.) may be recovered.

B. Both infective and noninfective *Strongyloides* larvae may be recovered, particularly in a heavy infection.

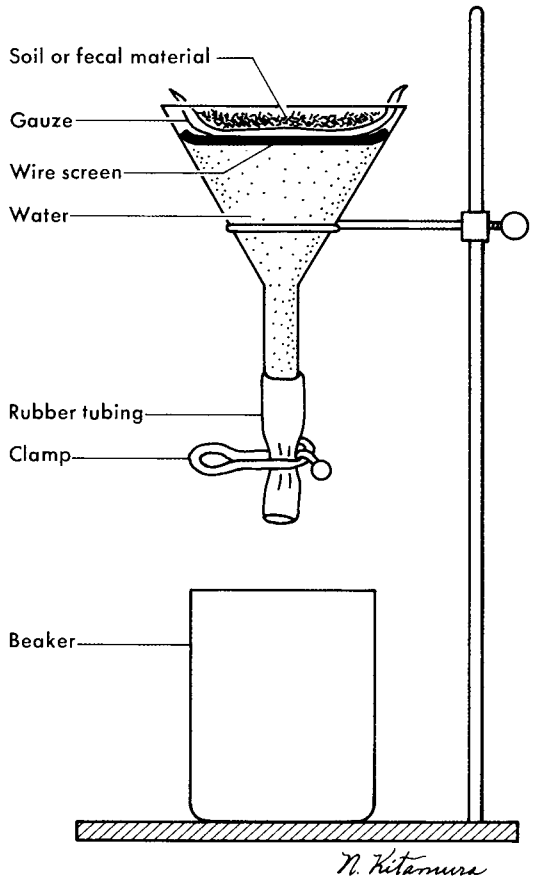


Figure 9.5.1–1 Diagram of the Baermann apparatus used for recovery of larval-stage nematodes (from reference 2).

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report your findings as "No larvae detected" if no larvae could be detected at the end of incubation.

B. Report larvae detected by fecal culture.
Example: *Strongyloides stercoralis* larvae detected by fecal culture.

VIII. PROCEDURE NOTES

A. It is often difficult to observe details in rapidly moving larvae. If desired, use slight heating or a drop of iodine or formalin to kill the larvae.

B. Preserved fecal specimens or specimens obtained after a barium meal are not suitable for processing by this method.

C. *Wear gloves when you perform this procedure.*

D. When you release the pinch clamp, do it slowly to prevent splashing.

E. For the same reason, hold the end of the tubing toward the bottom of the beaker.

F. Infective larvae may be found any time after the fourth day and occasionally after the first day in heavy infections. *Caution must be exercised in handling the fluid, gauze pad, and beaker to prevent accidental infection. Always remember to wear gloves.*

IX. LIMITATIONS OF THE PROCEDURE

A. This technique allows both parasitic and free-living forms of nematodes to develop. If specimens have been contaminated with soil or water containing these forms, it may be necessary to distinguish parasitic from free-living forms. This distinction is possible, since parasitic forms are more resistant to slight acidity than are free-living forms. Proceed as follows (2–4). Add 0.3 ml of concentrated hydrochloric acid per 10 ml of water containing the larvae (adjust the volume to achieve a 1:30 dilution of acid). Free-living nematodes are killed, while parasitic species live for about 24 h.

B. Specimens that have been refrigerated are not suitable for culture. Larvae of certain species are susceptible to cold.

REFERENCES

1. **Baermann, G.** 1917. Eine einfache Methode zur Auffindung vor Ankylostomum (Nematoden) Larven in Erdproben. *Meded. Geneesk Laborat. Weltever Feestbundel*, p. 41.
2. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
3. **Melyin, D. M., and M. M. Brooke.** 1985. *Laboratory Procedures for the Diagnosis of Intestinal Parasites*, p. 163–189. U.S. Department of Health, Education, and Welfare publication no. (CDC) 85-8282. U.S. Government Printing Office, Washington, DC.
4. **Shorb, D. A.** 1937. A method of separating infective larvae of *Haemonchus contortus* (Trichostrongylidae) from free living nematodes. *Proc. Helminthol. Soc. Wash.* **4:**52.
5. **Watson, J. M., and R. Al-Hafidh.** 1957. A modification of the Baermann funnel technique and its use in establishing the infection potential of human hookworm carriers. *Ann. Trop. Med. Parasitol.* **51:**15–16.

SUPPLEMENTAL READING

Ash, L. R., and T. C. Origel. 1987. *Parasites: a Guide to Laboratory Procedures and Identification.* ASCP Press, Chicago, IL.

Markell, E. K., D. T. John, and W. A. Krotoski. 1999. *Markell and Voge's Medical Parasitology*, 8th ed. W. B. Saunders Co., Philadelphia, PA.

9.5.2 "Culture" of Larval-Stage Nematodes: Harada-Mori Technique

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

This filter paper test tube culture technique was initially introduced by Harada and Mori in 1955 (2) and was later modified by others (3, 5). The technique employs a filter paper to which fecal material is added and a test tube into which the filter paper is inserted. Moisture is provided by adding water to the tube. The water continuously soaks the filter paper by capillary action. Incubation under suitable conditions favors hatching of ova and/or development of larvae. Fecal specimens to be cultured should not be refrigerated, since some parasites (especially *Necator americanus*) are susceptible to cold and may fail to develop after refrigeration.

II. SPECIMEN

Observe standard precautions.

The specimen must be fresh stool that has not been refrigerated.

III. MATERIALS

A. Reagents

None

B. Supplies

1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in. or larger)
3. Coverslips (22 by 22 mm; no. 1 or larger)
4. Filter paper (40 or 42 is fine; weight is not critical)
5. Wooden applicator sticks (nonsterile)
6. 15-ml-capacity conical tube
7. Forceps

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. To ensure reliable results, follow routine procedures for optimal collection and handling of specimens for parasitological examination.

B. If available, examine known positive and negative samples of stools (from laboratory animals) to make sure that the procedure is precise.

C. Review larval diagrams (any parasitology text) for confirmation of larval identification.

D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the

IV. QUALITY CONTROL *(continued)*

microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

E. Record all QC results.

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. Cut a narrow (3/8 by 5 in.) strip of filter paper, and taper it slightly at one end. Smear 0.5 to 1 g of feces in the center of the strip.

C. Add 3 to 4 ml of distilled water to a 15-ml conical centrifuge tube.

D. Insert the filter paper strip into the tube so that the tapered end is near the bottom of the tube. The water level should be slightly (0.5 in.) below the fecal spot. It is not necessary to cap the tube. However, a cork stopper or a cotton plug may be used (*see* Fig. 9.5.2–1).

E. Allow the tube to stand upright in a rack at 25 to 28°C. Add distilled water to maintain the original level (usually evaporation takes place over the first 2 days, but then the culture becomes stabilized).

F. Keep the tube for 10 days, and check daily by withdrawing a small amount of fluid from the bottom of the tube. Prepare a smear on a glass slide, cover with a coverslip, and examine with the 10× objective.

G. Examine the larvae for motility and typical morphological features to reveal whether hookworm, *Strongyloides*, or *Trichostrongylus* larvae are present.

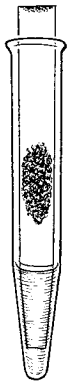


Figure 9.5.2–1 Diagram of Harada-Mori culture system (from reference 1).

VI. RESULTS

A. Larval nematodes (hookworm, *Strongyloides* spp., or *Trichostrongylus* spp.) may be recovered.

B. If *Strongyloides* organisms are present, free-living stages and larvae may be found after several days in culture.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report your findings as "No larvae detected" if no larvae could be detected at the end of incubation.

B. Report larvae detected by fecal culture.
Example: *Strongyloides stercoralis* larvae detected by fecal culture.

VIII. PROCEDURE NOTES

A. It is often difficult to observe details in rapidly moving larvae. If desired, use slight heating or a drop of iodine or formalin to kill the larvae.

B. Infective larvae may be found anytime after day 4 or even on day 1 in a heavy infection. Since infective larvae may migrate upward as well as downward on the filter paper strip, be careful when handling the fluid and the paper strip itself to prevent infection. Handle the filter paper with forceps.

C. It is important to maintain the original water level to keep optimum humidity.

D. Preserved fecal specimens or specimens obtained after a barium meal are not suitable for processing by this method.

E. *Wear gloves when you perform this procedure.*

IX. LIMITATIONS OF THE PROCEDURE

A. This technique allows both parasitic and free-living forms of nematodes to develop. If specimens have been contaminated with soil or water containing these forms, it may be necessary to distinguish parasitic from free-living forms. This distinction is possible since parasitic forms are more resistant to slight acidity than are free-living forms. Proceed as follows (4, 6). Add 0.3 ml of concentrated hydrochloric acid per 10 ml of water containing the larvae (adjust the volume to achieve a 1:30 dilution of acid). Free-living nematodes are killed, while parasitic species live for about 24 h.

B. Specimens that have been refrigerated are not suitable for culture. Larvae of certain species are susceptible to cold.

C. This method requires too much time to be clinically useful, but it can be used for field or survey studies where rapid results are not that important. Due to the time factor, this method, as well as the petri dish-filter paper slant method (procedure 9.5.3), may be replaced by the agar plate method (procedure 9.5.4), which is not only more rapid but also more sensitive.

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
2. **Harada, U., and O. Mori.** 1955. A new method for culturing hookworm. *Yonago Acta Med.* **1:**177–179.
3. **Hsieh, H. C.** 1962. A test-tube filter-paper method for the diagnosis of *Ancylostoma duodenale, Necator americanus,* and *Strongyloides stercoralis. W. H. O. Tech. Rep. Ser.* **255:**27–30.
4. **Melvin, D. M., and M. M. Brooke.** 1985. *Laboratory Procedures for the Diagnosis of Intestinal Parasites*, p. 163–189. U.S. Department of Health, Education and Welfare publication no. (CDC) 85-8282. U.S. Government Printing Office, Washington, DC.
5. **Sasa, M., S. Hayashi, H. Tanaka, and R. Shirasaka.** 1958. Application of test-tube cultivation method on the survey of hookworm and related human nematode infection. *Jpn. J. Exp. Med.* **28:**129–137.
6. **Shorb, D. A.** 1937. A method of separating infective larvae of *Haemonchus contortus* (Trichostrongylidae) from free living nematodes. *Proc. Helminthol. Soc. Wash.* **4:**52.

SUPPLEMENTAL READING

Ash, L. R., and T. C. Orihel. 1987. *Parasites: a Guide to Laboratory Procedures and Identification.* ASCP Press, Chicago, IL.

Markell, E. K., D. T. John, and W. A. Krotoski. 1999. *Markell and Voge's Medical Parasitology*, 8th ed. W. B. Saunders Co., Philadelphia, PA.

9.5.3 "Culture" of Larval-Stage Nematodes: Petri Dish-Filter Paper Slant

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

This alternative culture technique for recovery of nematode larvae was originally described by Little (1, 2). As in previously described techniques (procedure 9.5.2), sufficient moisture is provided by continuous soaking of filter paper in water. Stool material is placed on filter paper that has been cut to fit the dimensions of a standard (1- by 3-in.) microscope slide. The filter paper is then placed on a slanted glass slide in a glass or plastic petri dish containing water. This technique has the added advantage of allowing the microbiologist to look for nematode larvae and free-living stages of *Strongyloides stercoralis* in the fecal mass or the surrounding water by direct examination of the preparation with a dissecting microscope without having to sample the preparation.

II. SPECIMEN

Observe standard precautions.

The specimen must be fresh stool that has not been refrigerated.

III. MATERIALS

A. Reagents

None

B. Supplies

1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. Filter paper (40 or 42 is fine; weight is not critical)
5. Wooden applicator sticks (nonsterile)
6. Glass or plastic petri dish (100 by 15 mm)
7. Piece of glass rod or glass tubing cut to fit a petri dish (100 by 15 mm)

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Dissecting microscope with at least two different magnifications

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. To ensure reliable results, follow routine procedures for optimal collection and handling of specimens for parasitological examination.

B. If available, examine known positive and negative samples of stools (from laboratory animals) to make sure that the procedure is precise.

IV. QUALITY CONTROL *(continued)*

C. Review larval diagrams (any parasitology text) for confirmation of larval identification.

D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

E. Record all QC results.

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. Cut a filter paper strip (1 by 3 in.), and smear a film of 1 to 2 g of fecal material in the center of the strip.

C. Place the strip on a glass slide (1 by 3 in.). Place the slide at an incline (about 10°) at one end of the petri dish by resting the slide on a piece of glass rod or glass tubing.

D. Add water to the petri dish so that the bottom one-fourth of the slide is immersed in water. Cover the dish, and keep at 25 to 28°C. As needed, add water to maintain the original level (*see* Fig. 9.5.3–1).

E. Keep the dish for 10 days. Examine daily either by using the dissecting microscope or by withdrawing a small amount of fluid and placing it on a microscope slide. Add a coverslip, and examine microscopically with the 10× and 40× objectives.

F. Examine any larvae recovered for typical morphological features.

VI. RESULTS

A. Larval nematodes (hookworm, *Strongyloides* spp., or *Trichostrongylus* spp.)

B. If *Strongyloides* organisms are present, free-living stages and larvae may be found after several days of culture.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report your findings as "No larvae detected" if no larvae could be detected at the end of incubation.

B. Report larvae detected by fecal culture.
Example: *Strongyloides stercoralis* larvae detected by fecal culture.

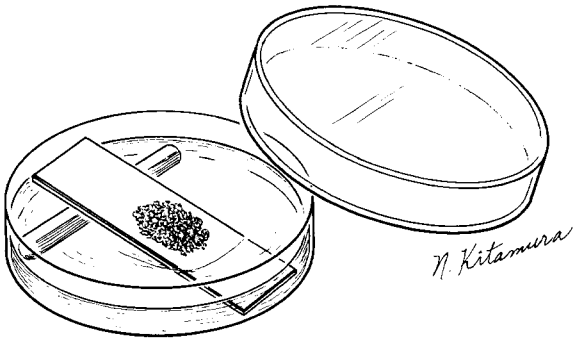


Figure 9.5.3–1 Diagram of petri dish-filter paper slant (from reference 1).

VIII. PROCEDURE NOTES

A. It is often difficult to observe details in rapidly moving larvae. If desired, use slight heating or a drop of iodine or formalin to kill the larvae.

B. Infective larvae may be found anytime after day 4. Since infective larvae may migrate upward as well as downward on the filter paper strip, be careful when handling the fluid and the paper strip itself to prevent infection.

C. *Wear gloves when handling the cultures.*

D. It is important to maintain the original water level to keep optimum humidity.

E. Preserved fecal specimens or specimens obtained after a barium meal are not suitable for processing by this method.

IX. LIMITATIONS OF THE PROCEDURE

A. This technique allows both parasitic and free-living forms of nematodes to develop. If specimens have been contaminated with soil or water containing these forms, it may be necessary to distinguish parasitic from free-living forms. The method for doing so depends on the fact that parasitic forms are more resistant to slight acidity than are free-living forms. Proceed as follows (3, 4). Add 0.3 ml of concentrated hydrochloric acid per 10 ml of water containing the larvae (adjust the volume to achieve a 1:30 dilution of acid). Free-living nematodes are killed, while parasitic species live for about 24 h.

B. Specimens that have been refrigerated are not suitable for culture. Larvae of certain species *(Necator americanus)* are susceptible to cold.

C. This method requires too much time to be clinically useful, but can be used for field or survey studies where rapid results are not that important. Due to the time factor, this method, as well as the Hara-Mori filter paper slant method (procedure 9.5.2), may be replaced by the agar plate method (procedure 9.5.4), which is not only more rapid but also more sensitive.

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology,* 5th ed. ASM Press, Washington, DC.
2. **Little, M. D.** 1966. Comparative morphology of six species of *Strongyloides* (Nematoda) and redefinition of the genus. *J. Parasitol.* **52:**69–84.
3. **Melvin, D. M., and M. M. Brooke.** 1985. *Laboratory Procedures for the Diagnosis of Intestinal Parasites,* p. 163–189. U.S. Department of Health, Education, and Welfare publication no. (CDC) 85-8282. U.S. Government Printing Office, Washington, DC.
4. **Shorb, D. A.** 1937. A method of separating infective larvae of *Haemonchus contortus* (Trichostrongylidae) from free living nematodes. *Proc. Helm. Soc. Wash.* **4:**52.

SUPPLEMENTAL READING

Ash, L. R., and T. C. Orihel. 1987. *A Guide to Laboratory Procedures and Identification.* ASCP Press, Chicago, IL.

Markell, E. K., D. T. John, and W. A. Krotoski. 1999. *Markell and Voge's Medical Parasitology,* 8th ed. W. B. Saunders Co., Philadelphia, PA.

9.5.4

"Culture" of Larval-Stage Nematodes: Agar Plate Culture for *Strongyloides stercoralis*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Agar plate cultures are recommended for the recovery of *Strongyloides stercoralis* larvae and tend to be more sensitive than some of the other diagnostic methods (1, 3, 4, 9). Stool is placed onto agar plates, and the plates are sealed to prevent accidental infections and held for 2 days at room temperature. As the larvae crawl over the agar, they carry bacteria with them, thus creating visible tracks over the agar. The plates are examined under the microscope for confirmation of larvae, the surface of the agar is then washed with 10% formalin, and final confirmation of larval identification is made via wet examination of the sediment from the formalin washings (Fig. 9.5.4–1).

In a study looking at the prevalence of *S. stercoralis* in three areas of Brazil, the diagnostic efficacy of the agar plate culture method (included in this procedure) was as high as 93.9%, compared to only 28.5 and 26.5% for the Harada-Mori filter paper culture and fecal concentration methods, when fecal specimens were processed using all three methods (8). Among the 49 positive samples, about 60% were confirmed as positive using only the agar plate method. These results indicate that the agar plate approach is probably a much more sensitive diagnostic method, and it is recommended for the diagnosis of strongyloidiasis.

It is important to remember that more than half of *S. stercoralis*-infected individuals tend to have low-level infections (10). The agar plate method continues to be documented as a more sensitive method than the usual direct smear or fecal concentration methods (6, 7). Daily search for furrows on agar plates for up to six consecutive days results in increased sensitivity for diagnosis of both *S. stercoralis* and hookworm infections. Also, a careful search for *S. stercoralis* should be made in all patients with comparable clinical findings before deciding on a diagnosis of idiopathic eosinophilic colitis, because consequent steroid treatment may have a fatal outcome by inducing widespread dissemination of the parasite (2).

Human T-cell leukemia virus 1 (HTLV-1) infection is endemic in a number of Latin American countries. HTLV-1-associated myelopathy/tropical spastic paraparesis and adult T-cell leukemia lymphoma are emerging diseases in the region. *S. stercoralis* hyperinfection syndrome and therapeutic failure in apparently healthy patients with nondisseminated strongyloidiasis may be markers of HTLV-1 infection (5).

II. SPECIMEN

Observe standard precautions.

The specimen must be fresh stool that has not been refrigerated.

III. MATERIALS

A. Reagents (*see* Appendix 9.5.4–1)

B. Supplies

1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. Plastic petri dish (100 by 15 mm)
5. Wooden applicator sticks (nonsterile)
6. Glass or plastic centrifuge tube (15 ml)
7. Plastic bags (large enough to hold several petri plates)
8. Metal forceps (no shorter than 3 to 4 in.)
9. Cellulose tape

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Dissecting microscope with at least two different magnifications

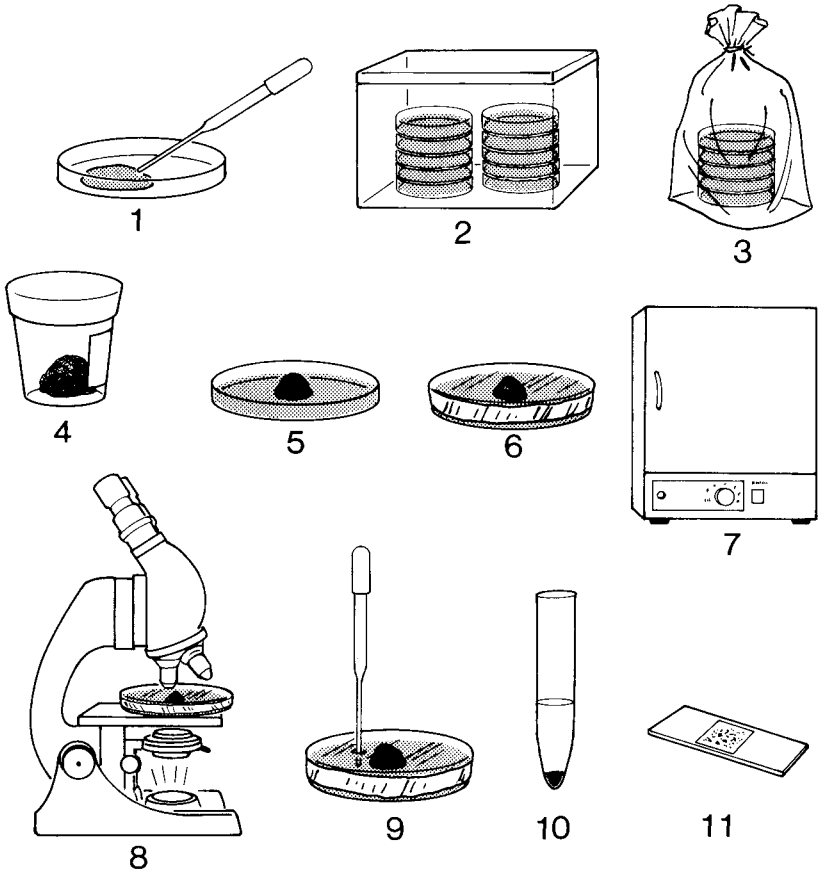


Figure 9.5.4–1 Agar culture method for *S. stercoralis.* (1) Agar plates are prepared; (2) agar is dried for 4 to 5 days on the bench top; (3) plates are stored in plastic bags; (4) fresh stool is submitted to the laboratory; (5) approximately 2 g of stool is placed onto an agar plate; (6) the plate is sealed with tape; (7) the culture plate is incubated at 26 to 33°C for 2 days; (8) the plate is examined microscopically for the presence of tracks (bacteria carried over agar by migrating larvae); (9) 10% formalin is placed onto agar through a hole made in the plastic with hot forceps; and (10) material from the agar plate is centrifuged and (11) examined as a wet preparation for rhabditiform or filariform larvae (high dry power; magnification, ×400). (Illustration by Sharon Belkin; from reference 3).

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. To ensure reliable results, follow routine procedures for optimal collection and handling of specimens for parasitological examination.

B. Examine agar plates to ensure that there is no cracking and the agar pour is sufficient to prevent drying. Also, make sure there is no excess water on the surfaces of the plates.

C. Review larval diagrams and descriptions (any parasitology text) for confirmation of larval identification.

D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. The calibration factors for all objectives should be posted on the microscope for easy access (multiplication factors can be pasted on the body of the microscope). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple

IV. QUALITY CONTROL *(continued)*

times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

E. Record all QC results (condition of agar plates).

V. PROCEDURE

A. Place approximately 2 g of fresh stool in the center of the agar plate (area of approximately 1 in. in diameter).

B. Replace the lid and seal the plate with cellulose tape or shrink seal.

C. Maintain the agar plate (right side up) at room temperature for 2 days.

D. After 2 days, examine the sealed plates through the plastic lid under the microscope for microscopic colonies that develop as random tracks on the agar and evidence of larvae at the ends of the tracks away from the stool (*see* Appendix 9.5.4–2).

◩ **NOTE:** It has been documented that daily search for furrows on agar plates for up to six consecutive days results in increased sensitivity for diagnosis of both *S. stercoralis* and hookworm infections (7). When trying to rule out strongyloidiasis in immunocompromised patients or in those who may receive immunosuppressives, it is recommended that two plates be set up, one that can be examined after 2 days and one that can be examined after the full 6 days.

E. With the end of hot forceps (heat the end of the forceps until able to melt plastic), make a hole in the top of the plastic petri dish.

F. Gently add 10 ml of 10% formalin through the hole onto the agar surface, swirl to cover the surface, and rinse the agar plate. Allow to stand for 30 min.

G. Remove the tape and lid of the agar plate. Pour the 10% formalin through a funnel into a centrifuge tube. Do not try and pour the formalin off directly into the centrifuge tube; the size of the tube opening is too small, and formalin will be spilled onto the counter.

H. Centrifuge formalin rinse fluid for 5 min at 500 $\times$ *g*.

I. Prepare wet-smear preparation from sediment, and examine using the 10$\times$ objective (low power) for the presence of larvae. If larvae are found, confirm identification using the 40$\times$ objective (high dry power).

VI. RESULTS

A. Larval nematodes (hookworm, *Strongyloides* spp., or *Trichostrongylus* spp.)

B. If *Strongyloides* organisms are present, free-living stages and larvae may be found after several days on the agar plates.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report your findings as "No larvae detected" if no larvae could be detected at the end of incubation and rinse procedure.

B. Report larvae detected by agar plate culture.
Example: *Strongyloides stercoralis* larvae detected by fecal culture.

VIII. PROCEDURE NOTES

A. It is often difficult to observe details in rapidly moving larvae. If desired, use slight heating or a drop of iodine or formalin to kill the larvae.

B. Infective larvae may be found anytime after the first or second day or even on the first day in a heavy infection. *Since infective larvae may be present on the agar, caution must be exercised in handling the plates once the cellulose tape is removed. Wear gloves when handling the cultures.*

C. It is important to maintain the plates upright at room temperature. Do not incubate or refrigerate at any time; this also applies to the fresh stool specimen.

VIII. PROCEDURE NOTES *(continued)*

D. Fresh stool is required for this procedure; preserved fecal specimens or specimens obtained after a barium meal are not suitable for processing by this method.

IX. LIMITATIONS OF THE PROCEDURE

A. This technique is successful if any larvae present are viable. If the fresh stool specimen is too old, larvae may not survive and a negative result will be reported.

B. Specimens that have been refrigerated or preserved are not suitable for culture. Larvae of certain species *(Necator americanus)* are susceptible to cold environments.

REFERENCES

1. **Arakaki, T., M. Iwanaga, F. Kinjo, A. Saito, R. Asato, and T. Ikeshiro.** 1990. Efficacy of agar-plate culture in detection of *Strongyloides stercoralis* infection. *J. Parasitol.* **76:**425–428.
2. **Corsetti, M., G. Basilisco, R. Pometta, M. Allocca, and D. Conte.** 1999. Mistaken diagnosis of eosinophilic colitis. *Ital. J. Gastroenterol. Hepatol.* **31:**607–609.
3. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
4. **Garcia, L. S.** 2009. *Practical Guide to Diagnostic Parasitology*, 2nd ed. ASM Press, Washington, DC.
5. **Gotuzzo, E., C. Arango, A. de Queiroz-Campos, and R. E. Isturiz.** 2000. Human T-cell lymphotropic virus-I in Latin America. *Infect. Dis. Clin. N. Am.* **14:**211–239, x–xi.
6. **Iwamoto, T., M. Kitoh, K. Kayashima, and T. Ono.** 1998. Larva currens: the usefulness of the agar plate method. *Dermatology* **196:**343–345.
7. **Jongwutiwes, S., M. Charoenkorn, P. Sitthichareonchai, P. Akaraborvorn, and C. Putaporntip.** 1999. Increased sensitivity of routine laboratory detection of *Strongyloides stercoralis* and hookworm by agar-plate culture. *Trans. R. Soc. Trop. Med. Hyg.* **93:**398–400.
8. **Kobayashi, J., H. Hasegawa, E. C. Soares, H. Toma, A. R. Dacal, M. C. Brito, A. Yamanaka, A. A. Foli, and Y. Sato.** 1996. Studies on prevalence of *Strongyloides* infection in Holambra and Maceio, Brazil, by the agar plate faecal culture method. *Rev. Inst. Med. Trop. Sao Paulo* **38:**279–284.
9. **Koga, K. S., C. Kasuya, K. Khamboonruang, M. Sukhavat, M. Ieda, N. Takatsuka, K. Kita, and H. Ohtomo.** 1991. A modified agar plate method for detection of *Strongyloides stercoralis. Am. J. Trop. Med. Hyg.* **45:**518–521.
10. **Uparanukraw, P., S. Phongsri, and N. Morakote.** 1999. Fluctuations of larval excretion in *Strongyloides stercoralis* infection. *Am. J. Trop. Med. Hyg.* **60:**967–973.

APPENDIX 9.5.4–1

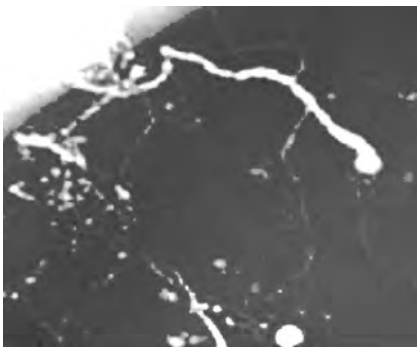
Include QC information on reagent container and in QC records.

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

Agar: 1.5% agar
0.5% meat extract
1.0% peptone
0.5% NaCl

◩ **NOTE:** Positive tracking on agar plates has been seen with a number of different types of agar. However, the most appropriate agar formula is that seen above.

APPENDIX 9.5.4–2



Tracking of *Strongyloides stercoralis* larvae on agar plate culture.

9.5.5 Determination of Egg Viability: Schistosomal Egg Hatching

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The diagnosis of schistosomal infections can be aided by this method. When eggs of *Schistosoma mansoni* or *Schistosoma japonicum* are scarce, their presence may be detected by allowing them to hatch. Upon dilution of a specimen with spring (nonchlorinated) water, eggs hatch within a few hours, after which the preparation is examined for free-swimming miracidia (1–4). Miracidia are phototropic; that is, they have a tendency to swim toward light. This procedure takes advantage of the organisms' phototropism. This is a very sensitive test for indirectly demonstrating small numbers of viable eggs in fecal specimens. This technique also allows one to determine the viability of the eggs. This is not a concentration technique; therefore, unless tests for egg viability are desired, there is seldom any point in using this technique to look for schistosome eggs in urine, in which they are readily concentrated by centrifugation. McMullen and Beaver (3) recommended the use of a sidearm flask, but an Erlenmeyer flask may be an acceptable substitute.

II. SPECIMENS

Observe standard precautions.

A. Fresh stool that has not been refrigerated
B. Urine (24 h) collected with no preservatives
C. Urine (random) collected with no preservatives

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

1. 0.85% NaCl
2. Spring water (nonchlorinated)

B. Supplies

1. Gauze
2. Funnel
3. Centrifuge tubes (50 ml)
4. 250-ml beaker
5. 500-ml sidearm flask or Erlenmeyer flask
6. Magnifying lens (hand lens)
7. Aluminum foil or brown paper

C. Equipment

1. Lamp (desk, gooseneck, or comparable)
2. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
3. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Make sure that water used in this procedure is chlorine free. Chlorine will affect the viability of miracidia.
B. Check saline solution for the presence of any free-living organisms (flagellates or ciliates).

IV. QUALITY CONTROL *(continued)*

C. If available, known positive and negative samples should be examined to make sure that the procedure is acceptable. Since this is not usually practical, review drawings and size measurements of schistosomal eggs and/or miracidia (any parasitology text).

D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

E. Record all QC results.

V. PROCEDURE

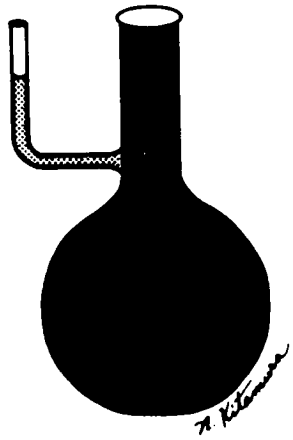


Figure 9.5.5–1 Diagram of a sidearm hatching flask (from reference 2).

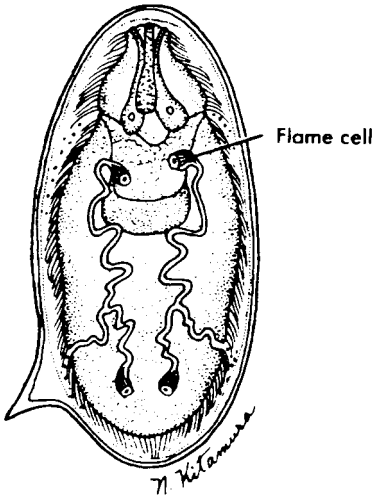


Figure 9.5.5–2 Diagram of a schistosomal miradicium within the egg shell (from reference 2).

A. Homogenize a stool specimen (40 to 50 g) in 50 to 100 ml of 0.85% NaCl.

B. Strain specimen through two layers of gauze placed on a funnel. Collect material in a centrifuge tube.

C. Allow the suspension to settle for 1 h. Pour off and discard the supernatant fluid, and repeat this process (steps V.B and V.C) at least twice.

D. Decant and discard the saline solution, suspend the sediment in a small quantity of chlorine-free (spring) water, and pour the suspension into a 500-ml sidearm flask or an Erlenmeyer flask (Fig. 9.5.5–1).

E. Add chlorine-free water to the flask so that the fluid level rises to 2 to 3 cm in the sidearm flask or to the top 2 cm of the Erlenmeyer flask. Cover the flask with aluminum foil or black paper, leaving the side arm of the flask exposed to light. If an Erlenmeyer flask is used, cover to 1 cm below the level of fluid in the neck of the flask.

F. Allow the flask to stand at room temperature for several hours or overnight in subdued light.

G. When ready for examination, place a bright light at the side of the flask opposite the surface of exposed water. Do not place the light against the glass to avoid generation of excess heat. As the eggs hatch, the liberated miracidia will swim to the upper layers and collect in the side arm (or neck region of an Erlenmeyer flask) (Fig. 9.5.5–2).

H. Examine the illuminated area with a magnifying lens (hand lens) to look for minute white organisms swimming rapidly in a straight line (placing a piece of dark cardboard behind the flask will help you see the white miracidia against the dark background).

VI. RESULTS

A. Living miracidia may be seen.
B. Failure to see living miracidia does not rule out schistosomiasis.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report as "Miracidia of schistosomes detected, indicating presence of viable eggs."
B. Report as "No miracidia of schistosomes detected; presence of eggs is not ruled out by this procedure."

VIII. PROCEDURE NOTES

A. Both urine and stool specimens must be collected without preservatives and should not be refrigerated prior to processing.
B. Hatching will not occur until the saline is removed and nonchlorinated water is added.
C. If a stool concentration is performed, use saline throughout the procedure to prevent premature hatching.
D. Make sure the light is not too close to the side arm or top layer of water in the Erlenmeyer flask. Excess heat will kill the miracidia.

IX. LIMITATIONS OF THE PROCEDURE

A. Absence of swimming miracidia does not rule out the presence of eggs. Nonviable eggs or eggs that did not hatch will not be detected by this method. Use microscopic examination of direct or concentrated specimens to demonstrate the presence or absence of eggs.
B. Egg viability can be determined by placing some stool or urine sediment (same material as that used for the hatching flask) on a microscope slide.
  1. Examine under 100× to locate eggs.
  2. Examine individual eggs under 400×, and look for moving cilia on the flame cells (primitive excretory system) (Fig. 9.5.5–2).
  3. Presence of moving cilia is proof of egg viability.
C. Coprozoic, free-living ciliates may be present in soil-contaminated water. Therefore, it may be necessary to perform the following steps to differentiate those forms from the parasitic miracidia (4).
  1. Transfer a few drops of the suspension containing the organisms to a slide (3 by 2 in.), and examine under the microscope.
  2. Add a drop of weak iodine solution (pale-tea color) or dilute methylene blue (pale blue).
  3. Parasitic miracidia will stop moving, but free-living forms will continue to move.

REFERENCES

1. **Chernin, E., and C. A. Dunavan.** 1962. The influence of host-parasite dispersion upon the capacity of *Schistosoma mansoni* miracidia to infect *Australorbis glabratus. Am. J. Trop. Med. Hyg.* **11:**455–471.
2. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
3. **McMullen, D. B., and P. C. Beaver.** 1945. Studies on schistosome dermatitis. IX. The life cycles of three dermatitis producing schistosomes from birds and a discussion of the subfamily Bilharziellinae (Trematodai Schistosomatidae). *Am. J. Hyg.* **42:**125–154.
4. **Melvin, D. M., and M. M. Brooke.** 1985. *Laboratory Procedures for the Diagnosis of Intestinal Parasites*, p. 179–181. U.S. Department of Health, Education and Welfare publication no. (CDC) 85-9393. U.S. Government Printing Office, Washington, DC.

9.5.6 Recovery of Scolices and Proglottids of Cestodes

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Occasionally, stool specimens have to be examined for the presence of scolices and gravid proglottids of cestodes for proper species identification. This procedure requires mixing a small amount of feces with water and straining the mixture through a wire screen to look for scolices and proglottids (1). The same procedure may be used to look for small adult nematodes and trematodes (2). Appearance of scolices after therapy is an indication of successful treatment. *If scolices have not been passed, they may be attached to the mucosa. The parasite is capable of producing more segments from the neck region of the scolex, and the infection continues.*

II. SPECIMEN

Observe standard precautions.

A. Fresh feces obtained prior to or after therapy

B. For posttherapy specimen, the patient must receive a saline purge immediately after taking niclosamide or praziquantel.

C. Stool specimen (24 h) collected in 10% formalin

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagent

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

0.85% NaCl

B. Supplies

1. Small-mesh (30/50 mesh) sieve or screen
2. Glass slides (1 by 3 in. or larger)
3. Wooden applicator sticks (nonsterile)
4. 500-ml beaker
5. Magnifying lens (hand lens)
6. Glass dish
7. Rubber bands (small)
8. Shallow pan

C. Equipment

1. Wood's lamp (optional)
2. Stereoscope (dissecting microscope)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. To ensure reliable results, follow routine procedures for optimal collection and handling of specimens for parasitological examination.

B. Review diagrams and sizes (any parasitology text) of proglottids and scolices of tapeworms.

V. PROCEDURE

A. Mix a 24-h stool specimen (fresh or preserved in 10% formalin) with water, and thoroughly break up the specimen to make a watery suspension.

B. Slowly strain small portions of the suspension (or the purged stool) through a double layer of screen wire or a sieve (one coarse-mesh screen placed over a fine-mesh screen).

C. Wash off the sediment remaining from each portion by running a slow current of water over it.

D. Examine the cleansed debris with a hand lens to look for scolices and proglottids (*Taenia* scolex is 0.5 to 1 cm long and 1 to 2 mm wide).

E. Repeat steps V.C and V.D for each portion of the suspension strained.

F. Collect the strained sediment in a glass dish, and place over a black surface to increase the contrast of organisms against the background.

G. Observe with a magnifying hand lens, and pick out pieces of worms with an applicator stick.

H. Rinse gravid proglottids and/or scolices with tap water, and place between two microscope slides separated at the edges by thin pieces of cardboard.

I. Fasten the preparation by placing rubber bands at each end of the slides so that the segments become somewhat flattened.

J. Use the low power of a dissecting microscope for determining the number of uterine branches and genital pores in the segments and the presence or absence of a rostellum of hooks on the scolex.

K. India ink injection of proglottid

1. Using a syringe (1 ml or less) and a 25-gauge needle, inject India ink into the central uterine stem of the proglottid, thus filling the uterine branches with ink, or inject ink into the uterine pore.

2. Rinse the proglottid in water or saline.

3. Blot the proglottid dry on paper towels, and press it between two slides.

4. Use the low power of a dissecting microscope for determining the number of uterine branches.

VI. RESULTS

A. Tapeworm proglottids may be recovered (either singly or with several attached together).

B. A scolex may or may not be seen.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report as "A search for adult worms reveals presence/absence of . . . [finding]."
Example: *Taenia saginata* scolex present.

B. Indicate species of gravid proglottid.
Example: *Taenia solium* proglottid

VIII. PROCEDURE NOTES

A. Remember that *T. solium* eggs are infective (cysticercosis), as are the eggs of *Hymenolepis nana.* Standard safety precautions should be followed.

B. *Wear gloves when performing this procedure.*

C. Specimens preserved in 70% alcohol are recommended.

D. After the identification has been made, leave the proglottid between the two slides (place a rubber band around the slides), dehydrate through several changes of ethyl alcohol (50, 70, 90, and 100%), clear in two changes of xylene, and mount with Permount for a permanent record. After xylene treatment, the proglottid will be stuck to one of the two slides. Do not try to remove it (will crack; very brittle), but merely put the Permount onto the proglottid and add the coverslip.

VIII. PROCEDURE NOTES *(continued)*

E. The proglottid of *T. solium* must be gravid, containing the fully developed uterine branches. If the proglottid is not fully developed (gravid), the branches may not be visible; when the uterine branches cannot be seen and/or counted, the proglottid cannot be accurately identified to the species level.

F. If the patient has received niclosamide or praziquantel, a purged specimen is required, and it should be immediately preserved in 10% formalin.

G. Wood's lamp may be used to search for scolices if the patient has been given quinacrine dyes. The worms, having absorbed the dye, will fluoresce at a wavelength of 360 nm. Also, after the use of guinacrine, tapeworm proglottids will appear yellow.

IX. LIMITATIONS OF THE PROCEDURE

A. Niclosamide or praziquantel therapy leads to dissolution of the tapeworm. Therefore, the scolex and other parts may be difficult to recover unless the patient receives a saline purge soon after taking the medication.

B. It may often be difficult to identify proglottids without staining. This may be achieved by staining with India ink (*see* item V.K above).

C. If the proglottid is not gravid or has started to disintegrate, it will be difficult to see the uterine branches, even after India ink injection.

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
2. **Melvin, D. M., and M. M. Brooke.** 1985. *Laboratory Procedures for the Diagnosis of Intestinal Parasites*, p. 187. U.S. Department of Health, Education and Welfare publication no. (CDC) 85-8282. U.S. Government Printing Office, Washington, DC.

9.5.7 Qualitative Fecal Fat

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The microscopic examination of stool with the addition of Sudan III is a very simple and quick technique and is the most widely used technique to screen for fat.

II. SPECIMEN

Observe standard precautions.

A. Fresh, unpreserved fecal material is needed. If there is a time delay prior to testing, then refrigerate the specimen.

B. Specimens greater than 48 h old or dried out should be discarded and another specimen should be collected.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

1. 95% Ethanol
2. Glacial acetic acid (36%, vol/vol)
3. Sudan III stain

B. Supplies

1. Disposable test tube, 15-ml size
2. Applicator sticks
3. Disposable plastic pipettes
4. Matches
5. Biohazard discard container
6. Glass slides, 1 by 3 in.
7. Coverslips, 22 by 22 mm, no. 1 or larger

C. Equipment

1. Bright-field microscope, with 10× and 40× objectives
2. Alcohol burning lamp or Bunsen burner
3. Refrigerator
4. Biological safety cabinet

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Run QC with each batch of patient tests as in the procedure below.

B. Positive control—mayonnaise: red-stained fat globules should be observed microscopically.

C. Negative control—water: no fat globules observed.

D. If QC is unacceptable, then the test must be repeated and documented on the corrective action sheet.

V. PROCEDURE

A. Mix a small amount of stool with an equal amount of 95% ethanol in a test tube. Mix well.
B. Add 2 drops of Sudan III stain to the stool-ethanol mixture. Mix well and let stand a few minutes.
C. Using a pipette, remove a drop from the test tube and place it on a slide. Cover with a coverslip.
D. Using the microscope, examine the slide for globules of fat stained red (neutral fat) and needle-like crystals (fatty acid).
E. Add several drops of glacial acetic acid to the test tube. Remove a drop from the test tube and place it on a slide. Cover with a coverslip.
F. Gently heat the slide over a flame. (Do not boil.)
G. Observe again on the microscope for globules of fat stained red.

VI. RESULTS

Red-stained fat globules or fatty acid crystals will be seen microscopically. Report specimen as "Fat not increased" or "Fat increased," depending on the number of globules seen.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. If fewer than 100 globules/high-power field (HPF) are seen before or after heating, report "Fat not increased."
B. If more than 100 globules/HPF are seen before or after heating, report "Fat increased."
C. If fatty acid crystals have been seen before heating and globules appear after heating, report "Fatty acids increased."

VIII. LIMITATIONS OF THE PROCEDURE

A. Just because large needle-like crystals form as the preparation cools after heating, it does not mean that the original globules were fatty acid. Sudan III will form very short needle-like crystals in bunches as it dries.
B. A very few, if any, neutral fat globules are seen in a normal stool specimen. The presence of large amounts of neutral fat should make one suspicious that the patient has ingested mineral oil or castor oil, thus causing a false positive.
C. Do not count the fat that is present in vegetable cells.

SUPPLEMENTAL READING

Drummey, B. S., J. A. Benson, Jr., and C. M. Jones. 1961. Microscopic examination of the stool for steatorrhea. *N. Engl. J. Med.* **264:**85–87.

Henry, J. B. 2001. *Clinical Diagnosis and Management by Laboratory Methods*, 20th ed. W. B. Saunders Company, Philadelphia, PA.

9.5.8 Reducing Substances (Clinitest)

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Clinitest is a reagent tablet test based on the classic Benedict's copper sulfate reduction reaction combining ingredients with an integral heat-generating system. Clinitest provides clinically useful information about carbohydrate metabolism by determining the amount of reducing substance in urine or stool. Reducing substances convert the cupric (Cu^{2+}) to cuprous (Cu^{+}) oxide and a change in solution color ranging from green to orange.

II. SPECIMEN

Observe standard precautions.

Unpreserved stool.

A. Place in the refrigerator in case there is a delay in testing.

B. Specimens >48 h old or dried out should be discarded and new specimens should be collected.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

1. Clinitest reagent tablets. (Store tablets at room temperature in a plastic bag.)
2. Deionized water
3. Chek-Stix positive control

B. Supplies

1. Disposable plastic test tubes with round bottoms
2. Applicator sticks
3. Disposable plastic transfer pipettes
4. Biohazard discard container
5. Disposable gloves

C. Equipment

Biological safety cabinet

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Run controls with each batch of patient tests as in the procedure below.

B. Positive control: Chek-Stix. The development of green, yellow, or orange color with yellow or red precipitate is considered a positive result.

C. Negative control: 0.5 ml of deionized water. A blue color development is considered a negative result.

D. If QC is unacceptable, the test must be repeated and documented on the corrective action sheet.

V. PROCEDURE

A. All testing on clinical specimens should be performed in a biological safety cabinet, wearing gloves and a lab coat.
B. Add 1 volume of stool to 2 volumes of deionized water. Mix thoroughly.
C. Using a disposable transfer pipette, transfer 15 drops of this suspension into a clean test tube.
D. Drop one Clinitest tablet reagent into the test tube.
E. Observe the reaction. Do not shake the tube while the chemical reaction is occurring.
F. Wait 15 s after the reaction stops, and gently shake contents to mix.
G. Compare the color of the liquid to the color chart in the package insert of the Clinitest tablet reagent.
H. Discard supplies in appropriate biohazard containers.

VI. RESULTS

A. Negative: clear to cloudy blue color
B. Positive: compare the liquid color to the color chart. Grade the degree of color development to the color chart (trace, 1+, 2+, 3+, or 4+). These results equate to the grams per deciliter of the reducing substance present in the sample.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Positive: report as trace (0.25 g/dl), 1+ (0.5 g/dl), 2+ (0.75 g/dl), 3+ (1.0 g/dl), or 4+ (greater than or equal to 2 g/dl).
B. Negative: report as negative.

VIII. PROCEDURE NOTES

A. Clinitest is not specific for glucose and will react with any reducing substance in stool, including lactose, fructose, galactose, and pentoses.
B. Interfering substances may affect results. Some interfering substances include salycilates, penicillin, large quantities of ascorbic acid, nalidixic acid, and cephalosporins.
C. Failure to observe the reaction at all times may lead to erroneously low results if reducing substances are present in large amounts. If more than 2% sugar is present, a rapid color change may occur during boiling, causing the color to pass rapidly through bright orange to a dark brown or greenish brown.

SUPPLEMENTAL READING

Bayer Corporation Diagnostic Division. September 1995. Clinitest package insert. Bayer Corporation Diagnostic Division, Elkhart, IN.

Davidson, G., and M. Mullinger. 1970. Reducing substances in neonatal stool detected by Clinitest. *J. Pediatr.* **46:**632–635.

Kerry, K. R., and C. M. Anderson. 1964. A ward test for sugar in feces. *Lancet* **i:**981.

9.6.1 Examination for Pinworm: Cellulose Tape Preparation

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The clear-cellulose-tape preparation is the most widely used procedure for the detection of human pinworm infections (1, 2, 4). Adult *Enterobius vermicularis* worms inhabit the large intestine and rectum; however, the eggs are not normally found in fecal material. The adult female migrates out the anal opening and deposits the eggs on the perianal skin, usually during the night. The eggs, and occasionally the adult female worms, stick to the sticky surface of the cellulose tape. These cellulose tape preparations are submitted to the laboratory, where they are examined under the microscope. Commercial collection systems are also available.

II. SPECIMEN

Observe standard precautions.

The specimen is collected from the skin of the perianal area first thing in the morning, before the patient has bathed or used the toilet. Preparations should be taken for at least 4 to 6 consecutive days with negative results before a patient is considered free of the infection.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagent

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

Xylene (or xylene substitute [ethyl acetate]) or toluene

B. Supplies

1. Clear cellulose tape (not Magic mend tape)
2. Tongue depressors
3. Glass slides (1 by 3 in., or larger if you prefer)

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

IV. QUALITY CONTROL *(continued)*

B. Known positive microscope slides, Kodachrome (2- by 2-in.) slides, and pictures of *Enterobius* eggs (with measurements) should be available for comparison with the clinical specimen. Images are also available online on various Web sites.

C. Record all QC results.

V. PROCEDURE

A. Place a strip of clear cellulose tape (adhesive side down) on a microscope slide as follows: starting ca. 1.5 cm from one end, run the tape toward the same end, and wrap the tape around the slide to the opposite end. Tear the tape even with the end of the slide. Attach a label to the tape at the end torn flush with the slide.

B. To obtain a sample from the perianal area, peel back the tape by gripping the labeled end, and, with the tape looped (adhesive side outward) over a wooden tongue depressor that is held firmly against the slide and extended about 2.5 cm beyond it, press the tape firmly several times against the right and left perianal folds (*see* Fig. 9.6.1–1).

C. Smooth the tape back on the slide, adhesive side down.

D. Label with patient name and date.

E. Submit the tapes and slides to the laboratory in a plastic bag.

F. Examine the slide directly under the low-power objective (10×) of the microscope. To make the eggs more visible, lift the tape from the slide and add a drop of xylene or toluene to the slide. Press the tape back down on the slide. Examine with low light intensity on low power.

VI. RESULTS

A. Typical pinworm eggs are thick shelled and football shaped, with one flattened side, and may contain a partially or fully developed larva.

B. Adult female worms are occasionally seen on the tape preparation. The worms are approximately 1 cm in length, are white or cream colored, and have a pointed tail.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism and stage. Do not use abbreviations.
Example: *Enterobius vermicularis* eggs present.

B. Report adult worms.
Example: *Enterobius vermicularis* adult worm present.

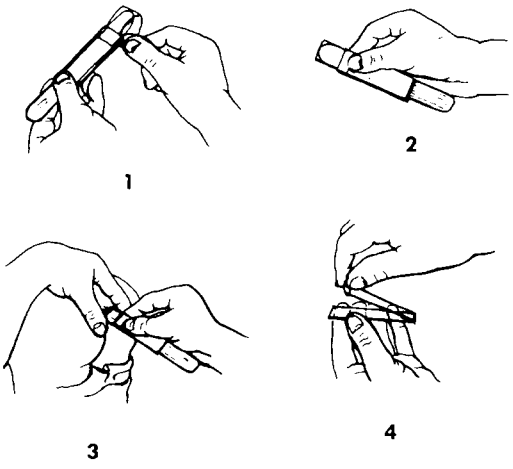


Figure 9.6.1–1 Collection of *E. vermicularis* eggs by the cellulose tape method (from reference 1).

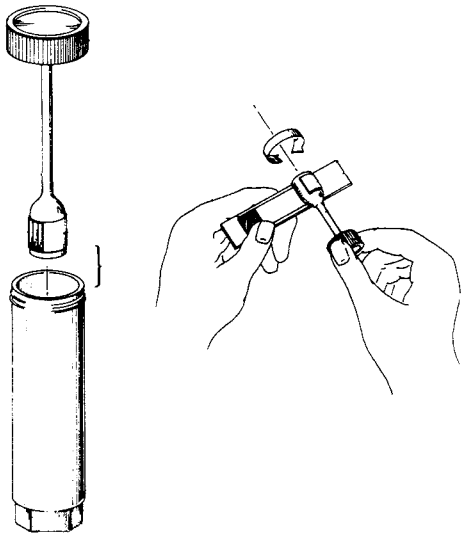


Figure 9.6.1–2 Diagram of a commercial kit (Evergreen Scientific) for use in sampling the perianal area for the presence of pinworm *(E. vermicularis)* eggs. On the left is the vial containing the sampler, which has sticky tape around the end. Once this is applied to the perianal area and eggs are picked up on the tape, the label area is placed at one end of the slide. The sticky tape is rolled down the slide and attaches to the glass. This device is easy to use and provides an area sufficient for adequate sampling. A minimum of four to six consecutive negative tapes are required to rule out a pinworm infection; most laboratories are accepting four rather than requesting the full six. (From reference 1.)

VII. REPORTING RESULTS *(continued)*

C. Report negatives.
Example: No *Enterobius vermicularis* eggs or adults seen.

VIII. PROCEDURE NOTES

A. Pinworm eggs are usually infectious. The use of glass slides and tapes may expose laboratory personnel to these eggs.
B. Some recommend the use of the pinworm collection kit (Evergreen Scientific, Los Angeles, CA) or the Swube (paddle with sticky adhesive coat; Becton Dickinson, Franklin Lakes, NJ) as a safer alternative (Fig. 9.6.1–2). The petrolatum swab is another alternative (3).
C. If opaque tape is submitted by mistake, a drop of immersion oil on the top of the tape will clear it enough to proceed with the microscopic examination.

IX. LIMITATIONS OF THE PROCEDURE

A. The female pinworm deposits eggs on the perianal skin only sporadically.
B. Without multiple tapes (taken consecutively, one each morning), it is not possible to determine if the patient is positive or negative for the infection.
C. Occasionally, a parent will bring in an adult worm collected from the perianal skin or from the surface of the stool. The identification of the adult worm (almost always the female) confirms the infection.

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
2. **Graham, C. F.** 1941. A device for the diagnosis of *Enterobius vermicularis*. *Am. J. Trop. Med.* **21:**159–161.
3. **Markey, R. L.** 1950. A Vaseline swab for the diagnosis of *Enterobius* eggs. *Am. J. Clin. Pathol.* **20:**493.
4. **Melvin, D. M., and M. M. Brooke.** 1982. *Laboratory Procedures for the Diagnosis of Intestinal Parasites*, 3rd ed. U.S. Department of Health, Education and Welfare publication no. (CDC) 82-8282. U.S. Government Printing Office, Washington, DC.

9.6.2 Sigmoidoscopy Specimen: Direct Wet Smear

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The direct smear (1, 2) is primarily used to detect motile parasites that are found in the colon (the organism in question is usually *Entamoeba histolytica* or *Entamoeba histolytica/E. dispar*). Specific ulcerated areas should always be sampled; in the absence of specific lesions, the mucosa is randomly sampled. On low-power (×100) examination of the smear, motility of trophozoites and/or human cells might be detected. At high dry power (×430), organisms might be tentatively identified on the basis of size, nucleus/cytoplasm ratio, appearance of the cytoplasm, and motility (saline only). The direct smear can be prepared either with 0.85% NaCl or with iodine (Lugol's or D'Antoni's). Presumptive findings using this procedure must be confirmed by some type of permanent stained smear.

II. SPECIMEN

Observe standard precautions.

A. The specimen may consist of mucosal lining, mucus, stool, and/or a combination of the three. The specimen will be taken by the physician and either prepared at bedside for immediate review or submitted to the laboratory for subsequent examination.

B. Prepare direct wet mounts on clean, new glass slides (1 by 3 in.). Depending on the specimen type, the following amounts should be used.

1. For mounts of mucus or similar material, place approximately 1 or 2 drops on the slide.

2. For mounts of stool, place approximately 1 or 2 drops on the slide.

3. If the material is very wet (watery), place 1 or 2 drops on the slide.

C. If the specimen must be transported to the laboratory, the material can be placed in a small amount of 0.85% NaCl (0.5 to 1.0 ml) to keep the specimen from drying out. These specimens should be transported to the laboratory within no more than 30 min from collection time and should be examined immediately. If this is not possible, then the material can be preserved and processed as a permanent stained smear (*see* procedure 9.6.3).

III. MATERIALS

A. Reagents (*see* Appendix 9.6.2–1)

B. Supplies

1. Disposable glass or plastic pipettes

2. Glass slides (1 by 3 in., or larger if you prefer)

3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)

4. Small tubes containing 0.5 to 1.0 ml of 0.85% NaCl

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)

2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.

3. Tabletop centrifuge (for tubes containing specimen and 0.85% NaCl if the specimen is submitted in saline)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check the direct-mount reagents each time they are used.
   1. The saline should be clear, with no visible contamination.
   2. The iodine should be a strong-tea color, and there should be crystals in the bottom of the bottle. Small aliquots of the stock solution should always be strong-tea color. If not, then discard and aliquot some stock solution into your dropper bottle.

B. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Known positive microscope slides, Kodachrome (2- by 2-in.) slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

D. Record all QC results.

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. To the drop or two of patient material on the slide, add 1 or 2 drops of 0.85% NaCl, mix with the corner of the coverslip or an applicator stick, and mount with a no. 1 coverslip (22 by 22 mm). The amount of saline will be determined by the specimen (less saline if the material is very liquid).

C. Examine the smear with the low-power (10×) objective with low light. View each field for a few seconds, looking for any organism motility. Any suspicious objects can be examined with the high dry (40×) power objective with low light.

D. Prepare a wet mount with Lugol's or D'Antoni's iodine (working solution) rather than saline. Another option would be to add a small drop of iodine at the side of the coverslip on the saline wet preparation. The iodine will diffuse into the saline suspension under the coverslip. However, if the specimen is thick or contains mucus, then capillary action pulling the iodine under the coverslip and into the saline may not occur, and a separate iodine mount may be required. The addition of iodine will give the material some color (organisms may be easier to see), but motility will be lost.

VI. RESULTS

A. Protozoan trophozoites may be confused with human cells (macrophages), so report any identification as presumptive until the permanent stained smears have been examined.

B. Also, the presumptive identification and quantitation of the human cells (macrophages, PMNs, eosinophils, and RBCs) could be obtained from the wet preparations. Consider this information presumptive also until the permanent stained smears have been examined.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism and stage (trophozoite, cyst, oocyst, etc.; do not use abbreviations); however, confirmation of species may require some type of permanent stained smear.
Example: *Entamoeba histolytica* trophozoites

B. Note and quantitate the presence of human cells.
Examples: Moderate WBCs, many RBCs, few macrophages

C. Call the physician if pathogenic organisms are identified.

D. Report negative results as a presumptive report (based on wet examination only) prior to the examination of the permanent stained smear.

E. See Table 9.6.2–1 for correct method of quantitation.

VIII. PROCEDURE NOTES

A. Remember that the iodine working solution should be a strong-tea color; if it is not, discard and prepare new working solution.

B. Final identification of some of the intestinal protozoa may be difficult (small size, confusion between organisms and human cells), and a permanent stained smear must be used as a confirmatory method and examined at $\times 1{,}000$ to see morphological details.

C. In saline, human cells and/or protozoan trophozoites may exhibit some motility.

D. In iodine, human cells and/or protozoan trophozoites may be seen (no motility).

E. Confirm presumptive findings (either positive or negative) with a permanent stained smear.

IX. LIMITATIONS OF THE PROCEDURE

A. Multiple areas of the mucosa should be examined (six smears are often recommended), and this technique should not take the place of the routine ova and parasite examination.

B. Wet preparations are normally not examined with oil immersion power ($100\times$). Consequently, use permanent stained smears to confirm morphology and organism (or human cell) identification.

C. If the specimen amount is limited, then do not do the wet preparation, but process the specimen that is available by using the permanent-stained-smear protocol (*see* procedure 9.6.3) to maximize the amount and clinical relevance of the information obtained.

D. Morphologic differentiation between the *Entamoeba histolytica/E. dispar* group and *Entamoeba coli* can be difficult, in addition to the problem of differentiating human cells from protozoa. Also, unless RBCs are seen, it will be impossible

Table 9.6.2–1 Quantitation of parasites, human cells, yeast cells, and artifacts in specimens from the intestinal tract[a]

Quantity	No. of protozoa, human cells, yeast cells, artifacts		No. of helminths/coverslip[b]
	Per 10 oil immersion fields ($\times 1{,}000$)[c]	Per 10 40$\times$ fields ($\times 400$)[d]	
Few	≤ 2	≤ 2	≤ 2
Moderate	3–9	3–9	3–9
Many	≥ 10	≥ 10	≥ 10

[a] In general, protozoa are not quantitated on the laboratory slip (exception: *Blastocystis hominis*), but human cells, yeast cells, and artifacts like Charcot-Leyden crystals are normally reported and quantitated.

[b] Wet preparation. Coverslips were 22 by 22 mm.

[c] Polyvinyl alcohol smear.

[d] Wet preparation.

IX. LIMITATIONS OF THE PROCEDURE *(continued)*

to differentiate organisms in the *E. histolytica/E. dispar* group from the actual pathogen, *E. histolytica.*

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
2. **Melvin, D. M., and M. M. Brooke,** 1985. *Laboratory Procedures for the Diagnosis of Intestinal Parasites.* U.S. Department of Health and Human Services publication no. (CDC) 85-8282. U.S. Government Printing Office, Washington, DC.

APPENDIX 9.6.2–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. 0.85% NaCl

1. Dissolve in distilled water in an appropriate glass flask by using a magnetic stirrer.

   sodium chloride (NaCl) ..... 850 mg
   distilled water ..... 100 ml

2. Store in a glass bottle.
3. Label as 0.85% NaCl with the preparation date and an expiration date of 6 months. Store at room temperature.

B. Modified D'Antoni's stock iodine

1. Dissolve in distilled water in an appropriate glass flask, using a magnetic stirrer.

   potassium iodide (KI) ..... 1.0 g
   powdered iodine crystals ..... 1.5 g
   distilled water ..... 100 ml

2. The D'Antoni's solution should be saturated with iodine, with some excess crystals left in the bottle. Store in a brown bottle at room temperature. The stock solution remains good as long as an excess of iodine crystals remains on the bottom of the bottle.
3. Label as D'Antoni's stock iodine with the preparation date and an expiration date of 1 year.
4. Small amounts of stock iodine solution can be aliquoted into brown dropper bottles for routine daily use. The expiration date will be from 30 to 60 days, depending on the amount of fading of the solution from the normal strong-tea color (smaller dropper bottles and use of clear glass result in a shorter expiration time). The use of a brown bottle will lengthen the expiration time.

C. Lugol's iodine solution

1. Dissolve in distilled water in an appropriate glass flask by using a magnetic stirrer.

   potassium iodide (KI) ..... 10.0 g
   iodine crystals ..... 5.0 g
   distilled water ..... 100 ml

2. The Lugol's solution should be saturated with iodine, with some excess crystals left in the bottle. Store in a brown bottle at room temperature. The stock solution remains good as long as an excess of iodine crystals remains on the bottom of the bottle.
3. Label as Lugol's stock iodine with the preparation date and an expiration date of 1 year.
4. Small amounts of stock iodine solution can be aliquoted into brown dropper bottles for routine daily use. The expiration date will be from 30 to 60 days, depending on the amount of fading of the solution from the normal strong-tea color (smaller dropper bottles and the use of clear glass result in a shorter expiration time). The use of a brown bottle will lengthen the expiration time.

9.6.3 Sigmoidoscopy Specimen: Permanent Stained Smear

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The permanent stained smear is used primarily to detect parasites in the colon (the primary organism in question is usually *Entamoeba histolytica* or *Entamoeba histolytica/E. dispar*). Specific ulcerated areas are always sampled; in the absence of specific lesions, the mucosa would be randomly sampled. On oil immersion (magnification of ×1,000) examination of the smear, protozoan trophozoites and/or cysts might be detected. Coccidian oocysts, helminth eggs or larvae, and/or human cells are also detected with this procedure. The permanent smear can be stained with trichrome or iron hematoxylin stains. Permanent stained smears usually confirm structures seen on wet specimen examinations (1).

II. SPECIMEN

Observe standard precautions.

The specimen may consist of mucosal lining, mucus, stool, and/or a combination of the three.

A. Prepare smears on clean, new glass slides (1 by 3 in.).
B. For mounts of mucus or similar material, place approximately 1 or 2 drops of specimen on the slide.
C. For mounts of stool, place approximately 1 drop on the slide.
D. If the material is very wet (watery), place 1 or 2 drops on the slide.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

1. Schaudinn's fixative
See Appendix 9.2.2–1, item A, for preparation instructions.
2. Polyvinyl alcohol (PVA) fixative
See Appendix 9.2.2–1, item B, for preparation instructions.

B. Supplies

1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm or larger; no. 1 thickness)

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Tabletop centrifuge

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check the fixatives weekly or when a new lot number is used (visual inspection). Use either fresh stool containing protozoa or negative stool seeded with human buffy coat cells to evaluate the efficacy of the fixatives after staining (*see* procedures 9.3.6 and 9.3.7). Cultured protozoa can also be used.

B. The Schaudinn's fixative should appear clear, without floating debris or crystals. Some crystal sediment on the bottom of the Coplin jar or dish is acceptable.

C. The PVA should be clear (slight milky or smoky color is acceptable). A slight precipitate on the bottom of the container is acceptable. The fluid should easily move in the bottle when it is inverted, and the viscosity of many of the available formulations will actually approach that of water.

D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

E. Known positive microscope slides, Kodachrome (2- by 2-in.) slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

F. Record all QC results.

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. If the specimen does not contain blood and/or mucus or is not "wet," gently smear a drop or two of patient material onto the slide, and immediately immerse the slide into the Schaudinn's fixative. There should be no problem with adherence to the slide. The fixation and staining times are identical to those for routine fecal smears (*see* procedures 9.2.2. V, 9.3.6, and 9.3.7 for specific directions).

C. If the material is bloody, contains a lot of mucus, or is a wet specimen, gently mix 1 or 2 drops of patient material with 3 or 4 drops of PVA fixative directly on the slide. Allow the smear to air dry for at least 2 h prior to staining. The fixation and staining times are identical to those for routine fecal smears (*see* procedures 9.2.2.V, 9.3.6, and 9.3.7 for specific directions).

D. Examine the stained smear with the oil immersion lens (100×) with maximum light. Examine at least 300 oil immersion fields of the smear.

VI. RESULTS

A. With either the trichrome or iron hematoxylin stain, the protozoan trophozoites and cysts will be easily seen. Oocysts will not be clearly delineated; if you see suspect organisms, then use additional procedures for confirmation (*see* procedures 9.4.1 and 9.4.2).

B. Helminth eggs or larvae may not be easily identified on the permanent stained smear, and you may need to do wet-mount examinations.

C. Human cells are readily identified (macrophages, PMNs, RBCs, etc.). Yeast cells (single cells, budding, and presence of pseudohyphae) can also be easily seen.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism and stage. Do not use abbreviations.
Example: *Entamoeba histolytica* trophozoites (contain ingested RBCs)
B. Note and quantitate the presence of human cells.
Examples: Moderate WBCs, many RBCs, few macrophages, etc.
C. Report and quantitate yeast cells.
Example: Moderate budding yeast cells and few pseudohyphae
D. Call the physician if pathogenic organisms are identified.
E. See Table 9.6.3–1 for correct method of quantitation.

VIII. PROCEDURE NOTES

A. Sigmoidoscopy specimens are submitted in order to help differentiate between inflammatory bowel disease and amebiasis. It is critical that the specimens be preserved immediately after being taken. Any delay could result in the disintegration of amebic trophozoites or distortion of human cells.
B. It is critical that permanent stained smears of this material be carefully examined with the oil immersion lens (100×).

IX. LIMITATIONS OF THE PROCEDURE

A. The more areas of the mucosa sampled, the more likely the organisms will be found. If only one or two smears are submitted for examination, the physician must be informed (recommendation is six smears from representative areas of the mucosa).
B. The examination of smears prepared at sigmoidoscopy does not take the place of routine ova and parasite examinations but serves as a supplemental procedure. Stools for routine examinations should also be submitted (a minimum of three specimens collected every other day or within no more than 10 days).
C. Morphological differentiation between the *Entamoeba histolytica/E. dispar* group and *Entamoeba coli* can be difficult, in addition to the problem of differentiating human cells from protozoa. Also, unless RBCs are seen, it will be impossible to differentiate organisms in the *E. histolytica/E. dispar* group from the actual pathogen, *E. histolytica.*

REFERENCE

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.

Table 9.6.3–1 Quantitation of parasites, human cells, yeast cells, and artifacts in specimens from the intestinal tract[a]

Quantity	No. of protozoa, human cells, yeast cells, artifacts		No. of helminths/coverslip[b]
	Per 10 oil immersion fields (×1,000)[c]	Per 10 40× fields (×400)[d]	
Few	≤2	≤2	≤2
Moderate	3–9	3–9	3–9
Many	≥10	≥10	≥10

[a] In general, protozoa are not quantitated on the laboratory slip (exception: *Blastocystis hominis*), but human cells, yeast cells, and artifacts like Charcot-Leyden crystals are normally reported and quantitated.

[b] Wet preparations. Coverslips were 22 by 22 mm.

[c] PVA smear.

[d] Wet preparation.

9.6.4 Duodenal Contents: String Test (Entero-Test Capsule)

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The Entero-Test capsule is usually administered and the string is retrieved by a physician. This test is used to procure specimens from the duodenum that are then examined for the presence of parasites. The Entero-Test is a gelatin capsule lined with silicone rubber that contains a spool of nylon string and a weight (Fig. 9.6.4–1). The end of the string is taped to the back of the patient's neck or the patient's cheek just before the capsule is swallowed with water. After swallowing the capsule, the patient is allowed to relax for 4 h. The patient is not allowed to eat during this time but is allowed to drink a small amount of water. As the capsule dissolves, the string unwinds and is carried by peristalsis to the duodenum, and the duodenal mucus adheres to the string. Any *Strongyloides* larvae (2), *Giardia* trophozoites (1–6), or *Cryptosporidium* (1, 5, 6) or *Cystoisospora* (*Isospora*) (1, 5, 6) oocysts that are present will also adhere to the string and will be pulled up with the string when it is removed. The specimen can be examined as a wet preparation or as a permanent stained smear. In rare instances, *Clonorchis* eggs may be recovered (1–3). This test is a less invasive substitute for duodenal aspiration (1–6).

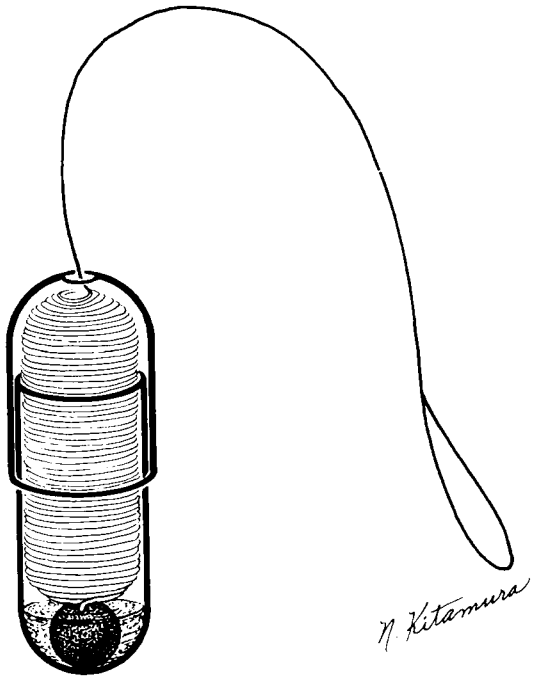


Figure 9.6.4–1 Diagram of Entero-Test capsule (from reference 2).

II. SPECIMEN

Observe standard precautions.

A. The physician must notify the laboratory when a capsule is swallowed so that a parasitologist is available to read the test 4 h later, when the string is removed.

B. The string is placed in a small, securely covered container that is transported in a plastic bag. Unless the specimen container is hand delivered to the microbiology unit, a petri dish should not be used because the top cannot be securely attached. A urine container with a screw-cap top is practical for this purpose.

C. The specimen must be transported immediately and read within 1 h (2).

D. If there is *any* delay anticipated in transport to the laboratory, a small amount of saline (~1 ml) can be added to keep the string moist.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

1. Schaudinn's fixative.
See Appendix 9.2.2–1, item A, for preparation instructions.

2. Polyvinyl alcohol (PVA) fixative.
See Appendix 9.2.2–1, item B, for preparation instructions.

3. 10% Formalin
See Appendix 9.2.2–1, item G, for preparation instructions.

B. Supplies

1. Disposable glass or plastic pipettes

2. Glass slides (1 by 3 in., or larger if you prefer)

3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)

4. Gloves

5. Urine container

6. Entero-Test capsule (adult or pediatric size) (*see* Appendix 9.10.6–1 at the end of this section for supplier)

7. Test tube (16 by 125 mm)

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)

2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check the fixatives weekly or when a new lot number is used (visual inspection). Use either fresh stool containing protozoa or negative stool seeded with human buffy coat cells to evaluate the efficacy of the fixatives after staining (*see* procedures 9.3.6 and 9.3.7). Cultured protozoa can also be used.

1. The Schaudinn's fixative should be clear, without floating debris or crystals. Some crystal sediment on the bottom of the Coplin jar or dish is acceptable.

2. The PVA should be clear (slightly milky or smoky color is acceptable). A slight precipitate on the bottom of the container is acceptable. The fluid should easily move in the bottle when it is inverted. The viscosity of many of the available formulations will actually approach that of water.

3. The 10% formalin should be clear, with no visible contamination.

B. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Known positive microscope slides, Kodachrome (2- by 2-in.) slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

D. Record all QC results.

V. PROCEDURE

A. *Gloves must be worn when handling this specimen.* Infectious *Strongyloides* larvae can penetrate the intact skin.

B. Record the color of the string. Yellow bile stain indicates that the string did reach the duodenum.

C. Place the specimen under the biosafety cabinet, hold the dry white end in one hand, and strip all the mucus off the string by gripping it between the thumb and index finger of the other hand and squeezing it all the way down to the end, so that the mucus goes into the screw-cap container.

D. Place 1 drop of mucus on a clean slide, and cover with a coverslip (22 by 22 mm). If the mucus is very viscous, add a drop of saline before adding the coverslip.

E. Store the remaining mucus in a transfer pipette placed in a labeled test tube (16 by 125 mm) so that it will not dehydrate.

F. Examine the entire coverslip under low power (100×) for eggs, larvae, or motile trophozoites, looking especially carefully at the mucus, where *Giardia* may be entangled.

G. Examine the mucus under high dry power (400×), since *Giardia* may be detectable only by the flutter of the flagella rather than by motility.

H. If there is enough specimen, gently smear a drop or two of patient material on two slides, and immediately immerse the slides in Schaudinn's fixative so that permanent stained slides may be made. If the specimen is not adequate for this, place the wet mount slide in a Coplin jar containing Schaudinn's solution after it has been read. The coverslip will float off and sink to the bottom, allowing the remaining material to be stained. The fixation and staining times are identical with those for routine fecal smears (*see* procedure 9.2.2 for specific directions).

I. If the material contains a lot of mucus or is a watery specimen, gently mix 1 or 2 drops of patient material with 3 or 4 drops of PVA fixative directly on the slide. Let the smear air dry for at least 2 h prior to staining. The fixation and staining times are identical to those for routine fecal smears (*see* procedure 9.2.2 for specific directions).

J. Place a drop of the mucus on one or more slides to be stained for coccidia or microsporidia and air dry. Repeat the wet-mount procedure (steps, V.D through G above) until all the remaining mucus is used.

K. Stain the air-dried slide(s) with modified acid-fast or modified trichrome stain, and examine as usual.

L. Examine the permanent stained smear with the oil immersion lens (100×) with maximum light. Examine at least 300 oil immersion fields on each smear.

M. If *Strongyloides* larvae are found, preserve the rest of the specimen in 10% formalin for teaching purposes.

VI. RESULTS

A. With either the trichrome or iron hematoxylin stain, the protozoan trophozoites and cysts will easily be seen. Oocysts will not be clearly delineated; if suspect organisms are seen, use additional procedures for confirmation (*see* procedures 9.4.1 and 9.4.2).

B. Coccidial oocysts will be visible on permanent stained smears (modified acid-fast procedures) (*see* procedures 9.4.1 and 9.4.2 for specific directions).

C. Helminth eggs or larvae may not be easily identified on the permanent stained smear but will be visible in the wet preparations.

D. Microsporidial spores will be visible on modified-trichrome-stained smears (*see* procedures 9.4.3 and 9.4.4 for specific directions).

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism and stage (trophozoite, cyst, oocyst, etc.). Do not use abbreviations. Confirmation of species may require some type of permanent stained smear.
Examples: *Giardia lamblia* trophozoites, *Strongyloides stercoralis* larvae
B. Call the physician if pathogenic organisms are identified.
C. Quantitate *Clonorchis sinensis* eggs if they are recovered.
D. If the results are negative on the wet smear examination, a preliminary report can be sent (based on wet examination only) prior to the examination of the permanent stained smear.
E. See Table 9.6.4–1 for correct method of quantitation.

VIII. PROCEDURE NOTES

Modified acid-fast methods (or immunoassay detection methods) will have to be used for the identification of *Cryptosporidium* spp. *Cystoisospora* (*Isospora*) spp. can be identified on the wet examination or from smears stained by modified acid-fast methods.

IX. LIMITATIONS OF THE PROCEDURE

A. Many of the parasites will be caught up in the mucus; therefore, it is very important to examine the specimen carefully under high dry power (400×) with low light in order to see the flutter of the *Giardia* flagella.
B. If the duodenal specimens from the Entero-Test capsule are normally examined as wet preparations, remember that some of the organisms (*Cryptosporidium* and *Cystoisospora* [*Isospora*] spp.) may be missed without additional permanent stains.

REFERENCES

1. **Beal, C. B., P. Viens, R. G. L. Grant, and J. M. Hughes.** 1970. A new technique for sampling duodenal contents. *J. Trop. Med. Hyg.* **19:**349–352.
2. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
3. **Gordts, B., W. Hemelhof, K. Van Tilborgh, P. Retore, S. Cadranel, and J. Butzler.** 1985. Evaluation of a new method for routine in vitro cultivation of *Giardia lamblia* from human duodenal fluid. *J. Clin. Microbiol.* **22:**702–704.
4. **Liebman, W. M., and P. Rosenthal.** 1980. Comparative study of stool examination, duodenal aspirations, and pediatric Entero-Test for giardiasis in children. *J. Pediatr.* **96:**278.
5. **Markell, E. K., D. T. John, and W. A. Krotoski.** 1999. *Markell and Voge's Medical Parasitology*, 8th ed., The W. B. Saunders Co., Philadelphia, PA.
6. **Whiteside, C. L.** 1984. Enteric coccidiosis among patients with the acquired immunodeficiency syndrome. *Am. J. Trop. Med. Hyg.* **33:**1065–1072.

Table 9.6.4–1 Quantitation of parasites, human cells, yeast cells, and artifacts in specimens from the intestinal tract[a]

Quantity	No. of protozoa, human cells, yeast cells, artifacts		No. of helminths/coverslip[b]
	Per 10 oil immersion fields (×1,000)[c]	Per 10 40× fields (×400)[d]	
Few	≤2	≤2	≤2
Moderate	3–9	3–9	3–9
Many	≥10	≥10	≥10

[a] In general, protozoa are not quantitated on the laboratory slip (exception: *Blastocystis hominis*); however, human cells, yeast cells, and artifacts like Charcot-Leyden crystals are normally reported and quantitated.

[b] Wet preparation. Coverslip was 22 by 22 mm.

[c] PVA smear.

[d] Wet preparation.

9.6.5 Duodenal Contents: Duodenal Aspirate

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Some organisms may be difficult to recover in the stool, particularly those normally found in the duodenum. An alternative approach to routine stool examinations is to sample the duodenal contents. Samples are obtained through the use of nasogastric intubation or the Entero-Test capsule (string test). Fluid from the duodenum is examined for the presence of *Strongyloides stercoralis* larvae (1, 2), *Giardia lamblia* trophozoites (1, 2), and *Cryptosporidium* and *Cystoisospora* (*Isospora*) oocysts. The specimen can be examined as a wet preparation or as a permanent stained smear. In rare instances, *Clonorchis sinensis* eggs may be recovered (1, 2). Material can also be examined as permanent stained smears (e.g., modified trichrome stain) for the presence of microsporidial spores.

II. SPECIMEN

Observe standard precautions.

Duodenal fluid must be transported immediately in a securely covered container placed in a plastic bag. A screw-cap urine container or plastic centrifuge tube with no preservatives is practical for this purpose.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

1. Schaudinn's fixative
   See Appendix 9.2.2–1, item A, for preparation instructions.
2. Polyvinyl alcohol (PVA) fixative
   See Appendix 9.2.2–1, item B, for preparation instructions.
3. 10% Formalin
   See Appendix 9.2.2–1, item G, for preparation instructions.

B. Supplies

1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. Gloves
5. Urine container
6. Centrifuge tubes (15 or 50 ml)

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Tabletop centrifuge

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check the fixatives weekly or when a new lot number is used (visual inspection). Use either fresh stool containing protozoa or negative stool seeded with human buffy coat cells to evaluate the efficacy of the fixatives after staining (*see* procedures 9.3.6 and 9.3.7). Cultured protozoa can also be used.

1. The Schaudinn's fixative should be clear, without floating debris or crystals. Some crystal sediment on the bottom of the Coplin jar or dish is acceptable.

IV. QUALITY CONTROL
(continued)

2. The PVA should be clear (slightly milky or smoky color is acceptable). A slight precipitate on the bottom of the container is acceptable. The fluid should easily move in the bottle when it is inverted. The viscosity of many of the available formulations will actually approach that of water.
3. The 10% formalin should be clear, with no visible contamination.

B. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Known positive microscope slides, Kodachrome (2- by 2-in.) slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

D. Record all QC results.

V. PROCEDURE

A. *Gloves must be worn when handling this specimen.* Infectious *Strongyloides* larvae can penetrate the intact skin.

B. Examine the specimen within 1 h after it is taken. Note the amount of yellow color, which indicates bile staining and confirms that the specimen is actually from the duodenum.

C. Centrifugation may be necessary to concentrate the mucus and any organisms present (500 $\times$ *g* for 10 min). Centrifugation should be routinely performed if the volume of fluid is $\geq$2 ml.

D. Place 1 drop of fluid on a clean slide, and cover with a coverslip (22 by 22 mm). If the specimen is very viscous, add a drop of saline before adding the coverslip.

E. Examine the entire coverslip under low power (100$\times$) for eggs, larvae, or motile trophozoites, looking especially carefully around the mucus, where *Giardia* may be entangled.

F. Examine the mucus present under high dry power (400$\times$), since *Giardia* may be detectable only by the flutter of the flagella rather than by motility.

G. If there is enough specimen, gently smear a drop or two of patient material on two slides, and immediately immerse the slides in Schaudinn's fixative so that permanent stained slides may be made. If the specimen is not adequate for this, place the wet-mount slide in a Coplin jar containing Schaudinn's solution, after it has been read. The coverslip will float off and sink to the bottom, allowing the remaining material to be stained. Fixation and staining times are identical to those for routine fecal smears (*see* procedure 9.2.2 for specific directions).

H. If the material contains a lot of mucus or is a watery specimen, gently mix 1 or 2 drops of patient material with 3 or 4 drops of PVA fixative directly on the slide. Let the smear air dry for at least 2 h prior to staining. The fixation and staining times are identical to those for routine fecal smears (*see* procedure 9.2.2 for specific directions).

I. Place a drop of the duodenal fluid on one or more slides to be stained for coccidia or microsporidia and air dry. Repeat the wet mount procedure (steps V.D through G above) until all the remaining mucus (after centrifugation) or sediment is gone.

J. Stain the air-dried slide(s) with modified acid-fast or modified trichrome stain, and examine as usual.

V. PROCEDURE *(continued)*

K. Examine the permanent stained smear with the oil immersion lens (100×) with maximum light. Examine at least 300 oil immersion fields on each smear.

L. If *Strongyloides* larvae are found, preserve the rest of the specimen in 10% formalin for teaching purposes.

VI. RESULTS

A. With either the trichrome or iron hematoxylin stain, the protozoan trophozoites and cysts will easily be seen. Oocysts will not be clearly delineated; if suspect organisms are seen, use additional procedures for confirmation (*see* procedures 9.4.1 and 9.4.2).

B. Coccidial oocysts will be visible on permanent stained smears (modified acid-fast procedures) (*see* procedures 9.4.1 and 9.4.2 for specific directions).

C. Helminth eggs or larvae may not be easily identified on the permanent stained smear but will be visible in the wet preparations.

D. Microsporidial spores will be visible on modified-trichrome-stained smears (*see* procedures 9.4.3 and 9.4.4 for specific directions).

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism and stage (trophozoite, cyst, oocyst, etc.). Do not use abbreviations. Confirmation of species may require some type of permanent stained smear.
Examples: *Giardia lamblia* trophozoites, *Strongyloides stercoralis* larvae

B. Call the physician if pathogenic organisms are identified.

C. Quantitate *Clonorchis sinensis* eggs if they are recovered.

D. If the results are negative on the wet smear examination, a preliminary report can be sent (based on wet examination only) prior to the examination of the permanent stained smear.

E. See Table 9.6.5–1 for the correct method of quantitation.

VIII. PROCEDURE NOTES

A. If you receive more than 2 ml of specimen, you must centrifuge the specimen (500 × *g* for 10 min) and examine the mucus or material in the bottom of the tube.

B. Modified acid-fast methods (or immunoassay detection methods) will have to be used for the identification of *Cryptosporidium* spp. *I. belli* can be identified by wet examination or from smears stained with the modified acid-fast methods.

Table 9.6.5–1 Quantitation of parasites, human cells, yeast cells, and artifacts in specimens from the intestinal tract[a]

Quantity	No. of protozoa, human cells, yeast cells, artifacts		No. of helminths/coverslip[b]
	Per 10 oil immersion fields (×1,000)[c]	Per 10 40× fields (×400)[d]	
Few	≤2	≤2	≤2
Moderate	3–9	3–9	3–9
Many	≥10	≥10	≥10

[a] In general, protozoa are not quantitated on the laboratory slip (exception: *Blastocystis hominis*), but human cells, yeast cells, and artifacts like Charcot-Leyden crystals are normally reported and quantitated.

[b] Wet preparation. Coverslips are 22 by 22 mm.

[c] PVA smear.

[d] Wet preparation.

IX. LIMITATIONS OF THE PROCEDURE

A. Many of the parasites will be caught up in the mucus; therefore, it is very important to centrifuge the specimen and concentrate this mucous material for examination. Centrifugation is mandatory if the specimen is more than 2 ml in volume.

B. Although duodenal aspirate specimens are normally examined as wet preparations, remember that some of the organisms may be missed without additional permanent stains (*Cryptosporidium* and *Cystoisospora* [*Isospora*] spp.).

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
2. **Markell, E. K., D. T. John, and W. A. Krotoski.** 1999. *Markell and Voge's Medical Parasitology*, 8th ed. The W. B. Saunders Co., Philadelphia, PA.

9.6.6 Urogenital Specimens: Direct Saline Mount

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Trichomonas vaginalis infections are primarily diagnosed by detecting live motile flagellates from direct saline (wet) mounts. Microscope slides made from patient specimens can be examined under low and high power for the presence of actively moving organisms (1).

II. SPECIMENS

Observe standard precautions.

A. Vaginal discharge
B. Urethral discharge
C. Penile discharge
D. Urethral-mucosa scrapings
E. First-voided urine with or without prostatic massage

Collect specimens with a platinum loop, cotton or Dacron swab, or speculum. Place these specimens in a small amount (<1.0 ml) of 0.85% NaCl in a test tube or on a microscope slide, and dilute them with a drop of 0.85% NaCl. If the specimen cannot be examined immediately, place the swab in Amies transport medium, which will keep the organisms viable for approximately 24 h. Collect urine specimens in a clean-catch urine collection container. Centrifuge the urine at 500 × *g*, and examine the sediment for *T. vaginalis.* Hold all specimens at room temperature, because refrigerator temperatures inhibit motility and have a deleterious effect on the organisms. Returning the specimen to room temperature will not reverse these deleterious morphological changes. Reject any specimens more than 24 h old.

III. MATERIALS

A. Reagents (*see* Appendix 9.6.6–1)
B. Supplies
1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. Small tubes containing 0.5 to 1.0 ml of 0.85% NaCl

C. Equipment
1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Tabletop centrifuge
4. Staining rack

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check the direct-mount reagents each time they are used.
   1. The saline should be clear, with no visible contamination.
   2. The iodine should be a strong-tea color, and there should be crystals in the bottom of the bottle. Small aliquots of the stock solution should always be strong-tea color. If not, then discard, and aliquot some stock solution into your dropper bottle.

B. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Known positive microscope slides, Kodachrome (2- by 2-in.) slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

D. Record all QC results.

V. PROCEDURE

A. Apply the patient's specimen to a small area on a clean microscope slide.

B. Immediately before the specimen dries, add 1 or 2 drops of saline with a pipette. If urine sediment is used, the addition of saline may not be necessary.

C. Mix the saline and specimen together with the pipette tip or the corner of the coverslip.

D. Cover the specimen with the no. 1 coverslip.

E. Examine the wet mount with the low-power (10×) objective and low light.

F. Examine the entire coverslip for motile flagellates. Suspicious objects can be examined with the high-power (40×) objective.

G. The organism is usually slightly larger than a PMN, and you should see flagellar movement.

VI. RESULTS

A. If motile flagellates (axostyle and undulating membrane) are seen, then the trophozoites of *T. vaginalis* are present.

B. If nonmotile organisms (axostyle) are visible after staining with D'Antoni's iodine, then the trophozoites of *T. vaginalis* are present.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism. Do not report the organism stage, since there is no known cyst stage for the trichomonads. The organisms do not need to be quantitated.
Example: *Trichomonas vaginalis* present.

B. If no flagellated organisms are seen, report the specimen as negative for *T. vaginalis.*
Example: No *Trichomonas vaginalis* seen.

VIII. PROCEDURE NOTES

A. It is very important that specimens to be examined for *T. vaginalis* be delivered to the laboratory within 1 h after collection.

B. After 1 h, organisms will lose their motility, particularly when they begin to dry out.

C. If a dry smear is delivered to the laboratory, salvage it by fixing the dry smear as you would a thin blood film (absolute methanol) and stain it with Giemsa at a 1:20 dilution for at least 20 min (*see* procedure 9.8.5). The stained organisms may be difficult to see, but if you can actually see and identify the organisms as *T. vaginalis*, that information may be clinically relevant.

D. Calgiswabs are not recommended (tight adherence of specimen to swab). Reject the specimen if submitted on this type of swab.

E. Swabs received in charcoal transport media are unacceptable because the charcoal particles make it difficult to visualize organisms.

F. When the specimen is examined microscopically, always confirm that no fecal contamination (artifacts, vegetable debris, etc.) is present. This type of contamination is rare and would probably be limited to a urine specimen. However, if a urine or other urogenital specimen was contaminated with fecal material, it is possible that *Pentatrichomonas hominis* (nonpathogen found in the intestinal tract) could be misidentified as *T. vaginalis*, an identification that implies sexual transmission.

G. Culture, immunoassay, and PCR methods are available for the detection of *Trichomonas vaginalis.*

IX. LIMITATIONS OF THE PROCEDURE

A. If the specimen is left at room temperature or held at refrigerator temperature for a prolonged period (usually >1 h), the organisms will round up, lose their motility, and eventually die. Motility may occasionally be enhanced by warming the specimen to 37°C, but this will not revive dying organisms.

B. Wet mounts have been reported to detect *T. vaginalis* in 75 to 85% of infected patients. *Alternative diagnostic methods may include culture, use of monoclonal antigen detection kits, use of permanent stained slides, and collection of a second sample for examination.*

C. If the patient has a *P. hominis* intestinal infection and the urogenital specimen becomes contaminated with fecal material, a false-positive *T. vaginalis* result may be reported, because *P. hominis* and *T. vaginalis* are similar in shape.

REFERENCE

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology,* 5th ed. ASM Press, Washington, DC.

SUPPLEMENTAL READING

Eschenbach, D., H. M. Pollock, and J. Schachter. 1983. *Cumitech 17, Laboratory Diagnosis of Female Genital Tract Infections.* Coordinating ed., S. J. Rubin. American Society for Microbiology, Washington, DC.

Holmes, K. K., P. Mardh, P. F. Sparling, and P. J. Wiesner. 1990. *Sexually Transmitted Diseases.* McGraw-Hill, New York, NY.

APPENDIX 9.6.6–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. 0.85% NaCl

1. Dissolve in distilled water in an appropriate glass flask by using a magnetic stirrer.

   sodium chloride (NaCl) ..... 850 mg
   distilled water ..... 100 ml

2. Store in a glass bottle.
3. Label as 0.85% NaCl with a preparation date and an expiration date of 6 months. Store at room temperature.

B. Modified D'Antoni's stock iodine

1. Dissolve in distilled water with an appropriate glass flask by using a magnetic stirrer.

   potassium iodide (KI) ..... 1.0 g
   powdered iodine crystals ..... 1.5 g
   distilled water ..... 100 ml

2. The D'Antoni's solution should be saturated with iodine, with some excess crystals left in the bottle. Store in a brown bottle at room temperature. The stock solution remains good as long as an excess of iodine crystals remains on the bottom of the bottle.
3. Label as D'Antoni's stock iodine with the preparation date and an expiration date of 1 year.
4. Small amounts of stock iodine solution can be aliquoted into brown dropper bottles for routine daily use. The expiration date will be from 30 to 60 days, depending on the amount of fading of the solution from the normal strong-tea color (small dropper bottles and the use of clear glass will result in a shorter expiration time). The use of a brown bottle will lengthen the expiration time.

9.6.7 Urogenital Specimens: Permanent Stained Smear (Giemsa)

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Trichomonas vaginalis infections are primarily diagnosed from direct saline (wet) mounts by detecting live motile flagellates. Permanently stained smears can be made from patient specimens for specific identification of the organism. Although a number of stains can be used, Giemsa and Papanicolaou stains are the ones most frequently used to diagnose *T. vaginalis* infections (1).

II. SPECIMEN

Observe standard precautions.

A. Vaginal discharge
B. Urethral discharge
C. Penile discharge
D. Urethral-mucosa scrapings
E. First-voided urine with or without prostatic massage

Collect specimens with a platinum loop, cotton-Dacron swab, or speculum. Place these specimens in a small amount (<1.0 ml) of 0.85% NaCl in a test tube, or smear them directly onto a microscope slide. Use a drop of 0.85% NaCl to dilute the direct smear when it is placed on the slide. Air dry slides prepared in this manner before transporting them to the laboratory. Place specimens collected with a cotton-Dacron swab in Amies transport medium if the specimen cannot be processed immediately. Organisms will remain viable for approximately 24 h in Amies transport medium. Collect urine specimens in a clean-catch urine collection container. Centrifuge the urine at 500 × *g* for 5 min, and examine the sediment for *T. vaginalis.* Hold all specimens at room temperature, because refrigerator temperatures have a deleterious effect on the organisms. Returning the specimen to room temperature will not reverse these deleterious morphological changes. Reject any specimens more than 24 h old.

III. MATERIALS

A. Reagents (*see* Appendix 9.6.7–1)
B. Supplies
1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. Small tubes containing 0.5 to 1.0 ml of 0.85% NaCl
5. 2 Coplin jars

C. Equipment
1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Tabletop centrifuge (for tubes containing specimen and 0.85% NaCl if the specimen is submitted in saline)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check the direct-mount reagents each time they are used.
- **1.** The saline should be clear, with no visible contamination.
- **2.** Giemsa stain
  A peripheral blood film may be used to quality control the Giemsa stain. For staining characteristics, see procedure 9.8.5.
- **3.** Phosphate buffer
  Check the buffer each time you use it. The buffer should be clear, with no signs of visible contamination or precipitates. The pH should be between 6.8 and 7.2.
- **4.** Review the Giemsa-stained control slide before searching the patient's specimen for the organism. If there was potential fecal contamination of the specimen, you may have to differentiate *T. vaginalis* from *Pentatrichomonas hominis.*

B. Known positive microscope slides, Kodachrome (2- by 2-in.) slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

C. The microscope should have been calibrated within the last 12 months, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

D. Record all QC results.

V. PROCEDURE

A. Apply patient's specimen to a small area of a clean microscope slide.

B. Immediately before the specimen dries, add 1 or 2 drops of saline with a pipette. If urine sediment is used, the addition of saline may not be necessary.

C. Mix the saline and specimen together with the pipette tip.

D. Air dry the slide.

E. Fix in absolute methanol for 1 min (Coplin jar or slide staining rack).

F. Place the slide in the Giemsa solution, and stain for the desired time, depending on the stain dilution used (20 min at 1:20 dilution).

G. Rinse the slide with tap water (gently running or in a Coplin jar) to remove excess stain solution.

H. Air dry the slide. Do not apply a coverslip.

I. Examine the slide with the oil (100×) objective.

J. Examine the entire smear for flagellates.

K. The organism is usually slightly larger than a PMN. The organism is 7 to 23 μm long and 5 to 15 μm wide. Differential characteristics to be observed include anterior flagella, undulating membrane, axostyle, and nucleus.

VI. RESULTS

If organisms (axostyle) are visible after staining with Giemsa stain, then the trophozoites of *T. vaginalis* are present.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the organism. Do not report the organism stage, since there is no known cyst stage for the trichomonads. The organisms do not need to be quantitated.
Example: *Trichomonas vaginalis* present.

B. If no flagellated organisms (axostyle and undulating membrane) are seen, report the specimen as negative for *T. vaginalis.*
Example: No *Trichomonas vaginalis* seen.

VIII. PROCEDURE NOTES

A. It is very important that specimens to be examined for *T. vaginalis* be delivered to the laboratory within 1 h after collection.

B. After 1 h, organisms will lose their motility, particularly when they begin to dry out, and the morphology on the permanent stained smear will be difficult to see.

C. If a dry smear is delivered to the laboratory, salvage it by fixing the dry smear as you would a thin blood film (absolute methanol) and staining it with Giemsa at a 1:20 dilution for at least 20 min (*see* procedure 9.8.5). The stained organisms may be difficult to see, but if you can actually see and identify the organisms as *T. vaginalis,* that information may be clinically relevant.

D. Calgiswabs are not recommended (tight adherence of specimen to swab). Reject the specimen if submitted on this type of swab.

E. Smears made from swabs received in charcoal transport media are unacceptable for staining. The charcoal particles make it difficult to visualize the organisms.

F. When the specimen is examined microscopically, always confirm that no fecal contamination (artifacts, vegetable debris, etc.) is present. This type of contamination is rare and would probably be limited to a urine specimen. However, if a urine or other urogenital specimen was contaminated with fecal material, it is possible that *P. hominis* (nonpathogen found in the intestinal tract) could be misidentified as *T. vaginalis,* an identification that implies sexual transmission.

G. Culture, immunoassay, and PCR methods are available for the detection of *T. vaginalis.*

IX. LIMITATIONS OF THE PROCEDURE

A. If the specimen is left at room temperature or held at a refrigerator temperature for a prolonged period (usually >1 h), the organisms will round up and eventually die.

B. If the patient has a *P. hominis* intestinal infection and the urogenital specimen becomes contaminated with fecal material, a false-positive *T. vaginalis* result may be reported, because *P. hominis* and *T. vaginalis* are similar in shape. The position of the undulating membrane will allow differentiation between *T. vaginalis* and *P. hominis.*

REFERENCE

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology,* 5th ed. ASM Press, Washington, DC.

SUPPLEMENTAL READING

Eschenbach, D., H. M. Pollock, and J. Schachter. 1983. *Cumitech 17, Laboratory Diagnosis of Female Genital Tract Infections.* Coordinating ed., S. J. Rubin. American Society for Microbiology, Washington, DC.

Holmes, K. K., P. Mardh, P. F. Sparling, and P. J. Wiesner. 1990. *Sexually Transmitted Diseases.* McGraw-Hill, New York, NY.

APPENDIX 9.6.7–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. 0.85% NaCl

1. Dissolve in distilled water in an appropriate glass flask by using a magnetic stirrer.

   sodium chloride (NaCl) ..... 850 mg
   distilled water ..... 100 ml

2. Store in a glass bottle.
3. Label as 0.85% NaCl with a preparation date and an expiration date of 6 months. Store at room temperature.

B. Giemsa stain

For preparation of Giemsa stain and phosphate buffer solutions, see procedure 9.8.5.

C. Absolute methanol

APPENDIX 9.6.7–2

Diagnostic differential characteristics

Characteristic	*T. vaginalis*	*P. hominis*
Size	7–23 µm long	5–15 µm long
	5–15 µm wide	7–10 µm wide
Shape	Pear	Pear
Flagella	4 anterior	3–5 anterior
Undulating membrane	Extends half of organism length	Extends entire length with free trailing flagella
Axostyle	Present	Present
Nucleus	Anterior end, oval	Anterior end, oval

9.6.8 Urine Concentration: Centrifugation

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Helminthic larval stages and eggs and some protozoa infecting humans may be found in the urine whether or not they cause pathologic sequelae in the urinary tract. Filariasis can be diagnosed from urine samples, and *Trichomonas vaginalis* and *Schistosoma haematobium* eggs can be detected in the urine. Microfilariae may be detected in the urine of heavily infected patients or of patients recently treated with diethylcarbamazine. Some of the microsporidia, such as *Encephalitozoon intestinalis*, can be found in the urine, particularly that of immunosuppressed patients, including those with AIDS.

II. SPECIMENS

Observe standard precautions.

Urine

A. *T. vaginalis*

Collection of first-voided urine, particularly after prostatic massage in male patients, is useful for the diagnosis of this infection.

B. *S. haematobium*

Collection of a midday urine specimen or a 24-h collection in a container without preservatives is recommended. Peak egg excretion occurs between noon and 3 p.m. In patients with hematuria, eggs may be found trapped in the blood and mucus in the terminal portion (last-voided portion) of the urine specimen.

C. Filariasis

Microfilariae may be detected in urine of patients with chyluria, of patients with very heavy filarial infections, and of patients treated with diethylcarbamazine.

D. Microsporidia

Microsporidial spores may be detected in concentrated urine of patients who are immunosuppressed, including those with AIDS. A number of different stains can be used, such as modified trichrome and optical brightening agents.

III. MATERIALS

A. Reagent (*see* Appendix 9.6.8–1)

B. Supplies

1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. Small tubes containing 0.5 to 1.0 ml of 0.85% NaCl
5. Conical centrifuge tubes (15 ml)
6. Erlenmeyer flask (1,000 ml)

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
3. Tabletop centrifuge (for tubes containing specimen and 0.85% NaCl if the specimen is submitted in saline)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check the direct-mount reagent each time it is used. The saline should be clear, with no visible contamination.

B. The microscope should have been calibrated within the last 12 months, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Known positive microscope slides, Kodachrome (2- by 2-in.) slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

D. Record all QC results.

V. PROCEDURE

A. If a 24-h urine sample was collected, allow the specimen to sediment for 2 h, and decant the major portion of the supernatant. There may be 100 to 200 ml of sediment left. If a first-voided urine specimen is received, use the entire specimen.

B. Place the remaining urine specimen (sediment) in centrifuge tubes.

C. Centrifuge the specimen at 500 $\times$ *g* for 10 min.

D. Decant the supernatant fluid.

E. With a pipette, mix and aspirate the sediment.

F. Place 1 drop of the sediment on a microscope slide.

G. Place a coverslip on top of the sediment.

H. Observe the specimen under the coverslip at magnifications of $\times$100 and $\times$400. Examine the entire coverslip at $\times$100 and at least half the coverslip at $\times$400.

VI. RESULTS

A. If motile flagellates are seen (axostyle and undulating membrane), then the trophozoites of *T. vaginalis* are present.

B. If live microfilariae are seen, then confirm or accomplish species identification by using permanent stains (1) (*see* procedures 9.8.5, 9.8.6, and 9.8.8).

C. If eggs of *S. haematobium* are seen, observe the eggs for live miracidia. If flame cell activity (motile cilia) is detected inside the miracidium larva, the miracidium is viable. A hatching test may also be used to determine if the eggs are viable (1) (*see* procedure 9.5.5).

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. *T. vaginalis*

Report the organism. Do not report the stage, since there is no known cyst stage for the trichomonads. The organisms do not need to be quantitated.

Example: *Trichomonas vaginalis* present.

B. Filariae

Report the presence of microfilariae. Genus and species should be reported if possible. The organisms do not need to be quantitated.

Example: *Wuchereria bancrofti* microfilariae present.

VII. REPORTING RESULTS *(continued)*

C. *S. haematobium*

If eggs are present, report the genus and species and whether the eggs are viable or nonviable.

Examples: *Schistosoma haematobium* eggs present (viable eggs seen).
Schistosoma haematobium eggs present (nonviable, eggshells only).

VIII. PROCEDURE NOTES

A. Specimens to be examined for *T. vaginalis* should be delivered to the laboratory as soon as possible after collection. Hold all specimens at room temperature because refrigerator temperatures will have a deleterious effect on *T. vaginalis.*

B. Species identification of the microfilariae from unstained preparations (urine sediment) may not be possible, and permanent stains may be necessary for further identification (1).

C. It is very important that all urine specimens (24-h and single-voided specimens) be collected with no preservatives. It is clinically important to determine whether the eggs are viable. This can be accomplished by examining eggs in the wet preparations at $\times 400$.

D. It is possible, although much less likely, that *Schistosoma mansoni* or *Schistosoma japonicum* eggs could also be recovered in urine. Therefore, the egg morphology must be carefully examined for accurate identification to species.

E. For the detection of *T. vaginalis*, filariae, and schistosomes, reject specimens, including midday urine samples, that are more than 24 h old.

F. Reject all 24-h urine specimens that are more than 48 h old.

G. When the specimen is examined microscopically, always confirm that no fecal contamination (artifacts, vegetable debris, etc.) is present. This type of contamination is rare and would probably be limited to a urine specimen. However, if a urine or other urogenital specimen was contaminated with fecal material, it is possible that *Pentatrichomonas hominis* (nonpathogen found in the intestinal tract) could be misidentified as *T. vaginalis*, an identification that implies sexual transmission.

IX. LIMITATIONS OF THE PROCEDURE

A. If the urine specimen is left at room temperature or held at a low temperature for a prolonged period, *T. vaginalis* may round up, become nonmotile, and eventually die.

B. If the patient has a *P. hominis* intestinal infection and the urogenital specimen becomes contaminated with fecal material, a false-positive *T. vaginalis* result may be reported because *P. hominis* and *T. vaginalis* are similar in shape.

C. Microfilariae can be identified to the species level only by making a permanent stained slide from the specimen.

REFERENCE

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.

SUPPLEMENTAL READING

Eschenbach, D., H. M. Pollock, and J. Schachter. 1983. *Cumitech 17, Laboratory Diagnosis of Female Genital Tract Infections.* Coordinating ed., S. J. Rubin. American Society for Microbiology, Washington, DC.

Holmes, K. K., P. Mardh, P. F. Sparling, and P. J. Wiesner. 1990. *Sexually Transmitted Diseases.* McGraw-Hill, New York, NY.

APPENDIX 9.6.8–1

Include QC information on reagent container and in QC records.

Reagent

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

0.85% NaCl

A. Dissolve in distilled water in an appropriate glass flask by using a magnetic stirrer.

sodium chloride (NaCl)	850 mg
distilled water	100 ml

B. Store in a glass bottle.

C. Label as 0.85% NaCl with a preparation date and an expiration date of 6 months. Store at room temperature.

9.6.9 Urine Concentration: Membrane Filter (Nuclepore)

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Microfilariae may be detected in the urine of heavily infected patients or of patients recently treated with diethylcarbamazine. Eggs of *Schistosoma haematobium* can also be recovered in urine specimens (2). Microfilariae and *S. haematobium* eggs can be easily concentrated by passing the specimen through a membrane filter. The filter can then be observed through a microscope.

II. SPECIMENS

Urine

Observe standard precautions.

A. Filariasis

Microfilariae may be detected in urine of patients with chyluria, of patients with very heavy filarial infections, and of patients treated with diethylcarbamazine. Collect specimens as first-voided specimens or as a 24-h collection in a container without preservatives.

B. *S. haematobium*

Collection of a midday urine specimen or a 24-h collection in a container without preservatives is recommended. Peak egg excretion occurs between noon and 3 p.m. In patients with hematuria, eggs may be found trapped in the blood and mucus in the terminal portion (last-voided portion) of the urine specimen.

III. MATERIALS

A. Reagent (*see* Appendix 9.6.9–1)

B. Supplies

1. Disposable glass or plastic pipettes
2. Glass slides (1 by 3 in., or larger if you prefer)
3. Coverslips (22 by 22 mm; no. 1, or larger if you prefer)
4. Small tubes containing 0.5 to 1.0 ml of 0.85% NaCl
5. Nuclepore membrane filters (25 mm) of 3-, 5-, and 8-μm pore sizes
6. Filter holder (25 mm) that attaches to syringe
7. Syringe (10 ml)
8. Forceps

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives (or the approximate magnifications for low-power, high dry power, and oil immersion examination)
2. Oculars should be 10×. Some workers prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check the direct-mount reagent each time it is used. The saline should be clear, with no visible contamination.

B. The microscope should have been calibrated within the last 12 months, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Known positive microscope slides, Kodachrome (2- by 2-in.) slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

D. Record all QC results.

V. PROCEDURE

A. If a midday urine sample is used, proceed to step V.B; if a 24-h urine sample was collected for *S. haematobium* diagnosis, allow the specimen to sediment for 2 h, and decant the major portion of the supernatant. There may be 100 to 200 ml of sediment left.

B. Thoroughly mix the urine specimen.

C. Draw 10 ml of urine into the syringe. If the urine is excessively cloudy or turbid, $<$10 ml may have to be used.

D. Attach the filter holder containing the filter to the syringe. For *S. haematobium,* use 8-μm-pore-size filters; for *Wuchereria bancrofti, Brugia malayi,* and *Loa loa,* use 5-μm-pore-size filters; and for *Mansonella* species, use 3-μm-pore-size filters.

E. Express the urine through the filter.

F. Wash the membrane with physiological saline by removing the filter holder, drawing 10 ml of saline into the syringe, reattaching the filter holder, and expressing the saline through the filter.

G. Repeat step V.F, but fill the syringe with air instead of saline, and express the air through the filter.

H. Remove the filter holder from the syringe.

I. Disassemble the filter holder to expose the filter.

J. Remove the filter from the holder with forceps.

K. Place the filter upside down on a microscope slide.

L. With a Pasteur pipette, add 1 drop of saline to moisten the filter.

M. Examine the filter for microfilariae and eggs at a magnification of $\times$100.

VI. RESULTS

A. If live microfilariae are seen, then confirm or accomplish species identification by using permanent stains (1) (*see* procedures 9.8.5, 9.8.6, and 9.8.8).

B. If eggs of *S. haematobium* are seen, observe the eggs for live miracidia. If flame cell activity (motile cilia) is detected inside the miracidium larva, the miracidium is viable. A hatching test may also be used to determine if the eggs are viable (1).

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Filariae

Report the presence of microfilariae. Genus and species should be reported if possible. The organisms do not need to be quantitated.

Example: *Wuchereria bancrofti* microfilariae present.

B. *S. haematobium*

If eggs are present, report the genus and species and whether the eggs are viable or nonviable.

Examples: *Schistosoma haematobium* eggs present (viable eggs seen).
Schistosoma haematobium eggs present (nonviable, egg shells only).

VIII. PROCEDURE NOTES

A. Species identification of the microfilariae may not be possible from unstained preparations (urine sediment), and permanent stains may be necessary for further identification (1). Microfilariae will measure 3 to 10 μm in width by 160 to 330 μm in length. Depending on the species, a sheath may or may not be present.

B. It is very important that all urine specimens (24-h and single-voided specimens) be collected with no preservatives. It is clinically important to determine whether the eggs are viable. This can be accomplished by examining eggs in the wet preparations at ×400.

C. For the detection of filariae and schistosomes, reject specimens, including midday urine samples, that are more than 24 h old.

D. Reject all 24-h urine specimens that are more than 48 h old.

E. If you accidently put the filter right side up, do not add more than 1 drop of saline (the organisms may accidentally float off the filter and onto the glass slide).

IX. LIMITATIONS OF THE PROCEDURE

A. Microfilariae can be identified to the species level only by making a permanent stained slide from the specimen. For a method that uses the membrane filter, see reference 1.

B. A hatching test may also be used to determine if the eggs are viable. For a hatching method, refer to procedure 9.5.5.

C. Infrequently, eggs of other *Schistosoma* species may be recovered in the urine.

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
2. **Peters, P. A., A. A. F. Mahmoud, K. S. Warren, J. H. Ouma, and T. K. Arap Siongok.** 1976. Field studies of a rapid, accurate means of quantifying *Schistosoma haematobium* eggs in urine samples. *Bull. W. H. O.* **54:**159–162.

SUPPLEMENTAL READING

Nathan, M. B., A. Lambourne, and S. Monteil. 1982. Evaluation of a membrane (Nuclepore) filtration method using capillary blood for the detection of microfilariae. *Ann. Trop. Med. Parasitol.* **76:**339–345.

APPENDIX 9.6.9–1

Include QC information on reagent container and in QC records.

Reagent

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

0.85% NaCl

A. Dissolve in distilled water in an appropriate glass flask by using a magnetic stirrer.

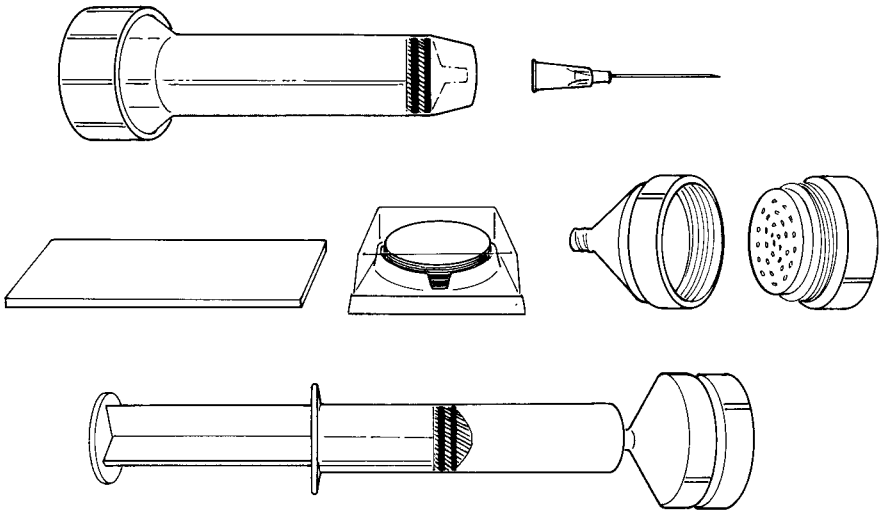
sodium chloride (NaCl)	850 mg
distilled water	100 ml

B. Store in a glass bottle.

C. Label as 0.85% NaCl with a preparation date and an expiration date of 6 months. Store at room temperature.

APPENDIX 9.6.9–2

Diagram of Nuclepore filtration system for the recovery of microfilariae. From top to bottom: syringe in sterile container (sterile syringe is not necessary) (top left) and needle (top right), slide (center left), package of filtration membranes (center middle), filter holder (center right, two pieces), and complete apparatus (bottom). (Illustration by Sharon Belkin.)



APPENDIX 9.6.9–3

Characterization of schistosome eggs

Species	Egg	
	Size	Shape
Schistosoma haematobium	112–170 by 40–70 μm	Elongate, terminal spine
Schistosoma japonicum	55–85 by 40–60 μm	Oval, minute lateral spine
Schistosoma mansoni	114–180 by 45–73 μm	Elongate, prominent lateral spine

9.7.1 Expectorated Sputum: Direct-Mount and Stained Preparations

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

A. A direct smear can be used to detect large or motile organisms from the lung. Parasites which can be detected and may cause pneumonia, pneumonitis, or Loeffler's syndrome include *Entamoeba histolytica, Paragonimus* spp., *Strongyloides stercoralis, Ascaris lumbricoides,* and hookworm. The smears can be examined with and without the addition of D'Antoni's or Lugol's iodine (*see* procedure 9.3.3).

B. Trichrome stains of material may aid in differentiating *E. histolytica* from *Entamoeba gingivalis,* and Giemsa stain may better define larvae and juvenile worms. Prepare a stain of material if organisms are found in examination of direct mounts which require additional differentiation.

C. Although *Cryptosporidium* spp. will be difficult to see in a direct mount, examination of smears stained with modified acid-fast stains (hot or cold method) may provide confirmation of pulmonary cryptosporidiosis.

D. In order to see microsporidial spores, centrifuged specimens stained with modified trichrome stains or optical brightening agents will be required (500 $\times$ *g* for 10 min).

II. SPECIMEN

Observe standard precautions.

A. Expectorated sputum specimens are collected after patient instruction in the appropriate measures to take to ensure quality specimens, including mouth wash before expectorating and exclusion of saliva from specimens.

B. Transport specimens to the laboratory in clean, closed containers. Select any blood-tinged, viscous areas for sampling.

C. If the specimen is uniformly mucoid, do the following.

1. Remove a 1.0-ml portion to a 15-ml conical tube.
2. Add 1.0 ml of mucolytic agent such as Sputolysin that has been prepared according to the manufacturer's instructions.
3. Incubate at room temperature for 15 min.
4. Add 2.0 ml of phosphate buffer (pH 6.8, 0.067 M).
5. Centrifuge the material at 1,000 $\times$ *g* for 5 min.
6. Decant supernatant, and use pelleted material to prepare wet mounts and smears.

III. MATERIALS

A. Reagents (*see* Appendix 9.7.1–1)

B. Supplies

1. Pasteur pipettes
2. 1- and 10-ml pipettes
3. Glass slides (2 by 3 in.)
4. Glass slides (1 by 3 in.)
5. 22- by 22-mm coverslips; no. 1 thickness
6. 15-ml conical centrifuge tubes
7. Coplin jars with lids
8. Protective gloves

C. Equipment

1. Centrifuge with carriers for 15-ml tubes with safety caps
2. Binocular microscope with 10×, 40×, and 100× (oil immersion) objectives; 50× oil objective optional but very helpful
3. Oculars should be 10×. Some may prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Saline (0.85% NaCl), clear and free of particulate material. If cloudy, discard.

B. Mucolytic agent free of contamination as determined by clear appearance. If cloudy, discard, and make new working solution.

C. Do a control trichrome stain for each new set of reagents with a specimen containing blood to see that WBCs stain with purple nuclei and blue-green cytoplasm (*see* procedure 9.3.6). If cells do not stain appropriately, change reagents.

D. With each new lot of Giemsa stain or new buffer, check stain with a specimen containing blood to see that RBCs stain grayish, WBC nuclei stain red-purple, and WBC cytoplasm stains bluish. If cells do not stain appropriately, check stock stain and buffer to find cause.

E. The microscope(s) should be calibrated, and the original optics used for the calibration should be in place on the microscope(s). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

F. Clean and check centrifuge for speed, and clean brushes once a year.

G. Maintain temperature of refrigerator where mucolytic-agent working solution is stored at 4°C (range, 2 to 8°C).

H. Store stock stain solutions in the dark. Store Giemsa stain tightly stoppered. Make fresh working solution each day. Use a clean, dry pipette to remove stain from stock stain container.

I. Known positive microscope slides, Kodachrome (2- by 2-in.) slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

J. Record all QC results.

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. Expectorated sputum (untreated with mucolytic agent)

1. With a Pasteur pipette, place 1 or 2 drops (50 μl) on one side of a glass slide (2 by 3 in.), and cover with a no. 1 coverslip (22 by 22 mm).
2. Place a second drop on the slide, add 1 drop of saline, and cover with a coverslip.

C. Material that has been treated with a mucolytic agent can be suspended in 100 μl of saline. Place 1 drop of the mixture on a slide (2 by 3 in.), and cover with a coverslip.

V. PROCEDURE *(continued)*

D. Reserve the specimen and remaining treated specimen for preparation of smears for staining should stains be required.

E. Examine the wet preparations field by field with low light and the 10× objective to detect eggs, larvae, oocysts, or amebic trophozoites.

F. If inconclusive, prepare smears of material for staining.

1. Place 1 drop of sediment in the center of each of three glass slides (1 by 3 in.), and spread the material with the tip of the pipette.

2. Place one slide in Schaudinn's fixative while wet, and dry the other two thoroughly.

3. Trichrome stain the slide fixed in Schaudinn's fixative (*see* procedure 9.3.6).

4. Fix the air-dried smears in methanol. Stain one with Giemsa (*see* procedure 9.8.5) and the other with a modified acid-fast stain (*see* procedures 9.4.1 and 9.4.2).

5. Put immersion oil on stained smears, and examine Giemsa-stained smear with the 10× objective and trichrome-stained smear with the 50× oil objective if available. Otherwise, use the 100× oil objective.

VI. RESULTS

A. Helminth larvae (rare) are more likely to be seen in the wet preparation with and/or without iodine (*see* procedures 9.3.3 and 9.3.4).

B. Protozoan trophozoites will generally be visible in the trichrome-stained smears.

C. Oocysts will be seen on the modified acid-fast preparation.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

Record any amebae, oocysts, larvae, or eggs detected by wet mounts.

A. Give genus and species of amebae after confirmation with permanent stain.

B. "No parasites found" in expectorated sputum are considered "normal flora"; therefore, call the physician if any organisms are detected.

VIII. PROCEDURE NOTES

In the United States, detection of parasites in expectorated sputum is rare. If characteristics of suspected parasites are not "classic," request another specimen for confirmation.

IX. LIMITATIONS OF THE PROCEDURE

If *Cryptosporidium* spp. are suspected, remember to centrifuge the sputum specimen for 10 min at 500 × *g*. Otherwise, the oocysts may not be found in the sediment used to prepare smears for modified acid-fast stains.

REFERENCES

1. **del Rio, C., J. Guarner, E. G. Honig, and B. A. Slade.** 1988. Sputum examination in the diagnosis of *Pneumocystis carinii* pneumonia in the acquired immunodeficiency syndrome. *Arch. Pathol. Lab. Med.* **112:**1229–1232.
2. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.

SUPPLEMENTAL READING

Melvin, D. M., and M. M. Brooke. 1975. *Laboratory Procedures for Diagnosis of Intestinal Parasites.* Department of Health, Education and Welfare publication no. (CDC) 76-8282. U.S. Government Printing Office, Washington, DC.

APPENDIX 9.7.1–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. Mucolytic agent such as Sputolysin Stat-Pack dithiothreitol solution (Behring Diagnostics, Inc.)

1. Store unopened at room temperature until expiration date published on package.
2. Store working solution at 4°C. Include the date made and the expiration date. Discard working solution after 48 h. Prepare working solution by removing 1.0 ml from the 10-ml bottle and diluting with 9.0 ml of sterile water.

B. Iodine reagents

1. D'Antoni's iodine (*see* procedure 9.3.3)
2. Lugol's iodine (*see* procedure 9.3.3)

C. Trichrome stain reagents

1. Schaudinn's fixative (*see* procedure 9.2.2)
2. Trichrome stain (*see* procedure 9.3.6)

D. Giemsa stain reagents

1. Giemsa stain (*see* procedure 9.8.5)
2. Phosphate buffer, pH 7.0 to 7.2 (*see* procedure 9.8.5)
3. Phosphate buffer plus Triton X-100 at 0.01% (*see* procedure 9.8.5)

Phosphate buffer solutions

pH	Na_2HPO_4 (9.5 g/liter) (anhydrous) (ml)	$NaH_2PO_4 \cdot H_2O$ (9.2 g/liter) (ml)	Distilled water (ml)
6.6	37.5	62.5	900
6.8	49.6	50.4	900
7.0	61.1	38.9	900
7.2	72.0	28.0	900

9.7.2 Aspirates and Bronchoscopy Specimens

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The examination of aspirated material for the diagnosis of parasitic infections is useful when routine specimens and methods have failed to demonstrate the organisms. Aspirates include liquid specimens collected from a variety of anatomic sites that delineate the types of organisms to be expected. Aspirates most commonly processed in the parasitology laboratory include fine-needle aspirates and duodenal aspirates. Fluid specimens collected by bronchoscopy include bronchoalveolar lavage (BAL) fluids and bronchial washings.

II. SPECIMENS

Observe standard precautions.

A. Fine-needle aspirates

When specimens are collected and sent to the laboratory for processing, slides must be stained appropriately for suspected organisms and examined microscopically. Suggested stains are Giemsa for *Toxoplasma gondii*, trichrome for amebae, and modified acid fast for *Cryptosporidium* spp.

B. Aspirates of cysts and abscesses

Aspirates to be evaluated for amebae may require concentration by centrifugation, digestion (streptokinase; *see* Appendix 9.7.2–1), microscopic examination for motile organisms in direct preparations, and cultures and microscopic evaluation of stained preparations (6).

C. Duodenal aspirates

Aspirates to be evaluated for *Strongyloides stercoralis, Giardia lamblia,* or *Cryptosporidium* spp. may require concentration by centrifugation prior to microscopic examination for motile organisms and permanent stains. In order to see microsporidial spores, centrifuged sediment stained with modified trichrome stains or optical brightening agents will be required.

D. Bone marrow aspirates

Aspirates to be evaluated for *Leishmania* amastigotes, *Trypanosoma cruzi* amastigotes, or *Plasmodium* spp. require Giemsa staining.

E. Fluid specimens collected by bronchoscopy

Specimens may be lavage fluids or washings, with BAL fluids preferred (7). Specimens are usually concentrated by centrifugation prior to microscopic examination of stained preparations (Sputolysin; *see* Appendix 9.7.2–1). Organisms discussed here which may be detected are *T. gondii, Cryptosporidium,* and the microsporidia (1, 4).

III. MATERIALS

A. Reagents (*see* Appendix 9.7.2–1)

B. Supplies

1. Pasteur pipettes
2. 1- and 10-ml pipettes
3. Glass slides (2 by 3 in.)
4. Glass slides (1 by 3 in.)
5. Coverslips (22 by 22 mm; no. 1 thickness)
6. Centrifuge tubes, 15 and 50 ml, conical
7. Glass Coplin jars
8. Plastic Coplin jar with lid
9. Stain rack
10. Graduated cylinder
11. Bleach
12. Protective gloves

C. Equipment

1. Centrifuge with carriers for 15- and 50-ml tubes with safety caps
2. Binocular microscope with 10×, 40× or 50× oil, and 100× oil immersion objectives
3. Fluorescence microscope with filters to obtain appropriate wavelength of light for fluorescent label used if immunostaining
4. Microwave oven or water bath and thermometer

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Incorporate control slides in all stain procedures.

1. Material containing RBCs and WBCs should be used as a control for Giemsa and trichrome staining.
2. Material containing *Cryptosporidium* should be used as a control for modified acid-fast staining.
3. Stains cannot be evaluated if controls do not stain appropriately.

B. Both Sputolysin and streptokinase working reagents should be stored at 4°C and made fresh after 48 h.

C. Incubate new lots of media to check for sterility, and inoculate samples with stock amebae to see that medium supports growth (*see* procedures 9.9.1 to 9.9.3 and 9.9.5).

D. Clean and check centrifuge for speed, and clean brushes once a year.

E. Maintain temperature of refrigerator where mucolytic agent working solution is stored at 4°C (range, 2 to 8°C).

F. With each new lot of Giemsa stain or new buffer, check stain with a specimen containing blood to see that RBCs stain grayish, WBC nuclei stain red-purple, and WBC cytoplasm stains bluish. If cells do not stain appropriately, check stock stain and buffer to find cause (*see* procedure 9.8.5).

G. The fluorescence microscope can be evaluated for correct light wavelength for label with commercially available QC slides.

H. Check incubator for temperature maintenance at 35 ± 2°C.

I. Known positive microscope slides, Kodachrome (2- by 2-in.) slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

J. Record all QC results.

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. Specimens that contain mucus may be treated with a mucolytic agent by adding a volume of agent equal to or one-half to two-thirds of the volume of specimen and incubating at room temperature for 15 min. Centrifuge at 1,000 × *g* for 5 min, and use sediment to prepare wet mounts and smears for staining.

C. Specimens that contain cell debris and proteinaceous material and that require digestion should be treated with streptokinase (1 part enzyme solution to 5 parts specimen) for 1 h at 35°C. Shake at intervals (every 15 min). Centrifuge at 1,000 × *g* for 5 min, and use sediment to prepare wet mounts and smears for staining.

V. PROCEDURE *(continued)*

D. Specimens that include significant amounts of blood require treatment with an agent to lyse RBCs. Add 1 volume of lysing agent per volume of specimen, and incubate at room temperature for 5 min.

E. Place representative samples of untreated and Lyse-treated specimens in 15-ml conical centrifuge tubes, and centrifuge at 1,000 × *g* for 5 min. For BAL or bronchial-washing specimens, which usually are 50 ml or more, place 20 to 24 ml of each specimen in a 50-ml conical centrifuge tube, and centrifuge as described above.

F. Decant supernatants from centrifuged samples into a disposal container containing disinfectant.

G. With a Pasteur pipette, remove drops of sediment for wet mounts, stain preparations, and culture.

H. For duodenal aspirates and aspirates from cysts or abscesses, place 1 drop of sediment on a glass slide (2 by 3 in.), add a drop of 0.85% NaCl, and cover with a no. 1 coverslip (22 by 22 mm).

I. Examine preparation field by field with low light until the entire mount has been examined.

J. If the wet mount is equivocal for a protozoan, place a drop of sediment on a glass slide (1 by 3 in.), and add a drop of polyvinyl alcohol (PVA) fixative. Mix the drops with a pipette, and spread the mixture into an even film (about 22 by 22 mm). Dry the preparation thoroughly, and stain with trichrome stain.

K. Trichrome stain (*see* procedure 9.3.6)

L. For material from cysts or abscesses, prepare cultures by adding 0.5 ml of material to a tube of culture medium for the recovery of amebae in accordance with procedure 9.9.1.

M. Aspirates of bone marrow may be submitted for diagnosis of leishmaniasis, trypanosomiasis, and occasionally for malaria. Material should be stained with Giemsa stain and examined with the 100× oil immersion objective.

N. Sediments from BAL or bronchial washings are examined in stained preparations. Three slides should be stained.

1. Use a Pasteur pipette to place drops of sediment from each specimen on at least four slides. With the pipette, spread the sediment into a thin, even film. For specimens treated with an agent to lyse RBCs, use sediment from both treated and untreated samples for smears.
2. Air dry slides, and fix in methanol. If slides are to be stained with immunospecific stains, fix according to package instructions.
3. Stain one slide with rapid Giemsa stain (Diff-Quik or Giemsa Plus).
   Rapid Giemsa stain procedure
   - **a.** Stain solutions should be kept in dropper bottles to avoid bacterial contamination. Place 1 or 2 drops of red stain solution 1 on specimen smear and control slide, hold for 10 s, and drain.
   - **b.** Add 1 or 2 drops of blue solution 2, hold for 10 s, drain, and rinse very briefly with deionized water.
   - **c.** Stand slides on end to drain and air dry.
   - **d.** Slides must be examined with oil or mounted with mounting medium.
4. Stain one slide with modified acid-fast stain (*see* procedure 9.4.1).
5. Stain one slide with trichrome stain.

O. Put oil on stained slides or mount. Examine stained slides with 50× oil immersion objective, if available; otherwise, use 100× oil objective. If any slides are equivocal, stain additional slides.

P. Slides of fine-needle aspirates from lung are stained as described above (step V.N) for BAL and bronchial washings. Fine-needle aspirates of lesions likely to contain amebae are stained as described above (steps V.H through L) for cysts and abscesses.

V. PROCEDURE *(continued)*

Q. Generally portions of aspirate specimens are shared with bacteriology, and it is always important to consider the importance of a Gram stain in ruling out bacterial etiology.

VI. RESULTS

A. Helminth larvae (rare) are more likely to be seen in the wet preparation (specimens from bronchoscopy) with and/or without iodine.
B. Protozoa will be visible in the trichrome- or Giemsa-stained smears.
C. Oocysts will be seen on the modified acid-fast preparation.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Organisms detected should be reported as follows.
  1. From fine-needle aspirates and aspirates of cysts and abscesses, report *T. gondii* and *Entamoeba histolytica* or *Acanthamoeba* spp. if morphology in stained preparations is classic. If ameba cultures are positive, species can be determined. Since the only *Entamoeba* sp. causing disseminated disease is *E. histolytica*, identification to the species level does not require positive culture.
  2. From bone marrow aspirates, report *Leishmania donovani, T. cruzi,* or *Plasmodium* spp. if forms demonstrated in stains are classic.
  3. From BAL specimens and bronchial washings, report *T. gondii* if characteristic tachyzoites are detected in Giemsa stain and *Cryptosporidium* spp. if characteristic oocysts are detected in modified acid-fast or immunospecific stains. Note the quality of the specimen. If only a few alveolar cells are present, failure to find organisms may not rule out infection.
B. Notify clinician if any organisms described are detected.

VIII. PROCEDURE NOTES

A. Fine-needle aspirates often vary from stick to stick. Examine Giemsa-stained slides with a low-power (10×) objective to find cellular areas of slides to screen carefully. If one stick has better material than others, use slides of this specimen for other staining.
B. With aspirates of cysts and abscesses, material may vary from thin, with few cells, to very thick. In thick material, select several samples, including bloody areas, for digestion. Organisms will often be found in the bloody material aspirated last.
C. With duodenal aspirates, if strongyloidiasis is expected, examine all of the specimen sediment in wet mounts with the 10× objective.
D. Aspirates of bone marrow are rarely submitted for examination for parasites. In the United States, splenic aspirations are very rarely performed.
E. With BAL specimens and bronchial washings, if *Cryptosporidium* spp. oocysts are found, report their detection, although their presence may not be clinically significant. *Toxoplasma* is being detected more frequently in BAL specimens, particularly those from human immunodeficiency virus-positive individuals (2).

REFERENCES

1. **Brinn, N. T.** 1983. Rapid metallic histological staining using the microwave oven. *J. Histotechnol.* **6:**125–129.
2. **Derouin, F., C. Sarfati, B. Beauvais, M. Iliou, L. Dehen, and M. Lariviere.** 1989. Laboratory diagnosis of pulmonary toxoplasmosis in patients with acquired immunodeficiency syndrome. *J. Clin. Microbiol.* **27:**1661–1663.
3. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
4. **Haley, L. D., and P. G. Standard.** 1973. *Laboratory Methods in Medical Mycology*, p. 100. Center for Disease Control, Atlanta, GA.
5. **Martin, W. J., II, T. F. Smith, D. R. Sanderson, W. M. Brutinel, F. R. Cockerill III, and W. W. Douglas.** 1989. Role of bronchoalveolar lavage in the assessment of opportunistic pulmonary infection: utility and complication. *Mayo Clin. Proc.* **62:**549–557.
6. **McQuay, R. M.** 1956. Charcoal medium for growth and maintenance of large and small races of *Entamoeba histolytica. Am. J. Clin. Pathol.* **26:**1137–1138.

APPENDIX 9.7.2–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. Staining reagents

1. Giemsa stain reagents (*see* procedure 9.8.5)
   a. Giemsa stain (*see* procedure 9.8.5)
   b. Phosphate buffer, pH 7.0 to 7.2 (*see* procedure 8.2)
   c. Phosphate buffer plus Triton X-100 at 0.01% (*see* procedure 9.8.5)
   d. Rapid Giemsa
      (1) Diff-Quik (Baxter Scientific)
      (2) Giemsa Plus (Trend Scientific)
2. Trichrome stain reagents (*see* procedure 9.3.6)
   a. Schaudinn's fixative (*see* procedure 9.2.2)
   b. PVA fixative (*see* procedure 9.2.2)
3. Acid-fast stain reagents
   a. Kinyoun's acid-fast stain (cold) (*see* procedure 9.4.1)
   b. Modified acid-fast stain (hot) (*see* procedure 9.4.2)

B. Culture media for *E. histolytica* (*see* procedure 9.9.1)

C. Materials for digestion

1. Sputolysin Stat-Pack dithiothreitol (Behring Diagnostics)
   Prepare (according to package directions) a 1:10 dilution with distilled water (include expiration date). Store no longer than 48 h in refrigeration.
2. Lysing agent (available from most major laboratory suppliers)
3. Streptokinase
   Reconstitute streptokinase and streptodornase (Veridase; Lederle Laboratory) as directed by manufacturer.

D. Diluent

0.85% NaCl

9.7.3 Biopsy Specimens

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Biopsy specimens are recommended for the diagnosis of tissue parasites. The procedures that may be used for this purpose in addition to standard histological preparations are impression smears and teased and squash preparations of biopsy tissue from skin, muscle, cornea, intestine, liver, lung, and brain. Tissue to be examined by permanent sections or electron microscopy should be fixed as specified by the laboratories which will process the tissue.

II. SPECIMENS

Observe standard precautions.

Tissue submitted in a sterile container on a sterile sponge dampened with saline may be used for protozoan cultures after mounts for direct examination or impression smears for staining have been prepared. If cultures for parasites will be made, use sterile slides for smear and mount preparation.

A. To prepare sterile slides for impression smears, lay six to eight glass slides (1 by 3 in.) on a paper towel, fold the towel (containing the slides) accordion fashion, tape, and autoclave using routine temperature and time frames (*see* procedure 9.8.4). An alternative method would be to soak the slides in 95% ethyl alcohol and flame them prior to use.

B. Use sterile (autoclaved or flamed) forceps for handling tissue.

C. Place tissue in a sterile petri dish to examine macroscopically and to select sample for microscopic evaluation. Minced tissue can be used if it is kept sterile.

1. If biopsy tissue is several millimeters to a centimeter in size, select from area differing from normal. For example, select gray consolidated or granulomatous portion of lung or ulcerated area of intestinal tissue.

2. If tissue is one or several small fragments a few millimeters in size that look alike, use one fragment. If tissue fragments look different, use one of each type for microscopic examination.

D. Prepare impression smears.

1. Blot tissue sample on sterile toweling. If the sample is large enough, cut the tissue, and use the cut surface to touch the slide.

2. Press tissue against slide, lift, and press again. Turn sample over and press against slide to make two more impressions. Keep impressions close together to speed screening. If several tissue samples were selected, make impressions in a row with each sample. For example, place sample 1 on top, sample 2 in the middle, and sample 3 at bottom, making impressions from left to right with each sample rather than from top to bottom. This scheme avoids differences in sample staining related to location.

II. SPECIMENS *(continued)*

3. Air dry smears, and fix in methanol for 1 min for subsequent Giemsa, methenamine-silver nitrate, and modified acid-fast staining. If the amount of tissue is sufficient, prepare multiple smears for each stain selected.
4. Place wet slide in Schaudinn's fixative for subsequent trichrome staining.
5. Fix slide according to manufacturer's directions for immunospecific staining.

E. Teased preparations

1. Place sample in the bottom of a plastic petri dish. Cover with 2 to 4 drops of saline (100 to 200 μl).
2. Gently tease tissue with needles, or hold tissue with forceps while pulling apart with a scalpel.
3. Put cover on dish, and leave at room temperature for 30 min.

F. Squash preparation

Cut selected tissue portions into very fine fragments with a scalpel. Place a fragment on a slide (1 by 3 in.), add 1 drop of saline, cover with a second slide (1 by 3 in.), and hold together with membrane clips (surgical supply company). If these are not available, use paper clips, but they are not as efficient.

G. Skin scrapings

Request that scrapings be sent between two glass slides or in a small vial.

H. Culture

Prepare cultures to demonstrate the following organisms.

1. *Entamoeba histolytica* (*see* procedure 9.9.1) (10)
2. *Acanthamoeba* spp. and *Naegleria* spp. (*see* procedure 9.9.2)
3. *Leishmania* spp. (*see* procedure 9.9.5)

I. Mouse passage for *Toxoplasma gondii* (19)

1. Grind tissue in 0.85% NaCl until a fine suspension results.
2. Intraperitoneally inject three to five mice of any laboratory strain that weigh ~20 g each with 0.2 to 0.4 ml of suspension.
3. House mice in isolation.
4. Check daily for signs of central nervous system (CNS) dysfunction. If CNS symptoms are detected, proceed to animal autopsy (*see* item V.F.4 below).

III. MATERIALS

A. Reagents (*see* Appendix 9.7.3–1)

B. Supplies

1. Pasteur pipette
2. Sterile glass slides (1 by 3 in.)
3. Petri dishes
4. Glass Coplin jars with lids
5. Plastic Coplin jars with lids
6. Pipettes, 1 and 10 ml
7. Scalpels
8. Forceps
9. Graduated cylinders
10. Stain rack
11. Protective gloves
12. Tissue grinder

C. Equipment

1. Microwave oven or water bath (80°C) and thermometer
2. Binocular microscope with 10×, 40×, or 50× oil, and 100× oil immersion objectives
3. Fluorescence microscope with filters to obtain appropriate wavelength of light for fluorescent label used if immunostaining

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Incorporate control slides in all stain procedures.

1. Material containing amebae should be used as a control for trichrome staining.
2. Material containing RBCs and WBCs may be used as a control for Giemsa staining (*see* procedure 9.8.5).
3. Material containing *Cryptosporidium* should be used as a control for modified acid-fast staining.
4. Stains cannot be evaluated if controls do not stain appropriately.

IV. QUALITY CONTROL *(continued)*

B. To check for sterility, incubate new lots of media and inoculate stock ameba cultures of *E. histolytica* to see that medium supports growth (*see* procedure 9.9.1).

C. In refrigerator where culture medium is stored, maintain temperature at 4°C. Remember, daily temperature recording is required by Clinical Laboratory Improvement Amendments of 1988 regulations.

D. With each new lot of Giemsa stain or new buffer, check stain with specimen containing blood to see that RBCs stain grayish, WBC nuclei stain red-purple, and WBC cytoplasm stains bluish. If cells do not stain appropriately, check stock stain and buffer to find cause.

E. The fluorescence microscope can be evaluated for correct light wavelength for label with commercially available QC slides (*see* Appendix 9.10.6–1).

F. Check incubator for temperature maintenance at 35 ± 2°C.

G. Known positive microscope slides, Kodachrome (2- by 2-in.) slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

H. Record all QC results.

V. PROCEDURE

A. Impression smears

Stain and examine to detect possible organisms according to specimen and clinical history (Table 9.7.3–1).

1. Giemsa stain (*see* procedure 9.8.5)

2. Modified acid-fast stain (*see* procedures 9.4.1 and 9.4.2)

3. Trichrome stain (*see* procedure 9.3.6)

4. Modified trichrome stain (*see* procedures 9.4.3, 9.4.4, and 9.4.5). However, many biopsy specimens for the microsporidia are sent for routine histology processing and staining with hematoxylin and eosin (H&E), tissue Gram stains (Brown and Brenn, Brown and Hopps), silver stains (Warthin-Starry), or periodic acid-schiff (PAS).

a. Follow package directions exactly.

b. Examine stained slides microscopically for suspected organisms. Send slides to referral laboratory if necessary.

B. Teased preparation

Skin snips for detection of microfilariae of *Onchocerca volvulus* and *Mansonella streptocerca*

1. Tease the small bit of tissue apart in a few drops of saline to release the microfilariae.

2. Remove drops of saline to a glass slide (1 by 3 in.), cover with no. 1 coverslip, and examine with low light for microfilariae.

3. For a permanent record, run alcohol under coverslip to fix filariae, partially dry, remove coverslip, and stain with Giemsa.

C. Squash preparation for muscle detection of *Trichinella spiralis*

Examine microscopically with low power (100×) and low light.

D. Scrapings of skin for scabies

Examine scrapings microscopically at low power (100×) and low light.

E. Inoculate cultures with ground tissue suspensions (to release organisms from the cells).

1. Place small tissue sample in sterile tissue grinder (Ten Broeck or Dounce) in 0.5 ml of sterile saline, and grind until tissue is dispersed.

2. Add several drops of ground tissue to culture medium as follows.

a. To NNN medium (*see* procedure 9.9.5), add drops at bottom of slant, where they will "pool" with condensed moisture on slant. Incubate at room temperature (isolation of *Leishmania* spp.).

Table 9.7.3–1 Stains for identifying parasites in various tissues[a]

Tissue	Possible parasite(s)	Stain(s)[b]
Lung	*T. gondii*	Giemsa, immunospecific (5)
Lung	*E. histolytica*	Trichrome (15), Giemsa
Lung	Microsporidia	Giemsa
Liver	*T. gondii, Leishmania donovani*	Giemsa
Liver	*Cryptosporidium* spp.	Modified acid fast (8), immunospecific (1, 14)
Liver	*E. histolytica*	Giemsa, trichrome
Brain	Free-living amebae	Giemsa, trichrome
Brain	*E. histolytica*	Giemsa, trichrome
Brain	*T. gondii*	Giemsa, immunospecific
Brain	Microsporidia	Acid fast, Giemsa, Gram (confirm with EM) (13)
Brain	*Encephalitozoon cuniculi*	Giemsa (confirm with EM)
Brain	Helminth larvae	
Skin	*Leishmania* spp.	Giemsa
Skin	*O. volvulus*	Giemsa
Skin	*M. streptocerca*	Giemsa
Intestine		
Small intestine	*Cryptosporidium* spp.	Modified acid fast (6), immunospecific (1, 14)
Jejunum	*Enterocytozoon bieneusi*	Giemsa (confirm with EM) (2, 3, 12)
Duodenum	*Giardia lamblia*	Trichrome
Colon	*E. histolytica*	Giemsa, trichrome
	Cryptosporidium spp.	Modified acid fast (6), immunospecific (1, 14)
Cornea, conjunctiva	*Microsporidium* spp., *Nosema* sp.	Acid fast, Giemsa (confirm with EM) (4)
Cornea, conjunctiva	*Acanthamoeba* sp.	Giemsa, trichrome, calcofluor for cysts (7)
Kidney	*Encephalitozoon* spp.	Giemsa (confirm with EM)
Muscle	*T. spiralis, Pleistophora* spp., *Trachepleistophora* spp.	Giemsa (confirm with EM)

[a] *Note:* In disseminated infections with microsporidia, spores and developing stages from a number of different species could be found throughout the body. Routine histology processing and staining (H&E, silver, Gram, PAS) are used, as are Giemsa, modified trichrome stains, and optical brightening agents. However, the modified trichrome stains (chromotrope) are more commonly used and recommended for clinical specimens other than tissues, such as stool, urine, eye specimens, etc.

[b] EM, electron microscopy.

V. PROCEDURE *(continued)*

b. To TYSGM-9 medium (*see* procedure 9.9.1), add several drops to the liquid medium, add 3 drops of the starch suspension, and incubate at a 45° to 50° angle at 35°C for 48 h (isolation of *E. histolytica*).

c. Add drops to center of seeded agar plate, and incubate at 35 to 37°C (isolation of *Acanthamoeba* spp. or *Naegleria* spp.).

F. Examine cultures.

1. TYSGM-9 medium for amebae (*see* procedure 9.9.1)

With a Pasteur pipette, remove 1 or 2 drops of material adhering to sides or bottom of tube, place on glass slide, cover with coverslip, and examine microscopically (100×) with low light.

V. PROCEDURE *(continued)*

2. Agar plate with lawn of *Escherichia coli* or *Enterobacter* for detection of free-living amebae (*see* procedure 9.9.2)
Examine microscopically at low power (100×) for changes in bacterial lawn, particularly patches and "tracks."

3. NNN agar for promastigotes of *Leishmania* spp. (11) (*see* procedure 9.9.5)
- **a.** With a Pasteur pipette, remove a drop of fluid from interface of agar slant and condensed moisture.
- **b.** Place on glass slide, and cover with coverslip.
- **c.** Examine microscopically (400×) with low light for motile promastigotes.

4. Mouse passage for detection of *T. gondii* (9)
- **a.** Wear canvas gloves to handle mice. Gardening gloves work well.
- **b.** Sacrifice mice.
- **c.** Pin mouse to board, spray with 70% ethyl alcohol, and open peritoneal cavity with sterile scissors.
- **d.** Remove fluid from the peritoneal cavity with sterile Pasteur pipette, and place in small tube or make slide directly.
- **e.** Make Giemsa-stained smears of peritoneal exudate, and examine microscopically at 1,000× for *T. gondii* tachyzoites.
- **f.** Place mice and all contaminated disposable materials in bag to be autoclaved and destroyed. Place nondisposable materials (scissors) in bag to be autoclaved prior to washing.

G. Correlate all examination results (wet mount, stains, and cultures) to determine presence of organisms.

VI. RESULTS

A. The majority of the protozoa will be found on the specimen permanent stained smears (impression smears, touch or squash preparations, or teased preparations).

B. When culture is used, permanent stained smears of cultural material may also reveal some protozoa (10).

C. Although infrequently used, material from animals (at autopsy) can be examined as both wet and permanent stained preparations for confirmation of protozoa.

D. Filarial infections may be confirmed by the recovery and identification of microfilariae in skin scrapings and/or biopsy samples.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

Report organisms detected if identifications are certain. If presumed parasites for which the laboratory has no corresponding reference material are found, confirmation by another laboratory is suggested.

VIII. PROCEDURE NOTES

A. Immunospecific staining reagents are rapidly becoming more available. Evaluations of monoclonal antibodies and staining techniques such as indirect fluorescence, direct fluorescence, and enzyme tagging have been variable. In using newly developed tests, closely follow directions, and include all possible control measures. In addition to new tests, there are "new" organisms.

B. Daily, more is being learned about the microsporidia. Most individuals working with new pathogens or developing new tests will gladly accept referral material. Check references for workers to whom material might be sent for confirmatory identification.

IX. LIMITATIONS OF THE PROCEDURE

Success in detection of parasites in tissue depends in part on the adequacy of the specimen. Biopsy specimens are often very tiny and may not be representative of the whole infectious process. The availability of more than one tissue sample taken by transbronchial biopsy enhances diagnosis. To optimize the yield from any tissue specimen, examine all areas, and use as many means of organism detection as possible. The tissue sample results from an invasive costly procedure and deserves the most exhaustive examination possible.

REFERENCES

1. **Arrowood, M. J., and C. R. Sterling.** 1989. Comparison of conventional staining methods and monoclonal antibody-based methods for *Cryptosporidium* oocyst detection. *J. Clin. Microbiol.* **27:**1490–1495.
2. **Cali, A., and R. L. Owen.** 1990. Intracellular development of *Enterocytozoon*, a unique microsporidian found in the intestine of AIDS patients. *J. Protozool.* **37**(2)**:**145–155.
3. **Canning, E. U., and W. S. Hollister.** 1990. *Enterocytozoon bieneusi* (Microspora): prevalence and pathogenicity in AIDS patients. *Trans. R. Soc. Trop. Med. Hyg.* **84:**181–186.
4. **Centers for Disease Control.** 1990. Microsporidian keratoconjunctivitis in patients with AIDS. *MMWR Morb. Mortal. Wkly. Rep.* **39:**188–189.
5. **Derouin, F., C. I. Sarfati, B. Beauvais, M. Iliou, L. Dehen, and M. Lariviere.** 1989. Laboratory diagnosis of pulmonary toxoplasmosis in patients with acquired immunodeficiency syndrome. *J. Clin. Microbiol.* **27:**1661–1663.
6. **Fayer, R., and B. L. P. Ungar.** 1986. *Cryptosporidium* spp. and cryptosporidiosis. *Microbiol. Rev.* **50:**458–483.
7. **Griffin, J. L.** 1978. Pathogenic free-living amoebae, p. 507–549. *In* J. P. Kreier (ed.), *Parasitic Protozoa*, vol. 15. Academic Press, Inc., New York, NY.
8. **Haley, L. D., and P. G. Standard.** 1973. *Laboratory Methods in Medical Mycology*, p. 100. Center for Disease Control, Atlanta, GA.
9. **Jones, F. E., D. E. Eyles, N. Coleman, and C. L. Gibson.** 1958. A comparison of methods for the isolation of *Toxoplasma* from suspected hosts. *Am. J. Trop. Med.* **7:**531–535.
10. **McQuay, R. M.** 1956. Charcoal medium for growth and maintenance of large and small races of *Entamoeba histolytica. Am. J. Clin. Pathol.* **26:**1137–1138.
11. **Novy, F. G., and W. J. MacNeal.** 1904. The cultivation of *Trypanosoma brucei. J. Infect. Dis.* **1:**1–30.
12. **Rijpstra, A. C., E. U. Canning, R. J. Van Ketel, J. K. M. Eeftinck Schattenkerk, and J. J. Laarman.** 1988. Use of light microscopy to diagnose small-intestinal microsporidiosis in patients with AIDS. *J. Infect. Dis.* **157:**827–831.
13. **Shadduck, J. A.** 1989. Human microsporidiosis and AIDS. *Rev. Infect. Dis.* **11:**203–207.
14. **Sterling, C. R., and M. J. Arrowood.** 1986. Detection of *Cryptosporidium* sp. infections using a direct immunofluorescent assay. *Pediatr. Infect. Dis.* **5:**S138–S142.
15. **Wheatley, W. B.** 1951. A rapid staining procedure for intestinal amoebae and flagellates. *Am. J. Clin. Pathol.* **21:**990–991.

SUPPLEMENTAL READING

Bryan, R. T. 1990. Microsporidia, p. 2130–2134. *In* G. L. Mandell, R. G. Douglas, and J. E. Bennett (ed.), *Principles and Practice of Infectious Diseases*, 3rd ed. Churchill Livingstone, New York, NY.

Bryan, R. T., A. Cali, R. L. Owen, and H. C. Spencer. 1991. Microsporidia: opportunistic pathogens in patients with AIDS, p. 1–26. *In* T. Sun (ed.), *Progress in Clinical Parasitology*, vol. 2. Field & Wood, Philadelphia, PA.

Garcia, L. S. 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.

Ignatius, R., S. Henschel, O. Liesenfeld, U. Mansmann, W. Schmidt, S. Koppe, T. Schneider, W. Heise, U. Futh, E. O. Riecken, H. Hahn, and R. Ulrich. 1997. Comparative evaluation of modified trichrome and Uvitex 2B stains for detection of low numbers of microsporidial spores in stool specimens. *J. Clin. Microbiol.* **35:**2266–2269.

Jelinek, T., S. Eichenlaub, and T. Loscher. 1999. Sensitivity and specificity of a rapid immunochromatographic test for diagnosis of visceral leishmaniasis. *Eur. J. Clin. Microbiol. Infect. Dis.* **18:**669–670.

Wittner, M., and L. M. Weiss. 1999. *The Microsporidia and Microsporidiosis.* ASM Press, Washington, DC.

APPENDIX 9.7.3–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. NaCl, 0.85%

B. Giemsa stain (*see* procedure 9.8.5)

C. Rapid Giemsa
1. Diff-Quick (Baxter Scientific)
2. Giemsa Plus (Trend Scientific)

D. Giemsa stain buffer with 0.01% Triton X-100 (*see* procedure 9.8.5)

E. Giemsa buffer (*see* procedure 9.8.4)

F. Methenamine-silver nitrate stain reagents (*see* procedure 8.3)

G. Trichrome stain reagents (*see* procedure 9.3.6)

H. Schaudinn's fixative (*see* procedure 9.2.2)

I. Kinyoun's modified acid-fast stain (*see* procedure 9.4.1)

J. Bleach

K. Culture media
1. *E. histolytica* (*see* procedure 9.9.1)
2. *Acanthamoeba* spp. and *Naegleria* spp. (*see* procedure 9.9.2)
3. *Leishmania* spp. (*see* procedure 9.9.5)

L. Modified trichrome stain (*see* procedures 9.4.3, 9.4.4, and 9.4.5)

9.8.1 Detection of Blood Parasites

I. PRINCIPLE

During some stages in their life cycle, *Plasmodium* spp. (malaria), *Babesia* spp., *Trypanosoma* spp., *Leishmania donovani*, and the filariae are detectable in human blood. *Plasmodium* and *Babesia* species are found within the RBCs; trypanosomes and microfilariae, the larval stage of filariae, are found outside the RBCs; and *Leishmania* amastigotes are occasionally found within monocytes. Trypanosomes and microfilariae, which frequently are present in low numbers, exhibit motility in freshly collected blood films, and this can aid in their detection. However, species identifications of all blood parasites are usually made from either or both of two types of stained blood films: a thin film and a thick film. These films can be made from whole or anticoagulated blood or from the sediment of a variety of procedures designed to concentrate trypanosomes and microfilariae in the blood. Although the films are clearest when stained with Giemsa stain, many infections are detected and diagnosed by using Wright's stain. Delafield's hematoxylin is used to enhance the morphological features of microfilariae.

Microscopic examination of stained blood films is best accomplished by beginning with a thorough search of both the thin and thick films with low-power magnification for microfilariae. If larvae are found, magnification at a higher power will reveal the finer morphological details necessary to make a definitive identification. Other blood parasites require examination with oil immersion magnification of both the thin and thick films. Trypanosomes, even those detected in thick films, are more frequently identified in the thicker portion of the thin film. *Plasmodium* and *Babesia* spp., being intracellular parasites, are detected in the thick film but are more readily identified in the thinner portion of the thin film. Depending on the experience of the microscopist, satisfactory examination usually requires 5 to 10 min for the thick film (about 100 fields) and 15 to 20 min for the thin film (about 200 to 300 fields) at $\times$1,000 (oil immersion) magnification. All species of parasites found in a blood specimen should be reported to the attending physician as soon as possible. Notification of appropriate governmental authorities should be made expeditiously where required by law (3–5).

II. THIN AND THICK BLOOD FILMS

A. Purpose

To date, stained blood films are the most reliable and efficient means for definitive diagnosis of nearly all blood parasites. They provide a permanent record and can be sent to a reference laboratory for consultation or verification of diagnosis. Ordinarily, when a laboratorian tests for blood parasites, two types of blood films are prepared: a thin film and a thick film (*see* Appendix 9.8.1–1). These can be made on the same microscope slide, that is, with the thin film on one end of the slide and the thick film on the other, or they can be made on separate slides. When malaria is suspected, the recommended procedure is to prepare a thin film on one slide, a thick film on another, and a combination of thin and thick films on a third slide. The thin film can be stained within a few minutes and will afford a quick diagnosis of malaria if the patient has a high degree of parasitemia; the thick film can be stained in a few hours and will afford a diagnosis of lighter infection; and the combination film is stained several hours later when the blood has dried longer, thus resulting in a better differential stain. The combination film is then used to verify the quick diagnosis and is kept as the permanent record. This three-slide procedure can be used for detecting all blood parasites.

II. THIN AND THICK BLOOD FILMS *(continued)*

B. Thin blood film (*see* procedure 9.8.2)

The thin film is identical to a differential WBC count film. It provides a good area for examining the morphology of parasites and RBCs and is used to confirm the identity of parasites that cannot be identified in thick films. Most parasitologists concur that a thin film must be used to differentiate *Plasmodium ovale* from *Plasmodium vivax* and *Babesia* species from the ring forms of *Plasmodium falciparum.* The thin film is also better for identifying *Trypanosoma cruzi,* because these organisms become distorted in thick films. The thin film, however, is less sensitive than the thick film in light parasitemias.

C. Thick blood film (*see* procedure 9.8.3)

The thick film essentially condenses into an area suitable for examination about 20 times more blood than the thin film. In this respect, the thick film is a concentration procedure. Here, the RBCs are lysed during the staining process so that only parasites, platelets, and WBCs remain visible. The thick film, then, has two advantages over the thin film: it saves time in examining the blood and increases the chance of detecting light infections. Therefore, the thick film is recommended for the routine *detection* of all parasites when the diagnostic stages occur in blood, and it can be used, in most instances, for identifying to the species level all microfilariae, the African trypanosomes, and all *Plasmodium* spp. except *P. ovale.*

D. Blood specimen

Blood for detecting parasites is obtained by either finger puncture or venipuncture. These procedures are described in detail in NCCLS publications (3, 4) and procedures 9.8.2 and 9.8.3. *Standard precautions must be used in the collecting, handling, and disposing of these blood samples.* If blood is obtained by finger puncture, care must be taken not to squeeze tissue juice into the sample and risk diluting a possible light parasitemia to below the level of detection.

E. Use of anticoagulants

Blood samples for malaria are preferably collected without anticoagulants, but if anticoagulants must be used for other testing, films for reliable staining of malaria parasites should be made immediately or within 1 h of collection. Trypanosomes and microfilariae are usually not affected by the use of anticoagulants. Although several anticoagulants have been used, EDTA (0.020 g/10 ml of blood) is recommended by most parasitologists for use with malaria parasites (*but see* Appendix 9.8.1–2). Heparin (0.002 g/10 ml of blood) and sodium citrate (0.050 g/10 ml of blood) are often used for concentration procedures for trypanosomes and microfilariae.

F. Collection guidelines

1. The blood sample must be labeled with the date and time of collection so that findings can be correlated with symptoms and with all other pertinent information. Some parasites appear more frequently in the blood during certain periods than during others. To accommodate this, blood for detection of parasites is usually collected as follows.

a. On admission or when first suspected of parasite infection

b. If no parasites are found in the first sample, blood is collected every 6 to 12 h until a diagnosis is made or infection is no longer suspected (usually 3 to 5 days). *Infection with a blood parasite cannot be ruled out by a single blood sample.*

c. If either trypanosomes or microfilariae are suspected, each sample should be concentrated by an appropriate technique in an effort to detect a low parasitemia.

d. *The examination of blood for malaria should be considered a stat request. Preparation and examination of smears and reporting of results should be performed on a stat basis.*

II. THIN AND THICK BLOOD FILMS *(continued)*

2. Blood films for parasites should be made on clean, standard, glass microscope slides (1 by 3 in.). All slides, even new "precleaned" ones, should be dipped in alcohol and polished with a lint-free towel to remove any grease or dirt before the preparations are made.

3. Instrument methods, for either preparing thin films or staining films, have not been reliable for blood parasites. At this time, manual techniques yield better results.

G. Staining of blood films

1. All blood films, regardless of the stain which will be used, should be stained as soon as possible after they are thoroughly dry. Prolonged storage may cause erratic staining.

2. After staining and thorough drying, films may be examined by using oil directly on the film, or a no. 1-thickness coverslip may be applied to the film by using a mounting medium (pH 7.0).

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
2. **Garcia, L. S., S. P. Johnston, A. J. Lindscott, and R. Y. Shimizu.** 2008. *Cumitech 46, Laboratory Procedures for Diagnosis of Blood-Borne Parasitic Diseases.* Coordinating ed., L. S. Garcia. ASM Press, Washington, DC.
3. **NCCLS.** 1991. *Procedures for the Collection of Diagnostic Blood Specimens by Skin Puncture*, vol. 11, no. 11, H4-A3. NCCLS, Villanova, PA.
4. **NCCLS.** 1991. *Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture*, vol. 11, no. 10, H3-A3. NCCLS, Villanova, PA.
5. **NCCLS.** 2000. *Laboratory Diagnosis of Blood-Borne Parasitic Diseases.* Approved guideline M15-A. NCCLS, Wayne, PA.

APPENDIX 9.8.1–1

Advantages of thick and thin blood films

Thin blood film advantages	Thick blood film advantages
RBC morphology (size, shape, stippling) can be seen after fixation with methanol prior to staining (Giemsa) or as a part of the staining process (Wright). RBCs are laked in the thick film.	Higher number of parasites per field than with the thin blood film. RBCs are laked, so WBCs, platelets, and parasites will be visible after staining.
Identification to *Plasmodium* species level easier, since the parasite can be seen within the RBC. The size of the parasites within the RBCs can provide information necessary for identification to the species level.	Phagocytized malaria pigment can be seen within the WBCs, even with a low parasitemia.
Parasitemia (%) can be calculated from the thin film; determination of parasitemia is mandatory for all species of *Plasmodium* and is particularly important to monitor therapy for *P. falciparum.* Parasitemia should be reported with every set of positive blood films for malaria or *Babesia.*	Stippling may be seen in a well-stained thick film. However, this will depend on how long the blood has been in contact with anticoagulant if not collected as a fresh specimen (finger stick).

APPENDIX 9.8.1–2

Potential problems using EDTA anticoagulant for the preparation of thin and thick blood films

Potential problem	Comments
Adhesion to the slide; blood falls off slide during staining.	Incorrect ratio of anticoagulant to blood; fill tube completely with blood (7 ml or pediatric draw tube).
Distortion of parasites; same type of distortion can also be seen after blood is refrigerated (not recommended).	Prolonged storage of blood in EDTA may lead to distortion (>1 h) and/or loss of parasites (4–6 h): trophozoites *(P. vivax)* and gametocytes *(P. falciparum)* tend to round up, thus mimicking *P. malariae.*
Change in ring form size	Ring forms of *P. falciparum* will continue to enlarge, thus resembling rings of the other species. Typical "small" rings will appear larger than usual.
Use of EDTA anticoagulant (primarily used by hematology). EDTA is the anticoagulant used by the hematology laboratory because the cellular components and morphology of the blood cells are preserved. 1. Blood smears for differentials from acceptable specimens should be prepared within 2 h of collection. 2. Blood counts from acceptable venipuncture specimens should be performed within 6 h of collection. Underfilling the EDTA blood collection tube can lead to erroneously low blood cell counts and hematocrits, morphological changes to RBCs, and staining alteration. Excess EDTA can shrink RBCs. Conversely, overfilling the blood collection tube will not allow the tube to be properly mixed and may lead to platelet clumping and clotting.	EDTA prevents coagulation of blood by chelating calcium. Calcium is necessary in the coagulation cascade, and its removal inhibits and stops a series of events, both intrinsic and extrinsic, which cause clotting. In some individuals, EDTA may cause inaccurate platelet results. These anomalies, platelet clumping and the formation of platelet satellites, may be the result of changes in the membrane structure occurring when the calcium ion is removed by the chelating agent, allowing the binding of preformed antibodies. Proper mixing of the whole-blood specimen ensures that EDTA is dispersed throughout the sample. Evacuated blood collection tubes with EDTA should be mixed by 8–10 end-to-end inversions immediately following venipuncture collection. Microcollection tubes with EDTA should be mixed by 10 complete end-to-end inversions immediately following collection. They should then be inverted an additional 20 times prior to analysis.
Loss of Schüffner's dots (stippling) in *P. vivax* and *P. ovale*	Schüffner's dots (true stippling) occur in both *P. vivax* and *P. ovale;* in the absence of stippling, identification to the species level may be much more difficult.
Prolonged storage of EDTA blood (room temp with stopper removed)	The pH, CO_2 and temp changes may reflect conditions within the mosquito. Thus, exflagellation of the male gametocyte may occur while still in the tube of blood prior to thin and thick blood film preparation. Microgametes may be confused with *Borrelia* or may be ignored as debris. If fertilization occurs between the male and female gametocytes, the ookinete may resemble a *P. falciparum* gametocyte (crescent shaped).
Release of merozoites from the schizonts into the blood. Normally, merozoites will not be found outside of the RBCs, in contrast to *Babesia* spp., with which rings may be seen outside of the RBCs.	Small rings may be seen outside of the RBCs or appear to be appliqué forms, thus suggesting *P. falciparum.* It will be important to differentiate these true rings (both cytoplasmic and nuclear colors) from platelets (uniform color).
Incorrect submission of blood in heparin	EDTA has less impact on parasite morphology than heparin.

9.8.2 Preparation of Thin Blood Films

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The thin film is prepared like that for a differential WBC count and provides an area for examination where the RBCs are neither overlapping nor distorted. Here the morphologies of parasites and infected RBCs are most typical.

II. SPECIMEN

Observe standard precautions.

The specimen usually consists of fresh whole blood collected by finger puncture or of whole blood containing EDTA (0.020 g/10 ml of blood) that was collected by venipuncture and is less than 1 h old (1–3). Occasionally a buffy coat (for leishmaniasis) or the sediment from a special concentration procedure (triple centrifugation for trypanosomes) is spread into a thin film.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagent

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

Absolute methanol (for Giemsa stain)

B. Supplies

1. Glass microscope slides (1 by 3 in.), alcohol washed (frosted on one side, preferably)

2. Glass marker

3. Blood collection supplies (if applicable)

C. Equipment

None

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Visually, the thin film should be rounded, feathered, and progressively thinner toward the middle of the slide.

B. There should not be any clear areas or smudges in the film itself (indicating that grease or fingerprints were on the glass).

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. Blood from finger puncture

After wiping off the first drop of blood, touch a clean glass microscope slide (1 by 3 in.) about ½ in. from the end to a small drop of blood (10 to 15 μl) standing on the finger, remove the slide from the finger, turn it blood side up, and place it on a horizontal surface.

Blood from venipuncture

Place a clean glass microscope slide (1 by 3 in.) on a horizontal surface. Place a small drop (10 to 15 μl) of specimen onto the center of the slide about ½ in. from the end.

C. Holding a second clean glass slide at a 40° angle, touch the angled end to the midlength area of the specimen slide.

D. Pull the angled slide back into the blood, and allow the blood to almost fill the end area of the angled slide.

E. Continuing contact with the blood under the lower edge, quickly and steadily move the angled slide toward the opposite end of the specimen slide until the blood is used up.

F. The result will be a thin film that is rounded, feathered, and progressively thinner toward the center of the slide.

G. Label the slide appropriately, and allow it to air dry while protected from dust for at least 10 min.

H. If the film will be stained with Giemsa, then after the film is completely dry, fix it by dipping the slide into absolute methanol, and allow the film to air dry in a vertical position. If the film will be stained with Wright's stain, it does not need to be fixed. Wright's stain contains the fixative and stain in one solution.

POSTANALYTICAL CONSIDERATIONS

VI. PROCEDURE NOTES

A. A diamond marking pen is recommended.

B. An indelible ink pen can be used.

C. Pencil can be used if the information is actually written in the thick part of the smear (where the original drop of blood was placed).

D. Do not use wax pencils; the material may fall off during the staining procedure.

E. Make sure the films are protected from dust (while drying).

VII. LIMITATIONS OF THE PROCEDURE

A. A light infection may be missed in a thin film, whereas the increased volume of blood present on a thick film may allow the detection of the infection, even with a low parasitemia.

B. If the smears are prepared from anticoagulated blood which is more than 1 h old, the morphology of both parasites and infected RBCs may not be typical.

C. You should be able to identify *Plasmodium vivax* and *Plasmodium ovale*, even in the absence of Schüffner's dots (stippling).

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
2. **Garcia, L. S., S. P. Johnston, A. J. Lindscott, and R. Y. Shimizu.** 2008. *Cumitech 46, Laboratory Procedures for Diagnosis of Blood-Borne Parasitic Diseases.* Coordinating ed., L. S. Garcia. ASM Press, Washington, DC.
3. **NCCLS.** 2000. *Laboratory Diagnosis of Blood-Borne Parasitic Diseases.* Approved guideline M15-A. NCCLS, Wayne, PA.

9.8.3 Preparation of Thick Blood Films

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The thick film samples more blood than the thin film and therefore is more likely to demonstrate a low parasitemia. RBCs are lysed during staining, making the preparation more or less transparent and leaving only parasites, platelets, and WBCs for examination (1–3).

II. SPECIMEN

Observe standard precautions.

The specimen usually consists of fresh whole blood collected by finger puncture or of whole blood containing EDTA (0.020 g/10 ml of blood) that was collected by venipuncture and is less than 1 h old. Heparin (2 mg/10 ml of blood) or sodium citrate (0.050 g/10 ml of blood) may be used as an anticoagulant if trypanosomes or microfilariae are suspected. The sediment from a concentration procedure for trypanosomes or microfilariae is frequently spread into a thick film that is stained, examined, and kept as a permanent record (*see* procedures 9.8.11 and 9.8.12).

Although both EDTA and heparin have been mentioned as anticoagulants, it is very important to request EDTA, rather than heparin, for the preparation and staining of blood films for malaria. However, heparin can be used for the concentration methods related to the recovery of trypanosomes and microfilariae. As a general rule, EDTA is the recommended anticoagulant for all parasitology blood work.

III. MATERIALS

A. Reagents
None

B. Supplies
1. Glass microscope slides (1 by 3 in.), alcohol washed (frosted on one end, preferably)
2. Glass marker
3. Blood collection supplies (if applicable)
4. Paper with newsprint-size print
5. Applicator sticks

C. Equipment
None

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Visually, the thick smear should be round to oval and approximately 2.0 cm across.
B. You should barely be able to read newsprint through the wet or dry film.
C. The film itself should not have any clear areas or smudges (indicating that grease or fingerprints were on the glass).

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. Blood from finger puncture

After wiping off the first drop of blood, touch a clean glass microscope slide (1 by 3 in.) to a large drop of blood standing on the finger, and rotate the slide on the finger until the circle of blood is nearly the size of a dime or a nickel (1.8 to 2.0 cm). Without breaking contact with the blood, rotate the slide back to the center of the circle. Remove the slide from the finger, quickly turn it blood side up, and place it on a horizontal surface. The blood should spread out evenly over the surface of the circle and be of a thickness that fine print (newsprint size) can just barely be read through it. If it is thicker than this, take the corner of a second clean slide or an applicator stick, and expand the size of the circle until the print is just readable. The final thickness of the film is important. If too thick, it might flake off while drying or wash off while staining. If too thin, the amount of blood available for examination is insufficient to detect a low parasitemia.

Blood from venipuncture

Place a clean glass microscope slide (1 by 3 in.) on a horizontal surface. Place a drop (30 to 40 μl) of blood onto the center of the side about ½ in. from the end. Using either the corner of another clean glass slide or an applicator stick, spread the blood into a circle about the size of a dime or a nickel (1.8 to 2.0 cm). Immediately place the thick film over some small print, and be sure that the print can just barely be read through it. If not, expand the size of the film until the print can be read. Three or four small drops of blood may be used in place of the large drop, and the small ones can be pooled into a thick film by using the corner of a clean slide or an applicator stick. Be sure that small print can be read through the film.

C. Allow the film to air dry in a horizontal position protected from dust for several hours (6 to 8 h) or overnight. Do not attempt to speed the drying process by applying any type of heat, because heat will fix the RBCs and they will not lyse in the staining process.

D. *Do not fix the thick film.* If thin and thick films are made on the same slide, do not allow the methanol or its vapors to contact the thick film by slightly tilting the slide when fixing the thin film.

E. Label the slide appropriately.

F. If staining with Giemsa will be delayed for more than 3 days or if the film will be stained with Wright's stain, lyse the RBCs in the thick film by placing the film in buffered water (pH 7.0 to 7.2) (*see* procedure 9.7.1) for 10 min. Then remove it from the water, and place it in a vertical position (thick film down) to air dry.

POSTANALYTICAL CONSIDERATIONS

VI. PROCEDURE NOTES

A. A diamond marking pen is recommended.

B. An indelible ink pen can be used.

C. Do not use wax pencils; the material may fall off during the staining procedure.

D. Make sure the films are protected from dust (while drying).

VII. LIMITATIONS OF THE PROCEDURE

A. If the smears are prepared from anticoagulated blood that is more than 1 h old, morphology of the parasites may not be typical, and the film may wash off the slide during the staining procedure.

VII. LIMITATIONS OF THE PROCEDURE *(continued)*

B. Identification to the species level, particularly between *Plasmodium ovale* and *Plasmodium vivax* and between the ring forms of *Plasmodium falciparum* and *Babesia* spp., may be impossible without examining the stained thin blood film. Also, *Trypanosoma cruzi* trypomastigotes are frequently distorted in thick films.

C. Excess stain on the film may be confusing and make the detection of organisms difficult.

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
2. **Garcia, L. S., S. P. Johnston, A. J. Lindscott, and R. Y. Shimizu.** 2008. *Cumitech 46, Laboratory Procedures for Diagnosis of Blood-Borne Parasitic Diseases.* Coordinating ed., L. S. Garcia. ASM Press, Washington, DC.
3. **NCCLS.** 2000. *Laboratory Diagnosis of Blood-Borne Parasitic Diseases.* Approved guideline M15-A. NCCLS, Wayne, PA.
4. **Wilcox, A.** 1960. *Manual for the Microscopical Diagnosis of Malaria in Man.* U.S. Public Health Service publication no. 796. U.S. Government Printing Office, Washington, DC. (Out of print.)

SUPPLEMENTAL READING

Ash, L. R., and T. C. Orihel. 1987. *Parasites: a Guide to Laboratory Procedures and Identification.* American Society of Clinical Pathologists, Chicago, IL.

Garcia, L. S. 2009. *Practical Guide to Diagnostic Parasitology,* 2nd ed. ASM Press, Washington, DC.

9.8.4 Combination Thick and Thin Blood Films (Can Be Stained as Either)

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The combination thick-thin blood film provides both options on one glass slide, and the slide can be stained as either a thick or thin blood film. If fixed prior to staining, then the smear will be read as a thin blood film; if RBCs are lysed during staining, the preparation will be read as a thick blood film (parasites, platelets, and WBCs). This combination blood film dries more rapidly than the traditional thick blood film, thus allowing staining and examination to proceed with very little waiting time for the slide(s) to dry (1–4).

II. SPECIMEN

Observe standard precautions.

The specimen usually consists of fresh whole blood collected by finger puncture or of whole blood containing EDTA (0.020 g/10 ml of blood) that was collected by venipuncture and is less than 1 h old. Heparin (2 mg/10 ml of blood) or sodium citrate (0.050 g/10 ml of blood) may be used as an anticoagulant if trypanosomes or microfilariae are suspected.

III. MATERIALS

A. Reagents
None

B. Supplies
1. Glass slides (1 by 3 in., or larger if you prefer), alcohol washed (frosted at one end, preferably)
2. Glass marker
3. Blood collection supplies (if applicable)
4. Paper with newsprint-size print
5. Applicator sticks

C. Equipment
None

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Visually, the smear should consist of alternating thick and thin portions throughout the length of the glass slide.
B. One should be able to barely read newsprint through the wet or dry film.
C. The film itself should not have any clear areas or smudges, indicating that grease or fingerprints were on the glass.
D. Blood from a finger puncture is not recommended, since the procedure does not lend itself to "stirring" to prevent fibrin strands.

V. PROCEDURE (Fig. 9.8.4–1)

A. *Wear gloves when performing this procedure.*
B. Place a clean 1- by 3-in. glass microscope slide on a horizontal surface.
C. Place a drop (30 to 40 μl) of blood onto one end of the slide about 0.5 in. from the end.

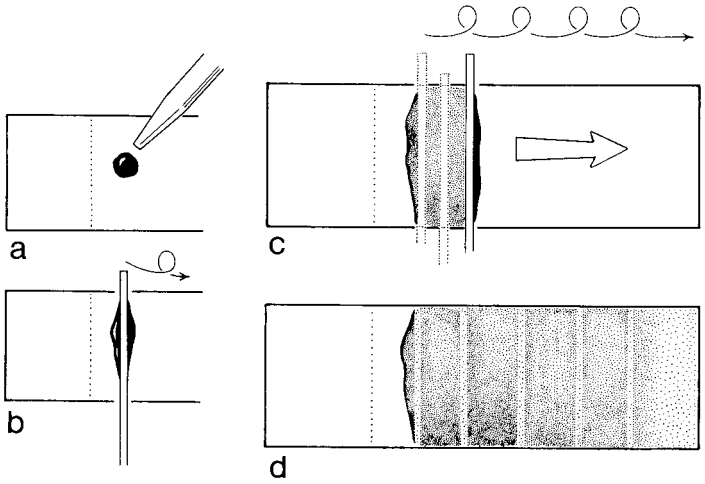


Figure 9.8.4–1 Method of thick-thin combination blood film preparation. (a) Position of drop of EDTA blood; (b) position of applicator stick in contact with blood and glass slide; (c) rotation of applicator stick; (d) completed thick-thin combination blood film prior to staining. (Illustration by Sharon Belkin; from reference 2.)

V. PROCEDURE *(continued)*

D. Using an applicator stick lying across the glass slide and keeping the applicator in contact with the blood and glass, rotate (do not "roll") the stick in a circular motion while moving the stick down the glass slide to the opposite end.

E. The appearance of the blood smear should be alternate thick and thin areas of blood that cover the entire slide.

F. Immediately place the film over some small print and be sure that the print is just barely readable.

G. Allow the film to air dry horizontally and protected from dust for at least 30 min to 1 h. Do not attempt to speed the drying process by applying any type of heat, because the heat will fix the RBCs and they subsequently will not lyse in the staining process.

H. This slide can be stained as either a thick or thin blood film.

I. Label the slide appropriately.

J. If staining with Giemsa (as a thick film) will be delayed for more than 3 days or if the film will be stained with Wright's stain, lyse the RBCs on the thick film by placing the slide in buffered water (pH 7.0 to 7.2) for 10 min, remove it from the water, and place it in a vertical position to air dry.

K. If staining with Giemsa (as a thin film), after the film is completely dry, fix it by dipping the slide into absolute methanol, and allow the film to air dry in a vertical position. If the film will be stained with Wright's stain, it does not need to be fixed. Wright's stain contains the fixative and stain in one solution.

POSTANALYTICAL CONSIDERATIONS

VI. PROCEDURE NOTES

A. A diamond marking pen is recommended.

B. An indelible ink pen can be used.

C. Pencil can be used if the information is actually written in the thickest part of the smear (where the original drop of blood was placed).

D. Do not use wax pencils; the material may fall off during the staining procedure.

E. Make sure the films are protected from dust (while drying).

VII. LIMITATIONS OF THE PROCEDURE

A. A light infection may be missed in a thin film, whereas the increased volume of blood present on a thick film may allow the detection of the infection, even with a low parasitemia.

B. If the smears are prepared from anticoagulated blood which is more than 1 h old, the morphology of both parasites and infected RBCs may not be typical.

C. If a tube of blood containing EDTA cools to room temperature and the cap has been removed, several parasite changes can occur. The parasites within the RBCs will respond as if they were now in the mosquito after being taken in with a blood meal. The morphology of these changes in the life cycle and within the RBCs can cause confusion when examining blood films prepared from this blood.

1. Stippling (Schüffner's dots) may not be visible.

2. The male gametocyte (if present) may exflagellate.

3. The ookinetes of *Plasmodium* species other than *Plasmodium falciparum* may develop as if they were in the mosquito and may mimic the crescent-shaped gametocytes of *P. falciparum.*

D. Identification to the species level, particularly between *Plasmodium ovale* and *Plasmodium vivax* and between the ring forms of *P. falciparum* and *Babesia* spp., may be impossible without examining one of the slides stained as a thin blood film. Also, *Trypanosoma cruzi* trypomastigotes are frequently distorted in thick films.

E. Excess stain deposition on the film may be confusing and make the detection of organisms difficult.

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
2. **Garcia, L. S.** 2009. *Practical Guide to Diagnostic Parasitology*, 2nd ed. ASM Press, Washington, DC.
3. **Garcia, L. S., S. P. Johnston, A. J. Lindscott, and R. Y. Shimizu.** 2008. *Cumitech 46, Laboratory Procedures for Diagnosis of Blood-Borne Parasitic Diseases.* Coordinating ed., L. S. Garcia. ASM Press, Washington, DC.
4. **NCCLS.** 2000. *Laboratory Diagnosis of Blood-Borne Parasitic Diseases.* Approved guideline M15-A. NCCLS, Wayne, PA.

9.8.5 Giemsa Stain

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Giemsa stain is used to differentiate nuclear and/or cytoplasmic morphology of platelets, RBCs, WBCs, and parasites (1–5). The most dependable stain for blood parasites, particularly in thick films, is Giemsa stain containing azure B. Liquid stock is available commercially, or the stock can be made from dry stain powder. Either must be diluted for use with water buffered to pH 6.8 or 7.0 to 7.2, depending on the specific technique used. Either should be tested for proper staining reaction before use. The stock is stable for years, but it must be protected from moisture because the staining reaction is oxidative. Therefore, the oxygen in water will initiate the reaction and ruin the stock stain. The aqueous working dilution of stain is good only for 1 day.

Although not essential, the addition of Triton X-100, a nonionic surface-active agent, to the buffered water used to dilute the stain enhances the staining properties of Giemsa (4) and helps to eliminate possible transfer of parasites from one slide to another (1). For routine staining of thin films and combination thin and thick films, a 0.01% (vol/vol) final concentration of Triton X-100 is best. For staining thick films for microfilariae, use a 0.1% (vol/vol) concentration.

II. SPECIMEN

Observe standard precautions.

The specimen may consist of a thin blood film that has been fixed in absolute methanol and allowed to dry, a thick blood film that has been allowed to dry thoroughly and is not fixed, or a combination of a fixed thin film and an adequately dried thick film (not fixed) on the same slide.

III. MATERIALS

A. Reagents (*see* Appendix 9.8.5–1)

B. Supplies (including those for preparing stock stain)

1. 3 or 4 Coplin jars, 50 ml
2. Pipette, 2 ml
3. 3 graduated cylinders, 50 ml
4. Mortar and pestle
5. Flask, Erlenmeyer, 500 ml with cotton plug
6. 2 flasks, volumetric, 1 liter
7. Bottle, brown, 150 to 200 ml
8. Bottles, clear, 100 ml and 3 1-liter
9. Bottle, airtight, 50 ml
10. Filter paper, Whatman no. 1
11. Funnel (glass) to hold filter paper

C. Equipment

1. Microscope, binocular with mechanical stage; low-power (10×), high dry power (40×), and oil immersion (100×) objectives; 10× oculars; calibrated ocular micrometer; light source equivalent to 20-W halogen or 100-W tungsten bulb; blue and white ground-glass diffuser filters
2. Timer, 1 h or more in 1-min increments
3. Water bath, 55 to 60°C
4. pH meter

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. The stock buffer solutions and buffered water should be clear, with no visible contamination.

B. Check the Giemsa stain reagents, including the pH of the buffered water, before each use. If Triton X-100 has been added to the buffered water, do not use a colorimetric method to determine the pH, because Triton X-100 interferes with the color indicators. Use a pH meter to test buffered water that contains Triton X-100. The buffered water is usable as long as the pH is within the limits listed for the procedure.

C. Prepare and stain films from "normal" blood, and microscopically evaluate the staining reactions of the RBCs, platelets, and WBCs.

1. Macroscopically, blood films appear purplish. If blue, the buffered water was too alkaline; if pink to red, the buffered water was too acid.

2. Microscopically, RBCs appear pinkish gray, platelets appear deep pink, and WBCs have purple-blue nuclei and lighter cytoplasm. Eosinophilic granules are bright purple-red, and neutrophilic granules are purple. Basophilic stippling within uninfected RBCs is blue.

3. Slight variations may appear in the colors described above depending on the batch of stain used and the character of the blood itself, but if the various morphological structures are distinct, the stain is satisfactory.

D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

E. Known positive microscope slides, Kodachrome (2- by 2-in.) projection slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

F. Record all QC results.

V. PROCEDURE

A. *Wear gloves when performing any of these procedures.*

B. Thin blood films (only)

1. Fix air-dried film in absolute methyl alcohol by dipping the film briefly (two dips) in a Coplin jar containing methyl alcohol.

2. Remove, and let air dry.

3. Stain with diluted Giemsa stain (1:20, vol/vol) for 20 min. For a 1:20 dilution, add 2 ml of stock Giemsa to 40 ml of buffered water containing 0.01% Triton X-100 in a Coplin jar (Triton X-100 optional).

4. Wash by briefly dipping the slide in and out of a Coplin jar of buffered water (one or two dips).

◩ **NOTE:** Excessive washing will decolorize the film.

5. Let air dry in a vertical position.

C. Thick blood films (only)

1. Allow film to air dry thoroughly for several hours or overnight. Do not dry films in an incubator or by heat, because this will fix the blood and interfere with the lysing of the RBCs.

◩ **NOTE:** If a rapid diagnosis of malaria is needed, thick films can be made slightly thinner than usual, allowed to dry for 1 h, and then stained.

V. PROCEDURE *(continued)*

2. *Do not fix.*
3. Stain with diluted Giemsa stain (1:50, vol/vol) for 50 min. For a 1:50 dilution, add 1 ml of stock Giemsa to 49 ml of buffered water containing 0.01% Triton X-100 (if staining microfilariae, use 0.1% Triton X-100) in a Coplin jar.
4. Wash by placing film in buffered water for 3 to 5 min.
5. Let air dry in a vertical position.

D. Combination thin and thick blood films

1. Allow the thick film to air dry thoroughly.
2. Fix the thin film by placing only the thin film in methyl alcohol (two dips). Be sure not to get the alcohol or its fumes on the thick film by slightly tilting the slide.
3. Let air dry in a vertical position with the *thick film up.* Be sure slide is thoroughly dry before staining. Introducing even a minute amount of methyl alcohol into the stain dilution will interfere with the lysing of the RBCs in the thick films.
4. Stain the entire slide with diluted Giemsa stain (1:50, vol/vol) for 50 min. Place the slide in the stain, *thick film down* to prevent the debris caused by dehemoglobinization from falling onto the thin film. For a 1:50 dilution, add 1 ml of stock Giemsa to 50 ml of buffered water containing 0.01% Triton X-100 in a Coplin jar.
5. Rinse the thin film by briefly dipping the film in and out of a Coplin jar of buffered water (one or two dips). Wash the thick film for 3 to 5 min. Be sure that the thick film is immersed but *do not allow the water to cover any part of the thin film.*
6. Let dry in a vertical position with the *thick film down.*

VI. RESULTS

A. If *Plasmodium* organisms are present, the cytoplasm stains blue and the nuclear material stains red to purple-red.

B. Schüffner's stippling and other inclusions in the RBCs infected by *Plasmodium* spp. stain red.

C. Nuclear and cytoplasmic colors that are seen in the malarial parasites will also be seen in the trypanosomes and any intracellular leishmaniae that are present.

D. The sheath of microfilariae may or may not stain with Giemsa, while the body will usually appear blue to purple.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report any parasite, including the stage(s) seen (do not use abbreviations).

Examples: *Plasmodium falciparum* rings and gametocytes, rings only
Plasmodium vivax rings, trophozoites, schizonts, and gametocytes
Wuchereria bancrofti microfilariae
Trypanosoma brucei gambiense/rhodesiense trypomastigotes
Trypanosoma cruzi trypomastigotes
Leishmania donovani amastigotes

B. Any laboratory providing malaria diagnoses should be able to identify *Plasmodium vivax* and *Plasmodium ovale,* even in the absence of Schüffner's stippling.

VIII. PROCEDURE NOTES

A. Blood films prepared from venipuncture blood when an anticoagulant is used must be prepared within 1 h of collection. Otherwise, certain morphological characteristics of both parasites and infected RBCs may be atypical. Also, thick blood films may wash off the slide during the staining procedure.

B. The correct pH for all buffered-water and staining solutions is also important. Solutions with the incorrect pH will prevent certain morphological characteristics (stippling) from being visible and will not give typical nuclear and cytoplasmic colors on the stained film.

C. Stain a QC slide each time patient blood films are stained. If several patient specimens are stained on the same day (using the same reagents), only one control slide need be stained and examined. The patient slide can serve as the QC slide; if the WBCs and RBCs exhibit typical colors, any parasites present would also stain correctly.

D. Report level of parasitemia when blood films are positive for *Plasmodium* spp. (*see* procedure 9.8.7).

IX. LIMITATIONS OF THE PROCEDURE

A. *Finding no parasites in one set of blood films does not rule out a parasitic infection.*

B. *Examine a minimum of 300 oil immersion (×1,000) fields before reporting no parasites found.*

C. Examine the entire smear under low power (100×) for the presence of microfilariae. Remember that the sheath may not be visible *(W. bancrofti).*

REFERENCES

1. **Brooke, M. M., and A. W. Donaldson.** 1950. Use of a surface-active agent to prevent transfer of malarial parasites between blood films during mass staining procedures. *J. Parasitol.* **36:**84.
2. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
3. **Garcia, L. S., S. P. Johnston, A. J. Lindscott, and R. Y. Shimizu.** 2008. *Cumitech 46, Laboratory Procedures for Diagnosis of Blood-Borne Parasitic Diseases.* Coordinating ed., L. S. Garcia. ASM Press, Washington, DC.
4. **Melvin, D. M., and M. M. Brooke.** 1955. Triton X-100 in Giemsa staining of blood parasites. *Stain Technol.* **30:**269–275.
5. **Wilcox, A.** 1960. *Manual for the Microscopical Diagnosis of Malaria in Man.* U.S. Public Health Service publication no. 796. U.S. Government Printing Office, Washington, DC. (Out of print.)

SUPPLEMENTAL READING

Ash, L. R., and T. C. Orihel. 1987. *Parasites: a Guide to Laboratory Procedures and Identification.* American Society of Clinical Pathologists, Chicago, IL.

Beaver, P. C., R. C. Jung, and E. W. Cupp. 1984. *Clinical Parasitology*, 9th ed. Lea and Febiger, Philadelphia, PA.

NCCLS. 2000. *Laboratory Diagnosis of Blood-Borne Parasitic Diseases.* Approved guideline M15-A. NCCLS, Wayne, PA.

APPENDIX 9.8.5–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. Stock Giemsa stain

Giemsa stain (azure B type) ……0.6 g
glycerin …… 50.0 ml
methyl alcohol, absolute (acetone free) .. 50.0 ml

1. Grind 0.6 g of stain powder with 10 to 15 ml of glycerin (freshly opened bottle, neutral) in a clean mortar. Pour the top third into a clean, dry 500-ml flask. Add more glycerin, and grind again. The grinding process should be thorough, to ensure mixing of stain powder and glycerin. Repeat until most of the powder has been mixed with the 50 ml of glycerin and the mixture has been poured into the flask.
2. Stopper or plug the flask, and place and secure it upright in a 55 to 60°C water bath for 2 h. The water in the bath should be above the level of the stain mixture. Shake gently at half-hour intervals.
3. After grinding powder and glycerin together in mortar, measure 50 ml of methyl alcohol (acetone free, neutral), and use it to wash the last bit of stain from the mortar. Pour the washing into a small, airtight bottle.
4. After 2 h, remove glycerin and stain powder mixture from water bath. Allow to come to room temperature, add alcohol washing from the mortar, and shake well.
5. Filter before use through Whatman no. 1 paper into a brown bottle. The stain can be used immediately, but it is preferable to let it stand about 2 weeks with intermittent shakings before the initial filtering.
6. Label appropriately, and store protected from light at room temperature. The shelf life is 36 months, providing QC criteria are met (stock solution).

B. Stock solution of Triton X-100 (10%)

Triton X-100 ……10 ml
distilled water ……90 ml

1. Combine above liquids in a bottle.
2. Mix thoroughly, label appropriately, and store at room temperature. This solution will keep indefinitely if tightly stoppered.

C. Stock buffers (for preparing buffered water)

1. Alkaline buffer, 0.067 M solution

Na_2HPO_4 (anhydrous) ……9.5 g
distilled water, to make ……1.0 liter

In a 1-liter volumetric flask, dissolve Na_2HPO_4 in about three-fourths of the water. Add water to make 1 liter of solution. Store in tightly stoppered bottle, and label appropriately. The shelf life is 24 months.

2. Acid buffer, 0.067 M solution

$NaH_2PO_4 \cdot H_2O$ ……9.2 g
distilled water, to make ……1.0 liter

In a 1-liter volumetric flask, dissolve $NaH_2PO_4 \cdot H_2O$ in about three-fourths of the water. Add water to make 1 liter of solution. Store in tightly stoppered bottle, and label appropriately. The shelf life is 24 months.

D. Buffered water (for diluting stain and washing films), pH 7.0 to 7.2

alkaline buffer (Na_2HPO_4) ……61 ml
acid buffer ($NaH_2PO_4 \cdot H_2O$) ……39 ml
distilled water ……900 ml

1. Combine above liquids in a 1-liter bottle.
2. Mix thoroughly, and test pH of solution. If pH is not 7.0 to 7.2, discard and remake. The solution can be used as long as the pH remains between 7.0 and 7.2.

APPENDIX 9.8.5–1 *(continued)*

E. Buffered water, pH 6.8 (called for by some commercial stains for diluting stain and washing films)

alkaline buffer (Na_2HPO_4) .....50 ml
acid buffer ($NaH_2PO_4 \cdot H_2O$) .....50 ml
distilled water .....900 ml

1. Combine above liquids in a 1-liter bottle.
2. Mix thoroughly, and test pH of solution. If pH is not 6.8 $\pm$ 0.1, discard and remake. The solution can be used as long as the pH is within the limits listed for the procedure.

F. Triton-buffered water solutions (optional)

1. For thin blood films or combination thin and thick blood films
   After determining the pH of the buffered water, add 1 ml of the stock 10% aqueous dilution of Triton X-100 to 1 liter of buffered water (pH 7.0 to 7.2, 0.01% final concentration). Label appropriately, and store in tightly stoppered bottle. The solution can be used as long as the pH is within limits listed for the procedure.
2. For thick blood films
   After determining the pH of the buffered water, add 10 ml of the stock 10% aqueous dilution of Triton X-100 to 1 liter of buffered water (pH 7.0 to 7.2, 0.1% final concentration). Label appropriately, and store in tightly stoppered bottle. The solution can be used as long as the pH is within limits listed for the procedure.

G. Methyl alcohol, absolute

9.8.6 Wright's Stain

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Wright's stain can be used to stain thin blood films for detecting blood parasites, but it is inferior to Giemsa for staining thick films. The liquid, ready-to-use stain is available commercially, or the stain can be made from dry stain powder and is ready for use in about a week. The staining reaction is somewhat similar to that of Giemsa and is achieved by using buffered water with a pH of 6.8. The stain contains the fixative and stain in one solution (1–3).

II. SPECIMEN

Observe standard precautions.

A. The specimen usually consists of a dry, unfixed thin blood film.

B. Thick blood films may be stained with Wright's stain if the RBCs are lysed before staining.

III. MATERIALS

A. Reagents (*see* Appendix 9.8.6–1)

B. Supplies

1. 2 Coplin jars, 50 ml, for dehemoglobinizing thick films
2. Mortar and pestle
3. Flask, volumetric, 1 liter
4. Bottle, brown, 500 ml
5. Bottle, tight stopper, 1 liter
6. Staining rack
7. 3 or 4 pipettes, disposable
8. 3 or 4 pipette bulbs
9. Gauze for wiping slides
10. Filter paper, Whatman no. 1
11. Funnel (glass) to hold filter paper

C. Equipment

1. Microscope, binocular with mechanical stage; low-power (10×), high dry power (40×), and oil immersion (100×) objectives; 10× oculars; calibrated ocular micrometer; light source equivalent to 20-W halogen or 100-W tungsten bulb; blue and white ground-glass diffuser filters
2. Timer, 1 h or more in 1-min increments
3. pH meter

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. The stock buffer solutions and buffered water should be clear, with no visible contamination.

B. Check the Wright's stain reagents, including the pH of the buffered water, before each use for diagnosis of blood parasites. The buffered water is usable as long as the pH is within the limits listed for the procedure.

C. Prepare and stain films from "normal" blood, and microscopically evaluate the staining reactions of RBCs, platelets, and WBCs.

1. Macroscopically, blood films appear pinkish purple. If blue, the buffered water was too alkaline; if pink to red, the buffered water was too acid.

IV. QUALITY CONTROL *(continued)*

2. Microscopically, RBCs appear tan to pinkish red, platelets appear deep pinkish red, and WBCs have bright blue nuclei and lighter cytoplasm.
3. Slight variations may appear in the colors described above depending on the batch of stain used and the character of the blood itself, but if the various morphological structures are distinct, the stain is satisfactory.

D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

E. Known positive microscope slides, Kodachrome (2- by 2-in.) slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

F. Record all QC results.

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. Thin blood films (only) (1)

1. Place air-dried films on a level staining rack.
2. Use a pipette to cover the surface of the slide with stain, adding stain drop by drop. Count the number of drops needed to cover the surface. Let stand 1 to 3 min (optimal staining time [to obtain correct color range and intensity] will vary with each batch of stain).
3. Add to the slide the same number of drops of buffered water as were used of stain for step V.B.2 and mix the stain and water by blowing on the surface of the fluid.
4. After 4 to 8 min, flood the stain from the slide with buffered water. Do not pour the stain off before flooding, or a precipitate will be deposited on the slide.
5. Wipe the underside of the slide to remove excess stain.
6. Let air dry in a vertical position.

C. Combination thin and thick blood films

1. Lyse the RBCs in the thick film by immersing it for 10 min in buffered water. *Be sure that the water does not touch the unfixed thin film.*
2. Remove the slide, and rinse the thick film by dipping in additional buffered water (two or three dips).
3. Let film air dry thoroughly.
4. Stain both thin and thick films with Wright's stain as directed for thin films.

VI. RESULTS

A. If *Plasmodium* organisms are present, the cytoplasm stains pale blue and the nuclear material stains red.

B. Schüffner's stippling in RBCs infected by malaria species usually does not stain or stains very pale red with Wright's stain.

C. Nuclear and cytoplasmic colors that are seen in the malarial parasites will also be seen in the trypanosomes and any intracellular leishmaniae that are present.

D. The sheath of microfilariae may or may not stain with Wright's stain, while the body will usually appear pale to dark blue.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report any parasite, including the stages found (do not use abbreviations).

Examples: *Plasmodium falciparum* rings and gametocytes, rings only
Plasmodium vivax rings, trophozoites, schizonts, and gametocytes
Plasmodium ovale rings and schizonts
Plasmodium malariae rings and developing trophozoites
Babesia sp. rings
Wuchereria bancrofti microfilariae
Trypanosoma brucei gambiense/rhodesiense trypomastigotes
Trypanosoma cruzi trypomastigotes
Leishmania donovani amastigotes

B. Any laboratory providing malaria diagnoses should be able to identify *Plasmodium vivax* and *Plasmodium ovale,* even in the absence of Schüffner's stippling.

VIII. PROCEDURE NOTES

A. Blood films prepared from venipuncture blood when an anticoagulant is used must be prepared within 1 h of collection. Otherwise, certain morphological characteristics of both parasites and infected RBCs may be atypical.

B. The correct pH for all buffered-water and staining solutions is also important. Solutions with the incorrect pH will prevent certain morphological characteristics (stippling) from being visible and will not give typical nuclear and cytoplasmic colors on the stained film.

C. Stain QC slide each time patient blood films are stained. If several patient specimens are stained on the same day (using the same reagents), only one control slide need be stained and examined.

D. If at any time the stain appears to contain particulate matter or stain, precipitate, filter prior to use.

E. Report level of parasitemia when blood films are positive for *Plasmodium* spp. (*see* procedure 9.8.7).

IX. LIMITATIONS OF THE PROCEDURE

A. *Finding no parasites in one set of blood films does not rule out a parasitic infection.*

B. Examine a minimum of 300 oil immersion ($\times$1,000) fields before reporting no parasites found.

C. Examine the entire smear under low power (100$\times$) for the presence of microfilariae. Remember that the sheath may not be visible *(W. bancrofti).*

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
2. **Garcia, L. S., S. P. Johnston, A. J. Lindscott, and R. Y. Shimizu.** 2008. *Cumitech 46, Laboratory Procedures for Diagnosis of Blood-Borne Parasitic Diseases.* Coordinating ed., L. S. Garcia. ASM Press, Washington, DC.
3. **Smith, J. W., and M. S. Barlett.** 1991. Diagnostic parasitology: introduction and methods, p. 701–716. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), *Manual of Clinical Microbiology,* 5th ed. American Society for Microbiology, Washington, DC.

SUPPLEMENTAL READING

Beaver, P. C., R. C. Jung, and E. W. Cupp. 1984. *Clinical Parasitology,* 9th ed. Lea and Febiger, Philadelphia, PA.

NCCLS. 2000. *Laboratory Diagnosis of Blood-Borne Parasitic Diseases.* Approved guideline M15-A. NCCLS, Wayne, PA.

APPENDIX 9.8.6–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label

A. Wright's stain

Wright's stain ……0.9 g
methyl alcohol, absolute
(acetone free) ……500.0 ml

1. Grind 0.9 g of stain powder with 10 to 15 ml of methyl alcohol (anhydrous, acetone free) in a clean mortar. Gradually add methyl alcohol while grinding. As the dye is dissolved in the alcohol, pour that solution off and add more alcohol to the mortar. Repeat the process until the 500 ml of alcohol is used up.
2. Store the stain solution in a tightly stoppered glass bottle (1 liter) at room temperature.
3. Shake the bottle several times daily for at least 5 days (aging process).
4. Before use, filter through Whatman no. 1 paper into a brown bottle (stock stain).
5. Label appropriately, and store protected from light at room temperature. The shelf life is 36 months, providing QC criteria are met.

B. Stock buffers (for preparing buffered water)

1. Alkaline buffer, 0.067 M solution

Na_2HPO_4 (anhydrous) ……9.5 g
distilled water, to make ……1.0 liter

In a 1-liter volumetric flask, dissolve Na_2HPO_4 in about three-fourths of the water. Add water to make 1 liter of solution. Store in tightly stoppered bottle, and label appropriately. The shelf life is 24 months. Note the date on label and in work record.

2. Acid buffer, sodium dihydrogen phosphate (monobasic), 0.067 M solution

$NaH_2PO_4 \cdot H_2O$ ……9.2 g
distilled water, to make ……1.0 liter

In a 1-liter volumetric flask, dissolve $NaH_2PO_4 \cdot H_2O$ in about three-fourths of the water. Add water to make 1 liter of solution. Store in tightly stoppered bottle, and label appropriately. The shelf life is 24 months. Note date on label and in work record.

C. Buffered water, pH 6.8 (called for by some commercial stains for diluting stain and washing films)

alkaline buffer (Na_2HPO_4) ……50 ml
acid buffer ($NaH_2PO_4 \cdot H_2O$) ……50 ml
distilled water ……900 ml

1. Combine above liquids in a 1-liter bottle.
2. Mix thoroughly, and test pH of solution. If pH is not 6.8 $\pm$ 0.1, discard and remake. The solution can be used as long as the pH is within limits listed for the procedure.

9.8.7 Determination of Parasitemia

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

It is important to report the level of parasitemia when blood films are examined and found to be positive for malaria parasites. Because of the potential for drug resistance in some of the *Plasmodium* species, particularly *Plasmodium falciparum*, it is important that every positive smear be assessed and the parasitemia reported exactly the same way for follow-up specimens as for the initial specimen (1–7). This allows the parasitemia to be monitored after therapy has been initiated. In cases where the patient is hospitalized, monitoring should be performed at 24, 48, and 72 h after initiating therapy. Generally the parasitemia will drop very quickly within the first 2 h; however, in cases of drug resistance, the level may not decrease but actually increase over time.

II. SPECIMEN

Observe standard precautions.

The specimen consists of stained thick or thin blood films that have been examined a minimum of 300 oil immersion fields per blood film to determine that the film is positive for malaria parasites.

III. MATERIALS

A. Reagents
None

B. Supplies
None

C. Equipment
Microscope, binocular with mechanical stage; low-power (10×), high dry power (40×), and oil immersion (100×) objectives; 10× oculars; calibrated ocular micrometer; light source equivalent to a 20-W halogen or 100-W tungsten bulb; blue and white ground-glass diffuser filters

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Prepare and stain films from "normal" blood, and microscopically evaluate the staining reactions of RBCs, platelets, and WBCs (normally done during staining procedure using Giemsa or Wright's stain).

B. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

V. PROCEDURE

A. Thin blood film: counting several hundred to 1,000 RBCs, report the percentage of infected RBCs per 100 RBCs counted (0.5%, 1.0%, etc.).

B. Thick or thin blood film: counting 100 WBCs (or more), report the number of parasites per 100 WBCs on the smear.

1. This figure can be converted to the number of parasites per microliter of blood; divide the number of parasites per 100 WBCs by 100, and multiply that figure by the number of WBCs per microliter of blood.

2. Depending on the parasitemia, 200 or more WBCs may have to be counted, so the denominator may vary (it may be 200 or even more).

3. *Using this method, blood for both the peripheral smears and cell counts must be collected at the same time.*

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS (Table 9.8.7–1)

A. Using the thin blood film method, report the percentage of parasite-infected RBCs per 100 RBCs counted.
Example: *Plasmodium falciparum*, parasitemia = 0.5%

B. Using the thick-thin blood film method, report the number of parasites per microliter of blood.
Example: *Plasmodium falciparum*, parasitemia = 10,000 per μl of blood

Table 9.8.7–1 Parasitemia determined from conventional light microscopy: clinical correlation[a]

% Parasitemia	No. of parasites/μl	Clinical correlation[b]
0.0001–0.0004	5–20	Number of organisms that are required for a positive thick film (sensitivity)
		Note: Examination of 100 TBF fields (0.25 μl) may miss infections up to 20% (sensitivity of 80–90%); at least 300 TBF fields should be examined before reporting a negative result.
		Note: Examination of 100 THBF fields (0.005 μl); at least 300 THBF should be examined before reporting a negative result. Both TBF and THBF should be examined for every specimen submitted for a suspected malaria case. *One set (TBF + THBF) of negative blood films does not rule out a malaria infection.*
0.002	100	Patients may be symptomatic below this level.
0.2	10,000	Level above which immune patients will exhibit symptoms
2	100,000	Maximum parasitemia of *P. vivax* and *Plasmodium ovale* (infect young RBCs only)
2–5	100,000–250,000	Hyperparasitemia, severe malaria,[c] increased mortality
10	500,000	Exchange transfusion may be considered, high mortality

[a] Adapted from references 3 and 7.

[b] TBF, thick blood film; THBF, thin blood film.

[c] World Health Organization criteria for severe malaria are parasitemia of >10,000/μl and severe anemia (hemoglobin <5 g/liter). Prognosis is poor if >20% of parasites are pigment-containing trophozoites and schizonts and/or if >5% of neutrophils contain visible pigment.

VII. PROCEDURE NOTES

A. It is critical that the same reporting method be used consistently for every subsequent set of blood films so that the parasitemia can be tracked for decrease or possible increase, indicating resistance.

B. Remember, drug resistance may not become evident for several days; the parasitemia may even appear to be dropping before it begins to increase again.

C. It is very important that any patient with *P. falciparum* infection be monitored; drug resistance has also been reported for *Plasmodium vivax* infections.

D. It is critical to remember that mixed malarial infections occur, many of which will include *P. falciparum.*

VIII. LIMITATIONS OF THE PROCEDURE

A. A light infection may be missed in a thin film, whereas the increased volume of blood present on a thick film may allow the detection of the infection, even with a low parasitemia.

B. If the smears are prepared from anticoagulated blood that is more than 1 h old, the morphology of both parasites and infected RBCs may not be typical.

C. It is important that good-quality blood films be examined and counted according to directions; poorly prepared and/or stained blood films will lead to incorrect assessments of the parasitemia (both too low and too high).

D. Follow-up counts are critical in monitoring the patient, and it is critical to understand the types of malaria resistance (*see* Table 9.8.7–2).

Table 9.8.7–2 Malaria resistance[a]

Resistance definition	Comments
Sensitive	From initiation of therapy, asexual parasites are cleared by day 6; no evidence of recrudescence up to day 28
	Peripheral blood films appear to go from positive to negative very quickly (can be a change from one draw to the second draw 6 h later).
Resistance type I	From initiation of therapy, asexual parasites have cleared for at least two consecutive days (the latest day being day 6); recrudescence follows.
	Parasite count initially drops and blood films appear to be negative; patient should be monitored for a period of days, particularly if drug-resistant *P. falciparum* is suspected.
Resistance type II	Within 48 h of initiation of therapy, marked reduction of asexual parasitemia to $<$25% of pretreatment count; however, no subsequent disappearance of parasitemia (smear positive on day 6)
	Patient appears to be improving; parasite count drops, but blood films always appear positive.
Resistance type III	Modest reduction in parasitemia may be seen; no change or increase in parasitemia seen during first 48 h after treatment; no clearing of asexual parasites
	In some cases, the parasite count continues to increase with no visible decrease at any time; blood films show overall parasite increase.

[a] Adapted from references 3 and 4.

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
2. **Garcia, L. S., S. P. Johnston, A. J. Lindscott, and R. Y. Shimizu.** 2008. *Cumitech 46, Laboratory Procedures for Diagnosis of Blood-Borne Parasitic Diseases.* Coordinating ed., L. S. Garcia. ASM Press, Washington, DC.
3. **Hansheid, T.** 1999. Diagnosis of malaria: a review of alternatives to conventional microscopy. *Clin. Lab. Haematol.* **21:**235–245.
4. **Homel, M., and H. M. Gilles.** 1998. Malaria, p. 361–409. *In* F. E. G. Cox, J. P. Krier, and D. Wakelin (ed.), *Topley & Wilson's Microbiology and Microbial Infections*, 9th ed. Arnold, London, United Kingdom.
5. **Milhouse, W. K., and D. E. Kyle.** 1998. Introduction to the modes of action of and mechanisms of resistance to antimalarials, p. 303–316. *In* F. E. G. Cox, J. P. Krier, and D. Wakelin (ed.), *Topley & Wilson's Microbiology and Microbial Infections*, 9th ed. Arnold, London, United Kingdom.
6. **NCCLS.** 2000. *Laboratory Diagnosis of Blood-Borne Parasitic Diseases.* Approved guideline M15-A. NCCLS, Wayne, PA.
7. **Wilkinson, R. J., J. L. Brown, G. Pasvol, P. L. Chiodini, and R. N. Davidson.** 1994. Severe falciparum malaria: predicting the effect of exchange transfusion. *Q. J. Med.* **87:**553–557.

9.8.8 Delafield's Hematoxylin Stain

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Microfilariae in an unstained preparation do not show sufficiently definite morphological characteristics to allow positive identification. Although Giemsa stain, used for the routine staining of blood parasites, can be used for microfilariae, Delafield's hematoxylin stain is widely used to demonstrate greater structural detail of microfilariae (1, 2). This stain especially enhances the nuclei and the sheath, if present. No one type of stain, however, will reveal all the details of morphology, and in some cases, more than one staining procedure may be necessary for species identification.

II. SPECIMEN

Observe standard precautions.

A. Delafield's hematoxylin stain can be used for thick or thin blood films.

B. It is most frequently used to stain the sediment of a concentration procedure (*see* procedures 9.8.10 and 9.8.11) designed to recover more microfilariae from the blood.

III. MATERIALS

A. Reagents (*see* Appendix 9.8.8–1)

B. Supplies

1. 4 or 5 Coplin jars, 50 ml
2. Glass or plastic Pasteur pipettes
3. Pipette, to deliver 0.05 ml
4. Beakers, 250 and 25 ml
5. Flask, Erlenmeyer, 500 ml
6. 3 graduated cylinders, 100, 500, and 50 ml
7. 2 bottles, 1,000 and 100 ml
8. Funnel (glass) to hold filter paper
9. Filter paper, Whatman no. 1
10. Coverslips, 22 by 22 mm or larger, no. 1 thickness
11. Mounting medium, pH 7.0

C. Equipment

1. Microscope, binocular with mechanical stage; low-power (10×), high dry power (40×), and oil immersion (100×) objectives; 10× oculars; calibrated ocular micrometer; light source equivalent to 20-W halogen or 100-W tungsten bulb; blue and white ground-glass diffuser filters
2. Timer, 1 h or more in 1-min increments
3. pH meter (or pH paper) to check ammonia water
4. Hot plate

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. If possible, check the stain procedure before each use for diagnosis of filariasis on blood known to contain microfilariae.

B. If human blood containing microfilariae is not available, try to obtain canine blood containing *Dirofilaria immitis* from a veterinarian. No sheath is present, but nuclei should be distinct, and the colors should be those described below.

IV. QUALITY CONTROL *(continued)*

C. If positive blood is not available, follow the procedure carefully in testing the specimen submitted for diagnosis. Be sure that the morphological features of the microfilariae found are distinct and that the colors are those described below.
  1. Macroscopically, the films appear bluish purple.
  2. Microscopically, the nuclei of the microfilariae are blue or purplish, and the sheath, if present, is a light purple. The cytoplasm is reddish. The R, anal, and excretory cells may or may not be visible, but if they are, they do not differ markedly from the nuclei. The inner body may be seen as a whitish structure.

D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

E. Known positive microscope slides, Kodachrome (2- by 2-in.) projection slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

F. Record all QC results.

V. PROCEDURE (adapted from reference 3)

A. *Wear gloves when performing this procedure.*

B. Prepare thick or thin blood films or films from sediments of concentrated blood, and dry thoroughly (*see* procedures 9.8.10 and 9.8.11).

C. If thick films are to be stained, lyse the RBCs by placing the film in distilled water for 10 min. Allow to air dry. This step is not necessary for thin blood films or films prepared from sediments.

D. Fix all films in absolute methanol for 5 min. Allow to air dry.

E. Stain in undiluted Delafield's hematoxylin for 10 to 15 min.

F. Destain with acid water (0.05% HCl) for 5 to 10 s.

G. Wash in water containing sufficient ammonia to yield an alkaline pH (~9.0 to 10.0) until a blue color appears in film.

H. Air dry.

I. Mount film with neutral mounting medium and coverslip (no. 1 thickness), or examine unmounted.

J. Examine the entire film with the low-power (10×) objective. Specific morphological details can be observed with the high dry power (40×) and oil immersion (100×) objectives.

VI. RESULTS

A. Microfilariae may be present. Based on the positions of the nuclei and the presence or absence of a sheath, they can be identified to the genus and species levels.

B. If other blood parasites are seen, it is recommended that routine thin and thick films be stained with Giemsa stain.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the genus and species of microfilariae present.
Example: *Wuchereria bancrofti* microfilariae present.

B. If sufficient morphological detail is not visible to allow generic and/or specific identification, report as follows.
Examples: Sheathed microfilariae present.
Unsheathed microfilariae present.
Microfilariae present.

VIII. PROCEDURE NOTES

A. It is important that the stock stain age for at least 1 month prior to use. During this time, it should be exposed to sunlight.

B. If the nuclei do not stain blue and the cytoplasm does not stain red, review proper aging of the stain.

C. One of the main benefits of this stain is improved visibility of the sheath.
1. The sheath of *W. bancrofti* will often not be seen using Giemsa stain.
2. The sheath of *W. bancrofti* is easily seen when Delafield's hematoxylin is used.

IX. LIMITATIONS OF THE PROCEDURE

A. If the blood specimen is too old or has not been processed properly, the final stain may not reveal clear nuclear and sheath detail.

B. Regardless of the quality of the stain, a low parasitemia may not be detected.

REFERENCES

1. **Ash, L. R., and T. C. Orihel.** 1987. *Parasites: a Guide to Laboratory Procedures and Identification.* American Society of Clinical Pathologists, Chicago, IL.
2. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology,* 5th ed. ASM Press, Washington, DC.
3. **Mackie, T. T., G. W. Hunter, and C. B. Worth.** 1945. *A Manual of Tropical Medicine.* The W. B. Saunders Co., Philadelphia, PA.

SUPPLEMENTAL READING

Garcia, L. S., S. P. Johnston, A. J. Lindscott, and R. Y. Shimizu. 2008. *Cumitech 46, Laboratory Procedures for Diagnosis of Blood-Borne Parasitic Diseases.* Coordinating ed., L. S. Garcia. ASM Press, Washington, DC.

NCCLS. 2000. *Laboratory Diagnosis of Blood-Borne Parasitic Diseases.* Approved guideline M15-A. NCCLS, Wayne, PA.

APPENDIX 9.8.8–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. Delafield's hematoxylin stain, hematoxylin solution

hematoxylin crystals	4.0 g
ethyl alcohol, 95%	25.0 ml
saturated aluminum ammonium sulfate solution	400.0 ml
methyl alcohol (acetone free)	100.0 ml
glycerin	100.0 ml

1. Dissolve 4.0 g of hematoxylin crystals in 25 ml of 95% ethyl alcohol.
2. Prepare 400 ml of saturated aluminum ammonium sulfate [$AlNH_4(SO_4)_2 \cdot 12H_2O$] solution by adding the aluminum ammonium sulfate crystals (~90 g) to 400 ml of very hot distilled water, stirring until no more crystals go into solution, cooling to allow excess alum to crystallize, and decanting the solution.

APPENDIX 9.8.8–1 *(continued)*

3. Mix the hematoxylin solution with the 400 ml of saturated aluminum ammonium sulfate solution in a loosely capped or cotton-plugged bottle. Keep in a light, airy location for 2 weeks.
4. After 2 weeks, mix 100 ml of methyl alcohol (acetone free) with 100 ml of glycerin, and add this mixture to the mixture from step A.3.
5. Bottle the complete mixture from step A.4, leave cap loose, and expose to direct sunlight for at least 1 month.
6. Filter before use through Whatman no. 1 paper into a clear glass bottle, stopper tightly, and store at room temperature.
7. Label appropriately. The shelf life is 36 months, providing QC criteria are met.

B. Acid water destain

HCl …… 0.05 ml
distilled water ……99.95 ml

1. Carefully dispense the concentrated hydrochloric acid into the distilled water.
2. Label and store in a tightly stoppered bottle, and label with an expiration date of 12 months.

C. Ammonia water

ammonium hydroxide (NH_4OH) ….. 3 to 5 drops
tap water …… 50.0 ml

1. Mix the two components in a Coplin jar. Discard remaining solution after use.
2. Check pH (~9.0 to 10.0).

D. Methyl alcohol (acetone free)

E. Glycerin

F. Distilled water

9.8.9 Concentration Procedures: Buffy Coat Concentration

Concentration procedures increase the number of organisms recovered from blood specimens submitted for diagnosis of trypanosomiasis, filariasis, and leishmaniasis. A concentration procedure should be performed routinely on all blood specimens submitted for examination for trypanosomes or microfilariae when the suspected organisms are not found in thick blood films or when organisms are so few that more are needed to make a positive identification of species (*see* Appendixes 9.8.9–1 and 9.8.9–2) (1–3).

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Leishmania donovani amastigotes are difficult to detect in blood specimens but may occasionally be found within monocytes by fractional centrifugation of comparatively large amounts of blood. The procedure may also be used to recover trypanosomes and microfilariae, both of which are found in the plasma.

II. SPECIMEN

Observe standard precautions.

Whole blood collected by using EDTA, heparin, or sodium citrate anticoagulant (regular venipuncture collection tubes are recommended—primarily the lavender/EDTA or green/heparin stoppers)

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

1. Methyl alcohol, absolute
2. Giemsa or hematoxylin stain (*see* procedures 9.8.5 and 9.8.8)

B. Supplies

1. 2 centrifuge tubes, glass, 12 ml (plastic tubes can be used, provided they are *clear* plastic)
2. Capillary pipette with bulb
3. Glass microscope slides, 1 by 3 in., alcohol washed (frosted at one end, preferably)
4. Coverslips, 22 by 22 mm or larger, no. 1 thickness
5. Microhematocrit tube(s) (for alternate procedure)
6. Blood collection supplies, if applicable

C. Equipment

1. Centrifuge with sealable carrier cups, speed calibrated
2. Microscope, binocular with mechanical stage; low-power (10×), high dry power (40×), and oil immersion (100×) objectives; 10× oculars; calibrated ocular micrometer; light source equivalent to 20-W halogen or 100-W tungsten bulb; blue and white ground-glass diffuser filters
3. Timer, 1 h or more in 1-min increments

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check calibration of centrifuge.

B. Perform the procedure on "normal" blood. The film should be composed almost exclusively of WBCs, which stain characteristically with Giemsa (*see* procedure 9.8.5). If parasites are present, they also should stain characteristically (*see* procedure 9.8.5).

C. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

D. Known positive microscope slides, Kodachrome (2- by 2-in.) projection slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

E. Record all QC results.

V. PROCEDURE (adapted from reference 4)

A. *Wear gloves when performing this procedure.*

B. Centrifuge the anticoagulated blood specimen in a sealed cup at 100 $\times$ *g* for 15 min.

C. Remove the thin creamy layer (buffy coat) between the RBCs and plasma with a capillary pipette, or transfer the creamy layer (buffy coat) and plasma to another tube, and centrifuge in a sealed cup at 300 $\times$ *g* for 15 min.

D. Examine buffy coat directly for motile trypomastigotes and microfilariae.

1. Place one-half drop of saline on a clean microscope slide.

2. Remove a drop of sediment, and mix it in the saline.

3. Add a coverslip, and examine for organism motility with the low-power (10$\times$) and high dry power (40$\times$) objectives.

E. Prepare thin films, dry, fix, and stain with Giemsa stain (*see* procedure 9.8.5 or, if for microfilariae, procedure 9.8.8).

VI. RESULTS

A. If present, *L. donovani* amastigotes will be found within the monocytes on a Giemsa-stained film. Nuclear material stains dark purple-red, the cytoplasm is light blue, and the kinetoplast may or may not be visible as a dark bluish purple structure.

B. Trypomastigotes will be found extracellularly (motile in the wet smear). Morphological detail will be seen in the Giemsa-stained film. The stain reaction is like that of *L. donovani;* the kinetoplast will be visible.

C. Microfilariae may be found in the wet smear. Morphological detail will be seen in a Giemsa- or hematoxylin-stained film. The stain reaction is typical for each stain (*see* procedures 9.8.5 and 9.8.8).

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the presence of organisms from the wet smear.

Examples: Trypomastigotes present.
Microfilariae present.

B. Report the genus and species of organisms from the Giemsa-stained film.

Examples: *Trypanosoma cruzi* trypomastigotes present.
Leishmania donovani amastigotes present.

VIII. PROCEDURE NOTES

A. If you need to add anticoagulant to blood, mix 9 ml of blood and 1 ml of 5% sodium citrate in a glass centrifuge tube. Then proceed with centrifugation.

B. This procedure can be performed in a microhematocrit tube if the tube is carefully scored and broken at the buffy coat interface and if the WBCs are prepared and stained as for a thin blood film.

C. Also, the tube can be examined microscopically (high dry magnification) at the buffy coat layer for motile trypomastigotes and microfilariae before the tube is scored and broken.

IX. LIMITATIONS OF THE PROCEDURE

A. When examined as a wet smear, the intracellular leishmaniae are very difficult to see.

B. Although trypomastigote and microfilarial motility may be visible on the wet smear, specific identification may be difficult.

REFERENCES

1. **Ash, L. R., and T. C. Orihel.** 1987. *Parasites: a Guide to Laboratory Procedures and Identification.* American Society of Clinical Pathologists, Chicago, IL.
2. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
3. **Garcia, L. S., S. P. Johnston, A. J. Lindscott, and R. Y. Shimizu.** 2008. *Cumitech 46, Laboratory Procedures for Diagnosis of Blood-Borne Parasitic Diseases.* Coordinating ed., L. S. Garcia. ASM Press, Washington, DC.
4. **Young, C. W., and H. Van Sant.** 1923. *Leishmania donovani* in the peripheral blood. *J. Exp. Med.* **38:**233.

APPENDIX 9.8.9–1

Advantages and disadvantages of buffy coat films

Buffy coat film advantages	Buffy coat film disadvantages
The volume of blood cells is considerably larger than with both thick and thin films prepared from whole blood.	It takes some practice to remove the correct blood layers from the centrifuged blood in order to prepare thick and thin films.
More sensitive than thick films for diagnosis of malaria; more likely to see infected RBCs.	Same potential problems may occur as with traditional thick or thin blood films
Malaria pigment, which is phagocytized by the WBCs, may be seen more easily in the concentrated WBCs.	Some organisms and/or stages of parasites might be damaged due to high centrifugation; however, centrifugation at 500 $\times$ *g* for 15 to 20 min should not cause damage.
Detection of parasitemia easier for larger stages such as schizonts and gametocytes	Increased number of platelets may be confusing in terms of parasite differentiation

APPENDIX 9.8.9–2

QBC Capillary Blood Tube

Recently, a centrifugation procedure which yields a buffy coat has been used to concentrate and detect *Plasmodium* spp. (1–3). The technique uses a commercially available capillary tube (QBC Capillary Blood Tube; Becton Dickinson, Franklin Lakes, NJ) which is coated with acridine orange stain and fitted with a buoyant plastic insert. The stain causes the malaria organisms to fluoresce, and the plastic insert forces the RBCs containing stained parasites, because they differ in buoyancy from uninfected RBCs, to be concentrated just under the buffy coat. After centrifugation, the tube is examined under the microscope at the plastic insert level for the presence of malaria parasites. Some laboratorians have been able to accurately identify species by using this method. At this time, however, this technique requires more trials, and appropriate thin and/or thick films are recommended for the positive identification of *Plasmodium* spp.

References

1. **Long, G. W., T. R. Jones, L. S. Rickman, R. Trimmer, and S. D. Hoffman.** 1991. Acridine orange detection of *Plasmodium falciparum* malaria: relationship between sensitivity and optical configuration. *Am. J. Trop. Med. Hyg.* **44:**402–405.
2. **Rickman, L. S., G. W. Long, R. Oberst, A. Caranban, R. Sangalang, J. I. Smith, J. D. Chulay, and S. L. Hoffman.** 1989. Rapid diagnosis of malaria by acridine orange staining of centrifuged parasites. *Lancet* **i:**68–71.
3. **Spielman, A., J. P. Perrone, A. Teklehaimanot, F. Balcha, S. C. Wardlaw, and R. A. Levine.** 1988. Malaria diagnosis by direct observation of centrifuged samples of blood. *Am. J. Trop. Med. Hyg.* **39:**337–342.

9.8.10 Concentration Procedures: Membrane Filtration Concentration

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Membrane filtration methods for recovering microfilariae from patients with light infections have been developed. These methods have an advantage over simple centrifugation methods in that large samples of blood (20 ml or more) can be used if necessary. The technique described here is one of the most efficient for the clinical laboratory when other procedures used to recover microfilariae are unsatisfactory (1–3).

Membrane filtration recovers most species of microfilariae; however, because of their smallness, *Mansonella perstans* and *Mansonella ozzardi* may not be recovered. Membranes of smaller pore size (3 μm) have been suggested for recovering these two species.

II. SPECIMEN

Whole blood collected by using EDTA, heparin, or sodium citrate anticoagulant

Observe standard precautions.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

1. Distilled water
2. Methyl alcohol, absolute
3. Giemsa or hematoxylin stain (*see* procedures 9.8.5 and 9.8.8)
4. Toluene

B. Supplies

1. Glass syringe, 15 ml (clear plastic is acceptable)
2. Nuclepore membrane filter, 25 mm, 5-μm porosity
3. Swinney filter adapter (attaches to syringe, holds filter)
4. Filter paper pad, 25 mm (used to support the membrane filter)
5. Glass microscope slides, 1 by 3 in.
6. Coverslips, 22 by 22 mm or larger, no. 1 thickness
7. Mounting medium

C. Equipment

Microscope, binocular with mechanical stage; low-power (10×), high dry power (40×), and oil immersion (100×) objectives; 10× oculars; calibrated ocular micrometer; light source equivalent to 20-W halogen or 100-W tungsten bulb; blue and white ground-glass diffuser filters

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. If possible, check the procedure by using human or canine blood containing microfilariae.

B. If positive blood is not available, follow the procedure carefully in testing the specimen submitted for diagnosis. Examine sediment thoroughly with low- and high-power magnification.

IV. QUALITY CONTROL *(continued)*

C. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

D. Record all QC results.

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. Draw 1 ml of fresh whole blood or anticoagulated blood into a 15-ml syringe containing 10 ml of distilled water.

C. *Gently* shake the mixture for 2 to 3 min to ensure that all blood cells are lysed.

D. Place a 25-mm Nuclepore filter (5-µm porosity) over a moist 25-mm filter paper pad, and place in a Swinney filter adapter (*see* illustration in Appendix 9.6.9–2 of procedure 9.6.9).

E. Attach the Swinney filter adapter to the syringe containing the lysed blood.

F. With gentle but steady pressure on the piston, push the lysed blood through the filter.

G. Without disturbing the filter, remove the Swinney adapter from the syringe, and draw approximately 10 ml of distilled water into the syringe. Replace the adapter, and gently push the water through the filter to wash the debris from the filter.

H. Remove the adapter again, draw the piston of the syringe to about half the length of the barrel, replace the adapter, and push the air in the barrel through the filter to expel excess water.

I. To prepare the filter for staining, remove the adapter, draw the piston about half the length of the barrel, and then draw 3 ml of absolute methanol into the syringe. Holding the syringe vertically, replace the adapter, and push the methanol followed by the air through the filter to fix the microfilariae and expel the excess methanol, respectively.

J. To stain, remove the filter from the adapter, place it on a slide, and allow it to air dry thoroughly. Stain with Giemsa stain as for a thick film (using 0.1% Triton X-100) (*see* procedure 9.8.5) or with Delafield's hematoxylin (*see* procedure 9.8.8).

K. To cover the stained filter, dip the slide in toluene *before* mounting the filter with neutral mounting medium and a coverslip. This will lessen the formation of bubbles in or under the filter.

VI. RESULTS

A. If present in the sample, microfilariae are concentrated and will appear on the wet membrane.

B. After being stained with Giemsa or Delafield's hematoxylin, the microfilariae will stain characteristically. The sheath, if present, may or may not stain with Giemsa.

VII. REPORTING RESULTS

A. Report the presence of organisms from the wet Nuclepore membrane.
Example: Microfilariae present.

B. Report the genus and species of organisms from the Giemsa- or hematoxylin-stained membrane.
Example: *Wuchereria bancrofti* microfilariae present.

VIII. PROCEDURE NOTES

A. Gently shake the water-blood mixture to ensure total lysis of blood cells. Some parasitologists prefer to use an aqueous solution of 10% Teepol (Shell Oil Co.) to lyse the blood cells.

B. Motile microfilariae may be seen on the membrane filter after washing with water (step V.G); however, low light will be necessary and the filter must be reassembled before fixing with methanol.

C. The membrane filter must be supported by the moistened filter pad to prevent rupture when the water is expelled through the membrane.

D. If you need to add anticoagulant to blood, mix 9 ml of blood and 1 ml of 5% sodium citrate in a clear plastic centrifuge tube. Then proceed with centrifugation.

IX. LIMITATIONS OF THE PROCEDURE

A. Giemsa or hematoxylin staining may be necessary to identify the organisms to the species level.

B. Identification of microfilariae on filters to the species level may be difficult.

REFERENCES

1. **Ash, L. R., and T. C. Orihei.** 1987. *Parasites: a Guide to Laboratory Procedures and Identification.* American Society of Clinical Pathologists, Chicago, IL.
2. **Dennis, D. T., and B. H. Kean.** 1971. Isolation of microfilariae: report of a new method. *J. Parasitol.* **57:**1146–1147.
3. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.

SUPPLEMENTAL READING

Beaver, P. C., R. C. Jung, and E. W. Cupp. 1984. *Clinical Parasitology,* 9th ed. Lea and Febiger, Philadelphia, PA.

Smith, J. W., and M. S. Bartlett. 1991. Diagnostic parasitology: introduction and methods, p. 701–716. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), *Manual of Clinical Microbiology*, 5th ed. American Society for Microbiology, Washington, DC.

9.8.11 Concentration Procedures: Knott Concentration

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

This technique is used to recover low numbers of microfilariae from blood. A solution is used to lyse the RBCs in a large blood sample, and the organisms are concentrated from the supernatant fluid by centrifugation (1–6). The disadvantage of this technique is that the microfilariae are killed and immobilized and are therefore not readily revealed by any motility.

II. SPECIMEN

Whole blood collected by using EDTA, heparin, or sodium citrate anticoagulant

Observe standard precautions.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagent

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

Formalin, aqueous, 2% (vol/vol) formaldehyde, liquid ……2 ml
distilled water …… 98 ml
Mix thoroughly. Store in stoppered bottle. Label appropriately. Shelf life is 24 months.

B. Supplies

1. Graduated cylinder, 100 ml
2. Pipette, 5 ml
3. Bottle, 100 ml
4. Capillary pipette with bulb
5. Glass microscope slides, 1 by 3 in.
6. Coverslips, 22 by 22 mm or larger, no. 1 thickness
7. Blood collection supplies, if applicable
8. Centrifuge tube, glass, 12 ml

C. Equipment

1. Centrifuge with sealable carrier cups, speed calibrated
2. Microscope, binocular with mechanical stage; low-power (10×), high dry power (40×), and oil immersion (100×) objectives; 10× oculars; calibrated ocular micrometer; light source equivalent to 20-W halogen or 100-W tungsten bulb; blue and white ground-glass diffuser filters
3. Timer, 1 h or more in 1-min increments

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check calibration of centrifuge.
B. If possible, check the procedure by using human or canine blood containing microfilariae with or without a sheath.
C. If positive blood is not available, follow the procedure carefully in testing the specimen submitted for diagnosis. Examine sediment thoroughly with low- and high-power magnification.
D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.
E. Known positive microscope slides, Kodachrome (2- by 2-in.) projection slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.
F. Record all QC results.

V. PROCEDURE

A. *Wear gloves when performing this procedure.*
B. Place 1 ml of fresh whole blood or anticoagulated blood in a centrifuge tube containing 10 ml of 2% formalin. Mix thoroughly.
C. Centrifuge for 5 min at 300 $\times$ *g*.
D. Pour off the supernatant fluid without disturbing the sediment.
E. Using a capillary pipette, transfer a portion of the sediment to a slide.
  1. Apply a coverslip, and examine microscopically under low-power (100$\times$) and high-power (400$\times$) magnification.
  2. If microfilariae are present, prepare a thick film from the remainder of the sediment, air dry, fix in absolute methanol for 5 min, air dry again, and stain with Giemsa or Delafield's hematoxylin (*see* procedures 9.8.5 and 9.8.8).

VI. RESULTS

A. If present in the sample, microfilariae are concentrated and will appear nonmotile in the wet smear.
B. After being stained with Giemsa or Delafield's hematoxylin, the microfilariae will exhibit diagnostic morphology and typical staining characteristics.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the presence of organisms from the wet smear.
  Example: Microfilariae present.
B. Report the genus and species of organisms from the Giemsa- or hematoxylin-stained film.
  Example: *Wuchereria bancrofti* microfilariae present.

VIII. PROCEDURE NOTES

A. If you need to add anticoagulant to blood, mix 9 ml of blood and 1 ml of 5% sodium citrate in a glass centrifuge tube. Then proceed with centrifugation.
B. Morphological details may not be visible prior to Giemsa or hematoxylin staining.

IX. LIMITATIONS OF THE PROCEDURE

A. Motility will not be visible after formalin fixation.

B. Identification to the species level may be difficult for most laboratorians without additional staining.

C. The blood-formalin mixture can be sent to a reference laboratory for staining and identification of microfilariae.

REFERENCES

1. **Ash, L. R., and T. C. Orihel.** 1987. *Parasites: a Guide to Laboratory Procedures and Identification.* American Society of Clinical Pathologists, Chicago, IL.
2. **Beaver, P. C., R. C. Jung, and E. W. Cupp.** 1984. *Clinical Parasitology,* 9th ed. Lea and Febiger, Philadelphia, PA.
3. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology,* 5th ed. ASM Press, Washington, DC.
4. **Garcia, L. S., S. P. Johnston, A. J. Lindscott, and R. Y. Shimizu.** 2008. *Cumitech 46, Laboratory Procedures for Diagnosis of Blood-Borne Parasitic Diseases.* Coordinating ed., L. S. Garcia. ASM Press, Washington, DC.
5. **Knott, J.** 1939. A method for making microfilarial surveys on day blood. *Trans. R. Soc. Trop. Med. Hyg.* **33:**191–196.
6. **NCCLS.** 2000. *Laboratory Diagnosis of Blood-Borne Parasitic Diseases.* Approved guideline M15-A. NCCLS, Wayne, PA.

9.8.12 Concentration Procedures: Triple Centrifugation Concentration

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

In many instances, the number of trypomastigotes (trypanosomes) present in peripheral blood is too small to be detected in direct wet mounts or stained films. Fractional centrifugation is an effective method of concentrating the organisms from a relatively large blood sample (1–5).

II. SPECIMEN

Observe standard precautions.

Whole blood collected by using EDTA, heparin, or sodium citrate anticoagulant. Make sure to fill the tube (containing the anticoagulant) completely, thus guaranteeing the correct anticoagulant/blood ratio.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagent

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

Giemsa or Wright's stain (*see* procedures 9.8.5 and 9.8.6)

B. Supplies

1. 3 centrifuge tubes, 12 or 15 ml
2. Capillary pipettes with bulb
3. Glass microscope slides, 1 by 3 in.
4. Coverslips, 22 by 22 mm or larger, no. 1 thickness
5. Blood collection supplies, if applicable

C. Equipment

1. Centrifuge with sealable carrier cups, speed calibrated
2. Microscope, binocular with mechanical stage; low-power (10×), high dry power (40×), and oil immersion (100×) objectives; 10× oculars; calibrated ocular micrometer; light source equivalent to 20-W halogen or 100-W tungsten bulb; blue and white ground-glass diffuser filters
3. Timer, 1 h or more in 1-min increments

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check calibration of centrifuge.

B. If possible, check the procedure by using blood containing trypanosomes. Positive blood may be available at universities and large research laboratories.

C. If positive blood is not available, follow the procedure carefully in testing the specimen submitted for diagnosis.

D. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the micro-

IV. QUALITY CONTROL *(continued)*

scope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

E. Known positive microscope slides, Kodachrome (2- by 2-in.) slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

F. Record all QC results.

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. Centrifuge the blood at 300 × *g* for 10 min.

C. Transfer the supernatant fluid to a second centrifuge tube, and centrifuge at 500 × *g* for 10 min.

D. Again, transfer the supernatant fluid to a clean tube, and centrifuge at 900 × *g* for 10 min.

E. Carefully decant the supernatant fluid, and examine about one-half of the sediment directly as a wet preparation for motile trypanosomes. Use the 10× objective for the examination; in order to see greater organism detail, use the 40× objective (do not use the 100× oil immersion objective on wet preparations).

F. From the remaining sediment, prepare thin films, and stain with Giemsa or Wright's stain.

VI. RESULTS

A. If present in the sample, trypomastigotes (trypanosomes) are concentrated.

B. After being stained with Giemsa or Wright's stain, the trypomastigotes will exhibit diagnostic morphology and typical staining characteristics.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Report the presence of organisms from the wet smear.
Example: Trypomastigotes present.

B. Report the genus and species of organisms from the Giemsa- or Wright-stained films.
Example: *Trypanosoma cruzi* or *Trypanosoma gambiense/rhodesiense* present.

VIII. PROCEDURE NOTES

A. Each time, remove the supernatant fluid carefully (do not remove any sediment).

B. Make sure the centrifuge is calibrated and the timing is begun when the centrifuge reaches the recommended speed.

C. Leave some sediment for the preparation of thin films for staining with Giemsa or Wright's stain.

IX. LIMITATIONS OF THE PROCEDURE

A. Although organism motility may be visible, morphological detail may not be.

B. Unless dividing trypomastigotes are found in the wet preparation, permanent staining with Giemsa or Wright's stain will be necessary to differentiate the species.

REFERENCES

1. **Ash, L. R., and T. C. Orihel.** 1987. *Parasites: a Guide to Laboratory Procedures and Identification.* American Society of Clinical Pathologists, Chicago, IL.
2. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
3. **Garcia, L. S., S. P. Johnston, A. J. Lindscott, and R. Y. Shimizu.** 2008. *Cumitech 46, Laboratory Procedures for Diagnosis of Blood-Borne Parasitic Diseases.* Coordinating ed., L. S. Garcia. ASM Press, Washington, DC.
4. **Markell, E. K., D. T. John, and W. A. Krotoski.** 1999. *Markell and Voge's Medical Parasitology*, 8th ed. W. B. Saunders Co., Philadelphia, PA.
5. **NCCLS.** 2000. *Laboratory Diagnosis of Blood-Borne Parasitic Diseases.* Approved guideline M15-A. NCCLS, Wayne, PA.

9.9.1 Parasite Culture: *Entamoeba histolytica*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Entamoeba histolytica, the agent of intestinal and hepatic amebiasis, can be cultivated in conjunction with the bacteria voided in feces by the infected patient. Although cultures for *E. histolytica* are not routinely offered by most clinical laboratories, this approach may be helpful when routine procedures have failed to provide a diagnosis. Polyxenic cultured organisms can also be used to produce intestinal and hepatic amebiasis in susceptible experimental hosts such as hamsters, guinea pigs, and rats. Axenic cultivation of organisms is invaluable for the following: (i) to study the biochemistry, physiology, and metabolism of the organisms in order to establish nutritional requirements of the parasites; (ii) to produce antigens of and monoclonal and polyclonal antibodies against *E. histolytica* for serological diagnosis as well as other immunologic studies; (iii) to differentiate pathogenic from nonpathogenic strains by using isoenzyme electrophoresis, monoclonal antibody, and/or DNA probes; (iv) to screen drugs in vitro to identify isolates susceptible and resistant to particular drugs so that advances in chemotherapy can be evaluated; (v) to infect experimental animals to produce the disease so that pathological processes can be understood; and (vi) to understand the organization of the parasite at the ultrastructural level.

II. SPECIMEN

Observe standard precautions.

A. The specimen may consist of stool, mucus, or a combination of the two.
B. The specimen should be as fresh as possible but never more than 24 h old.

III. MATERIALS

A. Reagents (*see* Appendix 9.9.1–1)
B. Supplies
1. Graduated Erlenmeyer flasks
2. Graduated cylinders (500 ml)
3. Volumetric flasks (1,000 and 2,000 ml)
4. Microscope slides (1 by 3 in. or larger)
5. Coverslips (no. 1; 22 by 22 mm or larger)
6. Sterile syringes (10 ml)
7. Sterile needles (20 gauge)
8. Sterile screw-cap tubes (16 by 125 mm)
9. Sterile cryovials or screw-cap vials (to hold 1 ml)
10. Box for vial storage in freezer
11. Disposable sterile Pasteur and serological pipettes
12. Filter paper, Whatman no. 1
13. Sterile filtration system (Seitz)
14. Membrane filters (0.22-μm pore size)
15. Nutrient agar plates
16. BHI broth
17. Thioglycolate broth
18. Methylene blue solution
19. Biohazard container of disinfectant for proper disposal of tubes, slides, and pipettes
20. Biohazard container for proper disposal of patient specimens
21. ATCC 30925 (*E. histolytica* HU-1:CDC)
22. ATCC 30015 (*E. histolytica* HK-9)
23. Laredo strain (*E. histolytica*-like, ATCC 30042) (can be maintained in culture at 25°C)
24. Any strain of *Entamoeba moshkovskii* (can be maintained in culture at 25°C)

III. MATERIALS *(continued)*

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives; phase-contrast and/or differential interference contrast optics preferred
2. Binocular inverted microscope with 4×, 10×, and 40× objectives; phase-contrast and/or differential interference contrast optics preferred (this microscope is recommended but not mandatory)
3. Oculars should be 10×. Some may prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
4. Tabletop centrifuge, preferably refrigerated (for centrifuging tubes containing patient specimens, e.g., watery stools, and for culture tubes)
5. Hot plate
6. Biological safety cabinet, type II
7. Magnetic stirrer and stir bar
8. pH meter
9. Drying oven or dry-heat sterilizer
10. Incubator that can be set at 35 ± 2°C

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check all reagents and media (PBS solution no. 8, rice starch suspension, Tween 80 solution, TYSGM-9 and TYI-S-33 media) each time they are used or periodically (once a week).

1. The media should be free of any signs of precipitation and bacterial and/or fungal contamination.
2. PBS solution no. 8, Tween 80 solution, and rice starch suspension should be clear, with no visible signs of contamination.

B. Maintain stock cultures of *E. histolytica* at 35°C (ATCC 30925 [strain HU-1: CDC] and ATCC 30015 [strain HK-9]). Maintain *E. histolytica*-like (ATCC 30042) Laredo and *E. moshkovskii* strains in culture at 25°C.

1. Transfer stock culture (ATCC 30925) every other day with TYSGM-9 medium. Transfer stock cultures held at 25°C once a month.
2. Transfer stock culture (ATCC 30015) once every 3 days with TYI-S-33 medium.
3. *E. histolytica* trophozoites are 10 to 60 μm long and demonstrate directional motility by extruding hyaline, fingerlike pseudopodia from the cytoplasm. Cysts are not usually found in cultures.
4. Trophozoites are uninucleate and the nucleus is characterized by finely granular, uniform, evenly distributed peripheral chromatin. The nucleolus is small and usually centrally located but may be eccentric.

C. The microscope(s) should be calibrated, and the original optics used for the calibration should be in place on the microscope(s). Post the calibration factors for all objectives on the microscope for easy access (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

D. Record all QC results.

V. PROCEDURE

Xenic and axenic culture

In Greek, *xenos* means stranger. Culture amebae grown in association with an unknown microbiota are called a xenic culture. If the amebae are grown in association with a single known bacterium, the culture is monoxenic; if the culture contains several identified bacteria, then it is polyxenic. If amebae are grown as pure culture without any bacterial associate, the culture is axenic.

V. PROCEDURE *(continued)*

A. *Wear gloves when performing this procedure.*

B. Xenic culture

1. Warm several tubes of TYSGM-9 medium in the incubator (35°C for 1 to 2 h).

2. Add 0.1 ml of stock antimicrobials to each tube of medium. Final concentrations of antimicrobials are 100 U of penicillin and 100 μg of streptomycin per ml.

3. After vortexing or vigorously shaking the tube, use a Pasteur pipette to add 3 drops of the starch suspension to each tube of the medium.

4. Place a pea-sized portion of stool sample into the bottom of the tube, and break up the stool gently with the pipette.

5. Tightly cap the tubes, and incubate at a 45 to 50° angle at 35°C for 48 h.

6. With an inverted microscope and the 10× objective, examine the tubes for the presence of amebae. If present, amebae will usually be seen attached to the underside of the tubes interspersed with the fecal material and rice starch. Sometimes it may be necessary to gently invert the tubes to disperse the stool material and rice starch to uncover the amebae. If you do not have an inverted microscope, proceed to step V.B.13.

7. If amebae are not seen, stand the tubes upright for about 30 min at 35°C.

8. With a Pasteur pipette, remove from the bottom of each tube 0.5 to 0.75 ml of sediment, and inoculate the sediment into fresh tubes containing rice starch and antibiotics. Centrifuge the original tubes at 250 × *g* for 10 min, decant the supernatant, and add 8 ml of fresh medium.

9. Incubate all tubes as described above (step V.B.5) for another 48 h.

10. Examine the tubes as before, and discard the tubes if amebae are still not seen. Report patient results as negative.

11. If amebae are present in small numbers, then chill the tube in ice water for 5 min, and centrifuge the tube for 5 min at 250 × *g*. Aspirate and discard the supernatant, and inoculate the sediment into a fresh tube as before.

12. If amebae are present in large numbers, then let the tube stand upright for 30 min, and remove about 0.5 ml of sediment from the bottom. Inoculate the sediment into fresh tubes as before.

13. If you do not have an inverted microscope, stand the tubes upright for about 30 min at 35°C. With a sterile pipette, remove about 0.5 ml of sediment from the bottom of the tube, and place a couple of drops onto each of two slides. Add 2 drops of methylene blue solution to one of the slides. Cover both slides with coverslips, and examine the slides under the microscope for amebae. Amebae may be rounded or have pseudopodial extrusions. The nucleus may be clearly seen in the methylene blue preparation. Proceed to steps V.B.7 through 12.

C. Axenic culture (used for research)

1. Remove tubes containing TYI-S-33 medium from 4°C and incubate at 35°C for 1 to 2 h.

2. With an inverted microscope, examine stock culture tubes of *E. histolytica* (HK-9 strain) for any signs of bacterial contamination (if present, tubes are no longer acceptable for use). Select one or several tubes showing good growth of amebae. Since the tubes are incubated in a slanted position, usually at an angle of 5° to 10°, a thick button of amebae will be seen at the bottom of the tube. Gently invert the tube once or twice to disperse the amebae uniformly, and examine the tubes again. A majority of the amebae should be attached to the tube walls and show pseudopodial motility. If you do not have an inverted microscope, examine organisms from the bottom of the tube (as a wet smear). If you can see pseudopodial motility, proceed to the next step (step V.C.3).

V. PROCEDURE *(continued)*

3. Immerse the tubes in a bucket of ice-cold water for about 5 to 10 min. This will dislodge the amebae from the tube walls. Invert the tubes several times in order to distribute the amebae.
4. With a Pasteur pipette, remove about 1.0 to 1.5 ml of culture medium; inoculate 0.5 to 1 ml into a fresh tube. Inoculate the rest of the fluid into nutrient agar, BHI broth, and thioglycolate broth for routine monitoring of bacterial contamination. Inoculate several tubes this way, and incubate the cultures slanted at a 5 to 10° angle at 35°C as before.
5. If amebic growth is not good but some amebae are attached to the tube walls, then use a serological pipette to remove about 10 ml of medium from the bottom, and add 10 ml of fresh medium.
6. If amebic growth is not good and only a few amebae are present along with a lot of debris, then centrifuge the tube at 250 × *g* for 10 min, aspirate the supernatant fluid, transfer the sediment to a fresh tube, and incubate the tube as before.

VI. RESULTS

Protozoan trophozoites and/or cysts may be recovered and identified.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. If the tubes containing the fecal material are positive for amebae after 48 h of incubation, then confirm the identification with a permanent stained smear.

B. To ascertain that the amebae cultured are *E. histolytica,* prepare a permanently stained smear (trichrome or iron hematoxylin), since *Entamoeba coli* may also be isolated in culture.

Example: *Entamoeba histolytica* present.

C. If the tubes do not show any amebae, then subculture the tubes as described above and incubate for an additional 48 h. If the tubes are still negative for amebae, then report the specimen as negative and discard the tubes.

Example: No *Entamoeba histolytica* isolated.

VIII. PROCEDURE NOTES

A. When initiating xenic cultures, inoculate stool samples into at least two tubes, one with and the other without antibiotics. In some cases, some component of the natural microbiota may be helpful or even necessary for the amebae to become established and to differentiate (possibly) into cysts.

B. If the culture tubes become contaminated during routine subculture and maintenance of the axenically grown amebae, try to eliminate the contaminant by adding 1,000 U of penicillin and 1,000 μg of streptomycin or 50 μg of gentamicin per ml. If, however, the contaminant happens to be *Pseudomonas* spp., it is probably better to discard the tube and use an uncontaminated tube for subculture purposes.

C. Vitamin mixture no. 13 used in the TYI-S-33 medium may be replaced with 10 ml of NCTC-109 medium (any supplier of tissue culture media). Although the final yield of amebae may not be as good as that obtained with medium containing the vitamin mix, the procedure is simpler and saves a lot of time and effort.

IX. LIMITATIONS OF THE PROCEDURE

A. Culture of *E. histolytica* serves only as a supplemental procedure and never replaces primary diagnosis by microscopic examination. Axenic culture is used for maintaining QC strains and for research purposes. Xenic cultures may be used as a supplemental diagnostic procedure.

B. Even when the culture system is within QC guidelines, a negative culture is still not definitive in ruling out the presence of *E. histolytica* (1–5).

REFERENCES

1. **Clark, C. G., and L. S. Diamond.** 2002. Methods for cultivation of luminal parasitic protists of clinical importance. *Clin. Microbiol. Rev.* **15:**329–341.
2. **Diamond, L. S.** 1983. Lumen dwelling protozoa: *Entamoeba*, trichomonads, and *Giardia*, p. 65–109. *In* J. B. Jensen (ed.), *In Vitro Cultivation of Protozoan Parasites.* CRC Press, Boca Raton, FL.
3. **Diamond, L. S.** 1987. *Entamoeba*, *Giardia* and *Trichomonas*, p. 1–28. *In* A. E. R. Taylor and J. R. Baker (ed.), *In Vitro Methods for Parasite Cultivation.* Academic Press, Orlando, FL.
4. **Diamond, L. S., C. G. Clark, and C. C. Cunnick.** 1995. YI-S, a casein-free medium for axenic cultivation of *Entamoeba histolytica*, related *Entamoeba*, *Giardia intestinalis* and *Trichomonas vaginalis. J. Eukaryot. Microbiol.* **42:**277–278.
5. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.

APPENDIX 9.9.1–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. TYSGM-9 medium

1. Nutrient broth

potassium phosphate, dibasic (K_2HPO_4)	2.8 g
potassium phosphate, monobasic (KH_2PO_4)	0.4 g
sodium chloride (NaCl)	7.5 g
casein digest peptone (BBL; catalog no. 97023)	2.0 g
yeast extract (BBL)	1.0 g
glass-distilled water	970.0 ml

The nutrient broth may be stored for several months at −20°C (without sterilization), according to L. S. Diamond (1, 2). Note the expiration date on the label and in the work record.

2. 5% Tween 80 solution
   a. With a magnetic stirrer, vigorously stir 95 ml of glass-distilled water in a bottle.
   b. Add 5 g of Tween 80 (very thick solution; must be weighed), and keep stirring for a few minutes. Avoid foam formation.
   c. Filter sterilize with a 0.22-μm-pore-size membrane.
   d. Aseptically dispense into a number of sterile screw-cap test tubes, with 10 ml per tube.
   e. Label as 5% Tween 80 solution with the preparation date and an expiration date of no longer than 1 month.
   f. Store at 4°C.
3. Phosphate-buffered solution (PBS no. 8), pH 7.2

sodium chloride (NaCl)	9.5 g
potassium phosphate, dibasic (K_2HPO_4)	3.7 g
potassium phosphate, monobasic (KH_2PO_4)	1.1 g
glass-distilled water to	1,000.0 ml

   a. Dissolve the salts in the distilled water by using a magnetic stirrer.
   b. Autoclave for 15 min at 121°C.
   c. When cool, label as PBS no. 8 with the preparation date and an expiration date of 3 months.

APPENDIX 9.9.1–1 *(continued)*

4. Rice starch
   For best results, use rice starch obtained from British Drug Houses Ltd. or Gailard Schlesinger, Inc. (ATCC can also be contacted for information).
   a. Dispense 500 mg of rice starch into each of several screw-cap tubes (16 by 125 mm). Do not tighten the caps.
   b. Place the tubes horizontally in a dry-heat sterilizer or an oven. Make sure that the rice is uniformly distributed loosely over the undersurface of the tubes.
   c. Heat the tubes for 2.5 h at 150°C.
   d. When cool, tighten caps and label as rice starch with the date of preparation and an expiration date of 3 months.
5. Rice starch suspension
   a. Add 9.5 ml of sterile PBS no. 8 to each tube of rice starch.
   b. Shake vigorously or use a vortex mixer to uniformly suspend the rice starch at the time of use.
6. Stock antimicrobial solution
   a. Using a sterile 6-ml syringe and 20-gauge needle, add 5 ml of sterile distilled water to a vial of penicillin G sodium (10^6 U).
   b. Using a 6-ml syringe and a 20-gauge needle, add 5 ml of sterile distilled water to a vial of streptomycin sulfate (10^6 μg/ml).
   c. Shake gently, and let stand for 30 min to dissolve the antimicrobials completely in the distilled water.
   d. Mix the two antimicrobials in a sterile graduated flask or cylinder, and bring the volume to 125 ml with distilled water. Stock concentrations of antimicrobials are 8,000 U of penicillin and 8,000 μg of streptomycin per ml.
   e. Filter sterilize the antimicrobial solution through a 0.22-μm-pore-size membrane filter, dispense the filtrate into a number of sterile screw-cap vials or sterile cryovials (1 ml per vial), and label as stock antimicrobial solution (Pen/Strep) with the preparation date and an expiration date of 1 year.
   f. Store at −20°C in a cryovial box.
7. Buffered methylene blue solution
   a. Solution A, 0.2 M acetic acid

      glacial acetic acid ……11.55 ml
      distilled water …… 988.45 ml

      Add the acetic acid to the water, mix, and store in a glass-stoppered bottle. Label with the date of preparation and an expiration date of 1 year.
   b. Solution B, 0.2 M sodium acetate

      sodium acetate ($NaC_2H_3O_2$) …… 16.4 g
      distilled water to ……1,000.0 ml

      Dissolve the sodium acetate in 400 ml of distilled water in a volumetric flask, bring the volume to 1,000 ml, mix well, and store in a glass-stoppered bottle. Label with the date of preparation and an expiration date of 1 year.
   c. Acetate buffer, pH 3.6

      solution A …… 46.3 ml
      solution B ……3.7 ml
      distilled water to ……100.0 ml

      Mix solutions A and B in a volumetric flask, and bring the volume to 100.0 ml with distilled water. pH should be 3.6. Store in a glass-stoppered bottle. Label with the date of preparation and an expiration date of 1 year.
   d. Methylene blue stain

      methylene blue dye …… 60.0 mg
      acetate buffer (from previous step [step A.7.c]) ……100.0 ml

      Dissolve the dye in the buffer, and store in a glass-stoppered bottle. Label with the date of preparation and an expiration date of 1 year.

APPENDIX 9.9.1–1 *(continued)*

8. Complete medium (TYSGM-9 medium)
   a. Place 200 mg of gastric mucin (U.S. Biochemical Corp.; catalog no. 16025) in 125-ml screw-cap bottle or Erlenmeyer flask.
   b. Add 97 ml of nutrient broth (*see* item A.1). Using a magnetic stirrer, stir vigorously for at least 1 h or until the medium becomes clear.
   c. Autoclave for 15 min at 121°C; cool to room temperature.
   d. In a biological safety cabinet, aseptically add 5.0 ml of heat-inactivated bovine serum.
   e. Add 0.1 ml of the 5% Tween 80 solution.
   f. In a biological safety cabinet, aseptically dispense 8 ml per tube into a number of sterile screw-cap tubes (16 by 125 mm).
   g. After vigorously shaking the tube, add 0.25 ml of rice starch solution.
   h. Store the tubes at 4°C for not more than 1 month.
   i. The final pH of the medium should be 7.2.

B. TYI-S-33 medium

1. Nutrient broth

potassium phosphate, dibasic (K_2HPO_4)	1.0 g
potassium phosphate, monobasic (KH_2PO_4)	0.6 g
sodium chloride (NaCl)	2.0 g
casein digest peptone (BBL; catalog no. 97023)	20.0 g
yeast extract (BBL)	10.0 g
glucose	10.0 g
L-cysteine-HCl	1.0 g
ascorbic acid	0.2 g
ferric ammonium citrate	22.8 mg
glass-distilled water to	870.0 ml

   a. Use a magnetic stirrer to dissolve the ingredients listed above in about 700 ml of glass-distilled water.
   b. Adjust pH to 6.8 with 1 N NaOH.
   c. Bring the volume to 870 ml with glass-distilled water.
   d. Filter through a no. 1 Whatman filter paper.
   e. Autoclave for 15 min at 121°C.
   f. Cool to room temperature.
   g. Store at 4°C.
2. Vitamin mixture no. 13
   a. Solution 1a

niacin	40 mg
p-aminobenzoic acid	180 mg

   Dissolve in glass-distilled water, and bring the volume to 125 ml.
   b. Solution 1b

nicotinamide	40 mg
pyridoxal hydrochloride	40 mg
pyridoxine	80 mg
calcium pantothenate	25 mg
choline chloride	830 mg
l-inositol	125 mg
thiamine hydrochloride	25 mg
vitamin B_{12}	12 mg

   Dissolve in about 100 ml of glass-distilled water, and bring the volume to 125 ml.
   c. Solution 1c

riboflavin	25 mg

   Using a magnetic stirrer, dissolve in about 100 ml of glass-distilled water with the dropwise addition of 1 N NaOH, and bring the volume to 450 ml with glass-distilled water.

APPENDIX 9.9.1–1 *(continued)*

d. Solution 1d

folic acid ……30 mg

Using a magnetic stirrer, dissolve in about 100 ml of glass-distilled water with the dropwise addition of 1 N NaOH, and bring the volume to 450 ml with glass-distilled water.

e. Solution 1e

D-biotin ……30 mg

Using a magnetic stirrer, dissolve in about 100 ml of glass-distilled water with the dropwise addition of 1 N NaOH, and bring the volume to 450 ml with glass-distilled water.

f. Solution 1

Mix all 5 solutions (a, b, c, d, and e). pH should be 6.5 to 7.0.

g. Solution 2a

DL-6,8-thiotic acid (oxidized form) ……. 100 mg
95% ethanol …… 50.0 ml

h. Solution 2b

Tween 80 (very thick solution, must be weighed) ……5.0 g
menadione sodium bisulfite ……30 mg
α-tocopherol acetate ……25 mg

Using a magnetic stirrer, dissolve in about 100 ml of distilled water, and bring the volume to 200 ml.

i. Solution 2c

Mix solutions 2a and 2b, and bring the volume to 300 ml with sterile glass-distilled water.

j. Solution 3

Combine solution 1 and solution 2c, and bring the volume to 2,000 ml with glass-distilled water. Filter sterilize the solution through a 0.22-μm-pore-size membrane filter.

Dispense in small volumes (e.g., 10 ml), and label as vitamin mix no. 13. Give the date of preparation on the label. Store at −20 or −70°C. The vitamin mix may be stored for several years in this fashion.

3. Complete medium (TYI-S-33 medium)

nutrient broth ……870.0 ml
bovine serum (inactivated at 56°C for 30 min) ……100.0 ml
vitamin mix no. 13 …… 20.0 ml

The final medium should have a pH of 6.6.

a. Aseptically dispense 13 to 14 ml per tube into a number of sterile screw-cap glass culture tubes (16 by 125 mm).
b. Label as TYI-S-33 medium with the date of preparation and an expiration date of no longer than 2 weeks.
c. Store at 4°C in a dark place, as the medium is extremely sensitive to light.

◩ **NOTE:** Casein digest peptone varies from lot to lot in its ability to support the growth of *E. histolytica.* Because of the difficulties in obtaining a uniformly good lot that supports good growth of *E. histolytica,* Diamond (3) has formulated a new medium (YI-S), which does not include casein digest peptone. YI-S medium is similar to TYI-S-33 medium. The major difference between the two media, however, is the increase in the concentration of yeast extract to 30 g (in YI-S medium) to compensate for the omission of casein digest peptone in the TYI-S-33 medium.

REFERENCES

1. **Clark, C. G., and L. S. Diamond.** 2002. Methods for cultivation of luminal parasitic protists of clinical importance. *Clin. Microbiol. Rev.* **15:**329–341.
2. **Diamond, L. S.** 1983. Lumen dwelling protozoa: *Entamoeba*, trichomonads, and *Giardia*, p. 69–109. *In* J. B. Jensen (ed.), *In Vitro Cultivation of Protozoan Parasites.* CRC Press, Boca Raton, FL.
3. **Diamond, L. S.** 1987. *Entamoeba, Giardia* and *Trichomonas*, p. 1–28. *In* A. E. R. Taylor and J. R. Baker (ed.), *In Vitro Methods for Parasite Cultivation.* Academic Press, Orlando, FL.

9.9.2 Parasite Culture: *Acanthamoeba* and *Naegleria* spp.

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Members of the genera *Acanthamoeba* and *Naegleria* are free-living soil and freshwater amebae known to cause human disease. Only one species of *Naegleria*, *Naegleria fowleri*, is known to cause primary amebic meningoencephalitis, which is almost always fatal, whereas several species of *Acanthamoeba* are known to cause fatal granulomatous amebic encephalitis and the nonfatal but vision-threatening *Acanthamoeba* keratitis (1–4). Members of both genera can be easily cultivated in the laboratory either monoxenically (with a single species of bacterium included in the culture medium) or axenically (containing no organisms other than the amebae). When the etiologic agent in CSF or other body fluids cannot be identified in suspected cases of amebic encephalitis, it is imperative to try to culture the organisms. Culture should be attempted even in those cases for which a presumptive diagnosis has been made on the basis of the morphologic features of the presumed agent, since these organisms can be mistaken for host cells. Axenic cultivation of the organisms is invaluable for the following: (i) studying the biochemistry, physiology, and metabolism of the organisms to determine their nutritional requirements; (ii) producing antigens for monoclonal and polyclonal antibodies to these amebae for serological diagnosis as well as for other immunologic studies; (iii) differentiating species within the genera by using isoenzyme electrophoresis, monoclonal antibody, and/or DNA probes; (iv) in vitro screening of drugs to identify isolates susceptible and resistant to particular drugs so that advances in chemotherapy can be made; (v) infecting experimental animals so that pathological processes involved in the disease state can be understood; and (vi) understanding the organization of the parasite at the ultrastructural level.

II. SPECIMENS

Observe standard precautions.

A. For both *Acanthamoeba* and *Naegleria* spp., the specimens usually consist of CSF, biopsy tissue, or autopsy tissue of the brain, and for *Acanthamoeba*, biopsy or autopsy tissue of the lungs, corneal scrapings or biopsy material, contact lenses and contact lens paraphernalia such as contact lens cases and solutions, skin abscess material, ear discharge, or feces can also be used.

B. Soil and water samples may also be processed for the isolation of these small free-living amebae.

C. For best results, process the specimens, especially CSF and tissue samples, for culture within 24 h, preferably sooner if possible.

D. The samples should never be frozen but may be refrigerated.

E. If the samples can be processed within 4 to 8 h, keep them at room temperature (24°C) until processed.

F. Collect all samples aseptically, and place them in sterile containers.

G. Collect at least 100 ml of water sample for the isolation of the amebae; the container should be large enough to have plenty of air space.

III. MATERIALS

A. Reagents (*see* Appendix 9.9.2–1)

B. Supplies

1. Nonnutrient agar plates, Nelson's and PYG media
2. Moist chamber
3. Microscope slides (1 by 3 in. or larger)
4. Coverslips (no. 1, 22 by 22 mm or larger)
5. Sterile 1× ameba saline
6. Sterile distilled water
7. Bacteriological loop
8. Fine spatula made of nichrome wire
9. 18- to 24-h-old culture of *Escherichia coli* or *Enterobacter aerogenes*, *Acanthamoeba castellanii* ATCC 30010, and *Naegleria gruberi* ATCC 30133
10. Sterile Pasteur and serological pipettes (1, 5, and 10 ml)
11. Sterile screw-cap test tubes (13 by 100 or 16 by 125 mm)
12. Volumetric flasks (1,000 ml)
13. Vaspar
14. Parafilm (American Can Co.) or equivalent

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives; phase-contrast and/or differential interference contrast optics preferred
2. Binocular inverted microscope with 4×, 10×, and 40× objectives; phase-contrast and/or differential interference contrast optics preferred (this microscope is recommended but not mandatory)
3. Oculars should be 10×. Some may prefer 5×; however, smaller magnification may make final organism identifications more difficult.
4. Tabletop centrifuge, preferably refrigerated (for centrifuging tubes containing patient specimens, contact lens solutions, and water samples)
5. Hot plate
6. Biological safety cabinet, type II
7. Magnetic stirrer and stir bar
8. pH meter

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check all reagents and media (ameba saline, distilled water, nonnutrient agar plates, Nelson's and PYG media) each time they are used or periodically (once a week).

1. The media should be free of any signs of precipitation and bacterial and/or fungal contamination.
2. Page's saline should be clear, with no visible sign of contamination.
3. Examine the nonnutrient agar plates under the 40× objective of an inverted or binocular microscope, and make sure that no fungal contamination has occurred.

B. Maintain stock cultures of *A. castellanii* and *N. gruberi* at 25°C.

1. Transfer stock cultures monthly with nonnutrient agar slants and Page's ameba saline.
2. *N. fowleri* is 10 to 35 μm long and demonstrates an eruptive locomotion by producing smooth hemispherical bulges. The cyst produces smooth walls (7 to 15 μm thick). The flagellate stage does not have a cytostome.
3. *Acanthamoeba* is 15 to 45 μm long and produces fine, tapering, hyaline projections called acanthapodia. It has no flagellate stage but produces a double-walled cyst with an outer wrinkled wall (10 to 25 μm thick).
4. Trophozoites of *Naegleria* and *Acanthamoeba* spp. are uninucleate and have a large, dense, central nucleolus.
5. Stain
   - **a.** Run a slide prepared from a stock strain of amebae in parallel with the patient slide.
   - **b.** Staining results are acceptable when the control amebae stain.
6. Culture
   - **a.** Plate both stock cultures onto fresh media, and incubate at 37°C in parallel with patient culture.
   - **b.** Culture results are acceptable when growth appears by day 7.

IV. QUALITY CONTROL
(continued)

7. Enflagellation
   a. Run *N. gruberi* in parallel with patient culture being observed for enflagellation.
   b. Test is acceptable when free-swimming, pear-shaped flagellates with two flagella are observed in 2 to 24 h on the control slide.

C. The microscope(s) should be calibrated, and the original optics used for the calibration should be in place on the microscope(s). Post the calibration factors for all objectives on the microscope for easy access (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

D. Known positive microscope slides, Kodachrome (2- by 2-in.) projection slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

E. Record all QC results.

V. PROCEDURE

Observe standard precautions.

A. *Wear gloves when performing this procedure.*

B. Remove the nonnutrient agar plates from the refrigerator, and place them in a 37°C incubator for 30 min.

C. Add 0.5 ml of ameba saline to a slant culture of *E. coli* or *E. aerogenes.* Gently scrape the surface of the slant (do not break the agar surface). Suspend the bacteria uniformly by gently pipetting with a Pasteur pipette, and add 2 or 3 drops of this suspension to the middle of the warmed agar plate. Spread the bacteria on the surface of the agar with a bacteriological loop.

D. Inoculate the specimen on the center of the agar plate as follows.

1. CSF

Centrifuge CSF at 250 × *g* for 10 min. With a sterile serological pipette, carefully transfer all but 0.5 ml of the supernatant to a sterile tube, and store at 4°C (for possible future use). Mix the sediment in the rest of the fluid, and use a Pasteur pipette to place 2 or 3 drops in the center of the nonnutrient agar plate that has been precoated with bacteria. After the fluid has been absorbed, seal the plates with a 5- to 6-in. length of 1-in.-wide Parafilm. Incubate the plate *upright* at 37°C.

2. Tissue

Triturate a small piece of the tissue (brain, lung, skin abscess, corneal biopsy, or similar specimens) in a small quantity (ca. 0.5 ml) of ameba saline. Process as described above. Corneal smear, ear discharge material, etc., may be placed directly on the agar surface and incubated as described above (step V.D.1).

3. Water sample

Water samples (10 to 100 ml) may be processed to isolate amebae. First, filter the water sample through three layers of sterile gauze or cheesecloth to remove leaves, dirt, etc. Next, (i) filter the sample through a sterile 5.0-μm-pore-size cellulose acetate membrane (47-mm diameter), invert the membrane over a nonnutrient agar plate precoated with bacteria, seal, and incubate the plates as described above (step V.D.1); or (ii) centrifuge the water sample for 10 min at 250 × *g*, aspirate the supernatant, suspend the sediment in about 0.5 ml of ameba saline, deposit this suspension in the center of the nonnutrient agar plate precoated with bacteria, seal, and incubate the plate at 37°C as before.

V. PROCEDURE *(continued)*

4. Soil
   Mix about 1 g of the soil sample with enough ameba saline (ca. 0.5 to 1 ml) to make a thick slurry. Inoculate this slurry in the center of the nonnutrient agar plate precoated with bacteria, and incubate as described above (step V.D.1).
5. Contact lens solutions
   Small volumes (ca. 1 to 2 ml) may be inoculated directly onto the nonnutrient agar plates precoated with bacteria. Centrifuge larger volumes (2 to 50 ml) as in step V.D.3, inoculate the sediment onto the center of the nonnutrient agar plate, and incubate it as before (step V.D.1).

E. Examine the plates microscopically for amebae (cysts or trophozoites) every day for 10 days. Thin linear tracks (areas where amebae have ingested bacteria) may be seen. If amebae are seen, circle that area with a wax pencil, carefully remove the Parafilm seal under a biological safety cabinet, open the lid of the petri dish, and carefully cut out the marked area from the agar by using a spatula that has been heated to red hot and cooled before use in order to prevent contamination (a sterile disposable scalpel can also be used). Transfer the piece facedown onto the surface of a fresh agar plate coated with bacteria, seal the plate with Parafilm, and incubate as before (step V.D.1).

F. Enflagellation experiment

1. Examine the plates every day for signs of amebae. If present, amebae will feed on bacteria, multiply, and cover the entire surface of the plate within a few days. Once the food supply is exhausted, the amebae will differentiate into cysts.
2. Mark the area containing a large number of amebic trophozoites with a wax pencil.
3. Using a bacteriological loop, scrape the surface of the agar at the marked area, and transfer several loopfuls of the scraping to a sterile tube containing about 2 ml of sterile distilled water. Alternatively, flood the surface of the agar plate with about 10 ml of sterile distilled water, gently scrape the agar surface with a loop, transfer the liquid to a sterile tube, and incubate at 37°C.
4. Periodically examine the tube with an inverted microscope for the presence of flagellates.
   - **a.** *N. fowleri*, the causal agent of primary amebic meningoencephalitis, undergoes transformation to a pear-shaped flagellate, usually with two flagella but occasionally with three or four flagella. The flagellate stage is a temporary nonfeeding stage and usually reverts to the trophozoite stage. *N. fowleri* trophozoites are typically amebalike and move in a sinuous way. They are characterized by a nucleus with a large, centrally located nucleolus. The trophozoites are also characterized by the presence of a contractile vacuole that appears once every 45 to 50 s and discharges its contents. The contractile vacuole looks like a hole or a dark depression inside the trophozoite and can easily be seen when examining the plate under the 10× or 40× objective. When the food supply is exhausted, *N. fowleri* trophozoites differentiate into spherical, smooth-walled cysts.
   - **b.** In contrast, *Acanthamoeba* spp., which cause keratitis and granulomatous amebic encephalitis, do not transform into the flagellate stage. *Acanthamoeba* trophozoites are characterized by the presence of fine, thornlike processes that are constantly extended and retracted. The trophozoites produce double-walled cysts characterized by a wrinkled outer wall (ectocyst) and a polygonal, stellate, oval, or even round inner wall (endocyst). The trophozoites are also characterized by the presence of the contractile vacuole, which disappears and reappears at regular intervals (45 to 50 s).
   - **c.** The cysts of both *Acanthamoeba* and *Naegleria* spp. are uninucleate.

VI. RESULTS

Protozoan trophozoites (amebic, flagellate forms) and/or cysts may be recovered and identified.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. Patient specimens

If a plate is positive for amebae and the amebae transform into flagellates, then the specimen should be reported as positive for *N. fowleri.* If the amebae do not transform into flagellates even after overnight incubation and if the trophozoites possess the characteristic acanthapodia, show a large centrally placed nucleolus in the nucleus on trichrome stain, and differentiate into the characteristic double-walled cysts, then report the specimen as positive for *Acanthamoeba.*

B. Contact lens solution

If the plates are positive for amebae and the amebae do not transform into flagellates but differentiate into cysts with an outer wrinkled ectocyst and an inner stellate, polygonal, oval, or round endocyst, then report the specimen as positive for *Acanthamoeba. Naegleria* spp. have not been isolated from contact lens solutions, but small amebae (e.g., hartmannellid or vahlkampfiid amebae, which produce smooth-walled cysts), probably contaminants, have occasionally been isolated from these solutions.

C. Water samples

Plates inoculated with water samples are usually positive for many genera and species of small free-living amebae (fresh water is their normal habitat). Therefore, report the sample as positive for small free-living amebae.

D. Notify the physician immediately if patient specimens are positive for *Acanthamoeba* or *Naegleria.*

VIII. PROCEDURE NOTES

A. Examine most patient specimens, especially CSF, microscopically as soon as they arrive in the laboratory.

1. Remove a small drop of the CSF sediment (step V.D.1), place it on a microscope slide, cover it with a no. 1 coverslip, seal the edges of the coverslip with Vaspar, and examine it immediately with a 10× or 40× objective (phase-contrast or differential interference contrast optics are preferred). If bright-field microscopy is used, reduce the illumination by adjusting the iris diaphragm.

a. *N. fowleri* trophozoites are highly motile and can be identified by their sinuous movement. A warmed penny applied to the bottom surface of the slide will activate the trophozoite. Occasionally a flagellate may be seen traversing the field.

b. *Acanthamoeba* trophozoites are rarely seen in the CSF. If present, they may be recognized by their characteristic acanthapodia, which are constantly extending and retracting. Both amebae, especially *Acanthamoeba,* may be recognized by the contractile vacuole.

2. Process and examine lens care solutions (opened) like CSF.

3. If very small amounts of tissue are received, reserve them for culture.

B. An alternative method for the preparation of agar plates would be to prepare agar deeps.

1. Aliquot 20 ml of the nonnutrient agar into screw-cap tubes (20 by 150 mm).

2. Autoclave the tubes at 121°C for 15 min.

3. Store at 4°C with a 12-month expiration date.

4. Prior to use, melt the agar deeps in boiling water.

VIII. PROCEDURE NOTES *(continued)*

5. Pour into a petri dish (100 by 15 mm).
6. Cool. Store at 4°C with a 3-month expiration date.

C. ATCC strains of *E. coli* or *E. aerogenes* are not necessary. Any routine clinical isolate or stock organism is acceptable.

D. Material from the surface of a positive agar plate can be removed, fixed, and stained by trichrome for microscopic examination at a higher magnification (×1,000).

IX. LIMITATIONS OF THE PROCEDURE

A. Always confirm results obtained with wet mounts by permanent stained smears (trichrome and iron hematoxylin) for nuclear characteristics in order to differentiate the amebae from host cells.

B. Organisms may not be recovered if appropriate centrifugation speeds and times are not used.

C. *Balamuthia mandrillaris* cannot be cultured using the agar plate method.

REFERENCES

1. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
2. **Krogstad, D. A., G. S. Visvesvara, K. W. Walls, and J. W. Smith.** 1991. Blood and tissue protozoa, p. 727–750. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), *Manual of Clinical Microbiology*, 5th ed. American Society for Microbiology, Washington, DC.
3. **Ma, P., G. S. Visvesvara, A. J. Martinez, F. H. Theodore, P.-M. Daggett, and T. K. Sawyer.** 1990. *Naegleria* and *Acanthamoeba* infections: review. *Rev. Infect. Dis.* **12:**490–513.
4. **Schuster, F. L.** 2002. Cultivation of pathogenic and opportunistic free-living amebas. *Clin. Microbiol. Rev.* **15:**342–354.

SUPPLEMENTAL READING

Martinez, A. J. 1985. *Free-Living Amebas: Natural History, Prevention, Diagnosis, Pathology, and Treatment of the Disease.* CRC Press, Inc., Boca Raton, FL.

Page, F. C. 1988. *A New Key to Fresh Water and Soil Gymnamoebae.* Fresh Water Biological Association, The Ferry House, Ambleside, Cumbria, United Kingdom.

Rondanelli, E. G. (ed.). 1987. *Amphizoic Amoebae: Human Pathology.* Piccin Nuova Libraria, Padua, Italy.

APPENDIX 9.9.2–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. Page's ameba saline (10×)

sodium chloride (NaCl)	1.20 g
magnesium sulfate ($MgSO_4 \cdot 7HOH$)	0.04 g
sodium phosphate, dibasic (Na_2HPO_4)	1.42 g
potassium phosphate, monobasic (KH_2PO_4)	1.36 g
calcium chloride ($CaCl_2 \cdot 2HOH$)	0.04 g
double-distilled water to	1,000 ml

1. Using a magnetic stirrer, dissolve the above ingredients in the order listed in distilled water in an appropriate glass flask or bottle.
2. Distribute 100 ml into each of 10 glass bottles.
3. Label as ameba saline (10×) with the preparation date and an expiration date of 3 months.
4. Sterilize by autoclaving at 121°C for 15 min.
5. Cool and store at 4°C.

APPENDIX 9.9.2–1 *(continued)*

B. Nonnutrient agar

10× ameba saline (from step A)	100.0 ml
Difco agar	15.0 g
double-distilled water	900.0 ml

1. Mix ameba saline with distilled water by using a magnetic stirrer to make 1× ameba saline. Add 15.0 g of agar to this solution, and dissolve with heat. Autoclave at 121°C for 15 min.
2. Cool to about 60°C, and aseptically pour into sterile plastic petri dishes (20 ml for 100- by 15-mm dish or 5 ml for 60- by 15-mm dish).
3. Label as nonnutrient agar plates with the preparation date and an expiration date of 3 months.
4. After the agar gels, store the agar plates in canisters at 4°C.

C. Nutrient agar, pH 6.8

beef extract	3.0 g
peptone or Gelysate pancreatic digest of gelatin	5.0 g
agar	15.0 g
distilled water	1,000.0 ml

1. Mix well, and autoclave at 121°C for 15 min.
2. Cool to 60°C, and dispense 5 ml per tube; slant to obtain a slant with a thick butt. Nutrient agar slants may be obtained from BBL (catalog no. 20970/20971).

D. Modified Nelson's medium for *N. fowleri*

Panmede (ox liver digest) (Difco)	10.0 g
glucose	10.0 g
10× ameba saline (from step A)	100.0 ml
double-distilled water to	900.0 ml

1. Add ameba saline to distilled water to make 1× ameba saline.
2. Dissolve the ingredients in ameba saline by using a magnetic stirrer.
3. Dispense 10 ml per tube into screw-cap tubes (16 by 125 mm).
4. Autoclave for 15 min at 121°C.
5. Cool, and label as Nelson's medium with the preparation date and an expiration date of 3 months.
6. Store at 4°C.
7. Add 0.2 ml of heat-inactivated fetal calf serum to each tube before inoculating with the amebae.

E. Peptone-yeast extract-glucose (PYG) medium for *Acanthamoeba* spp., pH 6.5 ± 0.2

Proteose Peptone (Difco)	20.0 g
yeast extract (Difco)	2.0 g
magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.980 g
calcium chloride ($CaCl_2$)	0.059 g
sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)	1.0 g
ferric ammonium sulfate $[Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O]$	0.02 g
potassium phosphate, monobasic (KH_2PO_4)	0.34 g
sodium phosphate, dibasic ($Na_2HPO_4 \cdot 7H_2O$)	0.355 g
glucose	18.0 g
distilled water to	1,000.0 ml

1. Dissolve all ingredients except $CaCl_2$ in about 900 ml of distilled water in a bottle or flask by using a magnetic stirrer.
2. Add $CaCl_2$ while stirring.
3. Bring the volume to 1,000 ml with distilled water.
4. Dispense 5 ml per tube into screw-cap tubes (16 by 125 mm).

APPENDIX 9.9.2–1 *(continued)*

5. Autoclave at 121°C for 15 min.
6. When tubes have cooled, label the tubes as *Acanthamoeba* medium with the preparation date and an expiration date of 3 months.
7. Store at 4°C.

F. Vaspar

This is a 1:1 mixture of petroleum jelly and paraffin.

1. Melt paraffin in a large (400-ml) beaker, and record the volume, e.g., 100 ml.
2. Gradually add petroleum jelly while stirring with a glass rod until the volume reaches 200 ml.
3. Remove mixture from heat, dispense about 10 to 15 ml into each of several 20-ml flasks or beakers, and cover with a piece of silver foil.
4. Store at room temperature.

9.9.3 Parasite Culture: *Trichomonas vaginalis*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Cultivation is the most sensitive method for the diagnosis of trichomoniasis; however, it may take 3 to 4 days to determine culture results (1–6). Axenic cultivation of the organism is invaluable for diagnosis and for studying the biochemistry, physiology, metabolism, immunology, and ultrastructure of the organism as well as for screening drugs in vitro so that advances in chemotherapy can be achieved. Axenic cultivation of the parasite also helps us understand the pathological processes in experimental animals and determine if it is possible to infect suitable experimental animals with organisms grown in culture and thus simulate the naturally occurring disease process. Along with culture, it is imperative that wet smears and/or stained smears be examined microscopically. If smears are positive, appropriate therapy can be instituted before culture results are available.

II. SPECIMENS

Observe standard precautions.

A. Specimens from women may consist of vaginal exudate collected from the posterior fornix on cotton-tipped applicator sticks or genital secretions collected on polyester sponges.

B. Specimens from men can include semen, prostatic fluid, urethral samples collected with polyester sponges, or urine.

C. Urine samples collected from the patient should be the first-voided specimen in the morning.

D. It is critical that clinical specimens be inoculated into culture medium as soon as possible after collection. Although collection swabs can be used, there are often problems with specimens drying prior to culture. Many laboratories are currently using the plastic envelope methods simultaneous transport and culture (*see* procedure 9.9.4).

III. MATERIALS

A. Reagents (*see* Appendix 9.9.3–1)

B. Supplies

1. Disposable sterile Pasteur and serological pipettes
2. Microscope slides (1 by 3 in. or larger)
3. Coverslips (no. 1, 22 by 22 mm or larger)
4. Volumetric flasks (100 ml)
5. Graduated cylinders (1,000 ml)
6. Flasks or beakers
7. Sterile and nonsterile screw-cap tubes (16 by 125 mm)
8. Büchner or other funnel
9. Filter paper, Whatman no. 1
10. Culture tube racks
11. Nutrient agar plates, BHI plates, and thioglycolate broth
12. Sterile cryovials or screw-cap vials (to hold 1 ml)
13. Box for vial storage in freezer
14. ATCC 30001 *(Trichomonas vaginalis)*

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives; phase-contrast and or differential interference contrast optics preferred

III. MATERIALS *(continued)*

2. Binocular inverted microscope with 10×, 40×, and 100× objectives; phase-contrast and differential interference contrast optics preferred (this microscope is recommended but not mandatory)
3. Oculars should be 10×. Some may prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
4. Tabletop centrifuge, preferably refrigerated (for tubes containing patient specimens, e.g., urine, and for culture tubes)
5. Water bath or heating block (range up to 80°C)
6. Freezer, −20°C
7. Magnetic stirrer and stir bar
8. pH meter

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check all reagents and media (at least once a week). All media, including Ringer's solution, should be free of any signs of precipitation and bacterial and/or fungal contamination.

B. The microscope(s) should be calibrated, and the original optics used for the calibration should be in place on the microscope(s). Post the calibration factors for all objectives on the microscope for easy access (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

C. Maintain stock cultures of *T. vaginalis* (ATCC 30001).

1. Transfer stock cultures weekly.
   - **a.** Always culture stock organisms at the same time a patient specimen is inoculated into culture medium.
   - **b.** If the stock organisms multiply and remain viable during the 96 h, report patient results.
2. Stain
   - **a.** Run a slide prepared from a stock strain of *T. vaginalis* in parallel with the patient slide.
   - **b.** Staining results are acceptable when the control organisms stain well.

D. Known positive microscope slides, Kodachrome (2- by 2-in.) projection slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.

E. Record all QC results.

V. PROCEDURE

A. *Wear gloves when performing this procedure.*

B. Inoculation of culture medium

1. Remove tubes containing culture medium from 4°C, and incubate at 37°C for 1 to 2 h.
2. Vigorously shake the cotton-tipped portion of the applicator stick containing the patient specimen in the medium, and then break off the tip with sterile forceps and drop it into the medium.
3. If the material is collected on polyester sponges, then drop the sponges into the medium and shake the tube.
4. Centrifuge urine samples for 10 min at 250 × *g*, aspirate the supernatant, and inoculate the sediment into the medium.
5. Examine the tubes daily for several days, and subculture if necessary. To subculture, first shake the tube to disperse the organisms uniformly, remove about 1 to 2 ml, and inoculate into a warmed, fresh tube.

V. PROCEDURE *(continued)*

6. Incubate the tubes in a slanted position (45° angle) at 37°C.
7. Incubate control tubes and those containing patient material for at least 72 to 96 h.
8. Examine the entire length of the tube. If the specimen is positive, *T. vaginalis* will be found freely swimming or attached to the tube walls.
9. Do not report negative results until 96 h.

C. To maintain stock cultures

1. With an inverted microscope, examine stock culture tubes of *T. vaginalis* for any signs of bacterial contamination. Select one or more tubes showing good growth. Since the tubes are incubated in a slanted position, usually at an angle of 45°, a thick button of organisms will be seen at the bottom of the tube. Gently invert the tube once or twice to disperse the trichomonads uniformly, and examine the tubes again. A large number of the organisms should be freely swimming, and a few of the organisms will be attached to the tube walls.
2. Immerse the tubes in a bucket of ice-cold water for about 5 to 10 min. This will dislodge the trichomonads from the tube walls. Invert the tubes several times in order to distribute the organisms.
3. With a sterile Pasteur pipette, remove about 1.0 to 1.5 ml; inoculate 0.5 to 1.0 ml into a fresh tube. Inoculate the rest of the fluid into nutrient agar, BHI, and thioglycolate broth for routine monitoring of bacterial contamination. Inoculate several additional tubes this way, and incubate the cultures slanted at a 45° angle at 37°C as before (step V.B.6).
4. If growth is poor and only few organisms are present along with a lot of debris, then centrifuge the tube at 250 $\times$ *g* for 10 min, aspirate the supernatant, transfer the sediment to a fresh tube, and incubate as before (step V.B.6).

VI. RESULTS

T. vaginalis trophozoites may be recovered and identified.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. If organisms are found prior to or at the end of 96 h, report the specimen as positive.
Example: Positive for *Trichomonas vaginalis*

B. If no trophozoites are seen after 4 days of incubation, then discard the tubes and report as negative.
Example: Negative for *Trichomonas vaginalis*

VIII. PROCEDURE NOTES

A. Cultivation is the most sensitive method for the diagnosis of trichomoniasis. Every effort, therefore, must be made to inoculate patient materials into culture medium. However, since this method may take 3 to 4 days and the patient materials may occasionally contain nonviable organisms, it is imperative that microscopic examination of wet smears and/or stained smears (Giemsa) also be performed.

B. Culture control organisms each time a patient specimen is inoculated into the culture medium.

C. Use the same medium for controls and patient specimen.

IX. LIMITATIONS OF THE PROCEDURE

A. Even though cultivation is the most sensitive method for the diagnosis of trichomoniasis, it may take 3 to 4 days to arrive at a diagnosis. Every effort, therefore, must be made to microscopically examine wet smears and or stained smears so that appropriate therapy can be instituted without delay in case of positive findings. In addition to culture, immunoassay and PCR kits are available for the detection of *T. vaginalis.*

B. Do not report results of patient specimens as positive unless control cultures are positive.

REFERENCES

1. **Clark, C. G., and L. S. Diamond.** 2002. Methods for cultivation of luminal parasitic protists of clinical importance. *Clin. Microbiol. Rev.* **15:**329–341.
2. **Diamond, L. S.** 1983. Lumen dwelling protozoa: *Entamoeba,* trichomonads, and *Giardia,* p. 65–109. *In* J. B. Jensen (ed.), *In Vitro Cultivation of Protozoan Parasites.* CRC Press, Boca Raton, FL.
3. **Diamond, L. S.** 1987. *Entamoeba, Giardia* and *Trichomonas,* p. 1–28. *In* A. E. R. Taylor and J. R. Baker (ed.), *In Vitro Methods for Parasite Cultivation.* Academic Press, Orlando, FL.
4. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology,* 5th ed. ASM Press, Washington, DC.
5. **Linstead, D.** 1990. Cultivation, p. 91–111. *In* B. M. Honigberg (ed.), *Trichomonads Parasitic in Man.* Springer-Verlag, New York, NY.
6. **McMillan, A.** 1990. Laboratory diagnostic methods and cryopreservation of trichomonads, p. 297–310. *In* B. M. Honigberg (ed.), *Trichomonads Parasitic in Man.* Springer-Verlag, New York, NY.

APPENDIX 9.9.3–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. Ringer's solution

sodium chloride (NaCl) ……0.6 g
sodium bicarbonate ($NaHCO_3$) …… 0.01 g
potassium chloride (KCl) …… 0.01 g
calcium chloride ($CaCl_2$) …… 0.01 g
double-distilled water to ……100.0 ml

Dissolve the ingredients in the order listed, and bring the volume to 100.0 ml with distilled water.

B. Liver infusion

double-distilled water ……330.0 ml
Bacto liver infusion powder (Difco) …… 20.0 g

1. Place the distilled water in a large beaker.
2. Add the liver infusion powder.
3. Infuse for 1 h at 50°C.
4. Raise the temperature to 80°C for 5 min to coagulate the protein.
5. Filter through a Whatman no. 1 filter paper with a Büchner funnel.

C. Methylene blue solution

methylene blue ……0.5 g
glass-distilled water ……100.0 ml

Mix well until dissolved.

APPENDIX 9.9.3–1 *(continued)*

D. Cysteine-peptone-liver-maltose (CPLM) complete medium

Bacto Peptone (Difco)	32.0 g
Bacto Agar (Difco)	1.6 g
cysteine HCl	2.4 g
maltose	1.6 g
Bacto liver infusion (Difco)	320.0 ml
Ringer's solution	960.0 ml

1. Mix Ringer's solution and liver infusion in a large beaker by using a magnetic stirrer.
2. Add peptone, maltose, cysteine HCl, and agar in that order, and heat the mixture until dissolved.
3. Add 0.7 ml of aqueous methylene blue.
4. Adjust pH to 5.8 to 6.0 with 1 N NaOH or 1 N HCl.
5. Dispense 8-ml volumes into culture tubes.
6. Autoclave at 121°C for 15 min.
7. Aseptically add 2 ml of human serum (heat inactivated at 56°C for 30 min and cooled) per tube. Horse serum is recommended as a replacement for human serum, particularly when considering safety issues such as handling human blood and blood products.
8. Label as CPLM medium with the preparation date.
9. Store at room temperature. Use as long as the amber zone, indicating an anaerobic condition, persists.

E. Diamond's Trypticase-yeast extract-maltose (TYM) complete medium

Trypticase (BBL)	20.0 g
yeast extract (BBL)	10.0 g
maltose	5.0 g
L-cysteine HCl	1.0 g
L-ascorbic acid	0.2 g
potassium phosphate, dibasic (K_2HPO_4)	0.8 g
potassium phosphate, monobasic (KH_2PO_4)	0.8 g
Bacto Agar (Difco)	0.5 g
double-distilled water	900.0 ml

1. Dissolve the buffer salts in the distilled water by using a magnetic stirrer.
2. Add the remaining ingredients except the agar in the order given, one at a time, until dissolved.
3. Adjust pH to 6.0 with 1 N HCl.
4. Add agar, and heat to dissolve.
5. Autoclave at 121°C for 15 min.
6. Cool to 45°C and add 100 ml of sterile bovine, sheep, or horse serum that has been heat inactivated for 30 min at 56°C.
7. Aseptically dispense 10-ml volumes into screw-cap tubes (16 by 125 mm).
8. Label as TYM medium with the preparation date and an expiration date of 10 days.
9. Store at 4°C.

F. Diamond's complete medium (modified by Klass)

Trypticase (BBL)	24.0 g
yeast extract (BBL)	12.0 g
maltose	6.0 g
cysteine HCl	1.2 g
ascorbic acid	0.24 g
double-distilled water	900.0 ml

1. Dissolve the ingredients one at a time in the order given.
2. Adjust pH to 6.0 with 1 N HCl or 1 N NaOH.
3. Dispense in 12.5-ml aliquots into screw-cap tubes (16 by 125 mm).
4. Autoclave at 121°C for 15 min.
5. When cool (50°C), add 1 ml of sterile inactivated horse serum and 0.5 ml of antibiotic mixture to each tube.

APPENDIX 9.9.3–1 *(continued)*

6. Label as modified TYM medium with the date of preparation and an expiration date of 3 weeks.

G. Antimicrobial mixture

sodium penicillin G ……1,000,000 U
streptomycin sulfate ……1,000,000 µg
amphotericin B (Fungizone) ……2,000 µg
sterile double-distilled water …… 50.0 ml

1. Mix thoroughly. Concentration of stock solution is

penicillin …… 20,000 U/ml
streptomycin …… 20,000 µg/ml
amphotericin B ……40 µg/ml

2. Dispense 1 ml of the antimicrobial mixture into sterile screw-cap vials or sterile cryovials.
3. Label as antimicrobial solution with the date of preparation and an expiration date of 1 year.
4. Store at −20°C in cryoboxes.

H. Serum substitutions

Bovine, sheep, or horse serum can be substituted in step D.7.

9.9.4 Parasite Culture: InPouch TV System for *Trichomonas vaginalis*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Trichomoniasis is a sexually transmitted infection caused by the flagellated protozoan *Trichomonas vaginalis.* It is recognized as one of the most prevalent forms of sexually transmitted disease (STD) worldwide, with over 180 million cases occurring annually. The CDC estimates that 3 million cases occur in the United States on a yearly basis. *T. vaginalis* can produce premature rupture of membranes in pregnancy and can also cause nongonococcal urethritis in males.

Cultivation is the most sensitive method for the diagnosis of trichomoniasis; however, it may take 3 to 4 days to determine culture results. The InPouch TV system has demonstrated a greater sensitivity than either the saline wet mount or Hollander's, Trichosel, or modified Diamond's media (1–6). The InPouch TV serves first as a specimen transport container and growth chamber during incubation, then as a viewing chamber during microscopy (using a plastic clip that fits onto the microscope stage). It consists of a clear, gas-impermeable, plastic pouch that is double chambered. The medium contains Trypticase, Proteose Peptone, yeast extract, maltose and other sugars, amino acids, salts, and antifungal and antimicrobial agents in normal saline phosphate buffer. An inoculum containing 1 to 10 organisms is sufficient to cause a positive test if the specimen is inoculated immediately after collection.

II. SPECIMENS

Observe standard precautions.

A. Specimens from women may consist of vaginal exudate collected from the posterior fornix using sterile cotton or Dacron swabs.

B. Specimens from men can include semen, prostatic fluid, and urethral samples collected with a cotton swab on a wire handle.

C. Although urine specimens are not recommended as the most likely to yield positive culture results, urine specimens collected from the patient should be the first-voided specimen in the morning.

D. Patient specimens that are contaminated with birth control foams or jellies will result in decreased recovery of trichomonads.

III. MATERIALS

A. Reagents

1. InPouch kits [BioMed Diagnostics, Inc.; (800) 964-6466] stored vertically at 15 to 25°C up to 1 year from date of manufacture.

2. *Warning:* This product contains a chemical known to cause cancer, birth defects, or other reproductive harm.

B. Supplies

1. Viewing slide holder (provided in the kit)

2. Disposable protective gloves

C. Equipment

Microscope, binocular with mechanical stage; low-power (10×), high dry power (40×), and oil immersion (100×) objectives; 10× oculars; calibrated ocular micrometer; light source equivalent to a 20-W halogen or 100-W tungsten bulb; blue and white ground-glass diffuser filters

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Inoculate a pouch from each new lot with 1 drop from a culture of actively growing *T. vaginalis* and incubate at 37°C. Observe for actively motile organisms at 3 days.
B. An InPouch containing cloudy medium should not be used.
C. The organism is maintained in subculture medium, purchased separately, where it will remain viable for 7 days.
D. If product does not meet standards, inform the parasitologist or supervisor, record all QC results.
E. The microscope should be calibrated, and the objectives and oculars used for the calibration procedure should be used for all measurements on the microscope. Post the calibration factors for all objectives on the microscope for easy access (multiplication factors can be pasted right on the body of the microscope) (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.

V. PROCEDURE

A. Inoculation of InPouch TV: *wear gloves when performing this procedure.*
1. Remove InPouch TV from box.
2. Fold pouch back over itself to reduce the folded crease. Make sure that the liquid in the upper chamber is below the closure tape to prevent fluid from leaking upon opening.
3. Tear open the pouch at the notch just above the white closure. Open the pouch sufficiently to admit the swab, by pulling apart the (white) tape's middle tabs of the closure tape.
4. Insert swab containing the specimen into the liquid of the pouch's upper chamber (Fig. 9.9.4–1 and 9.9.4–2).
5. Squeeze the swab to express specimen by gently pressing it between the walls of the upper chamber. Discard swab in a biohazard waste container.

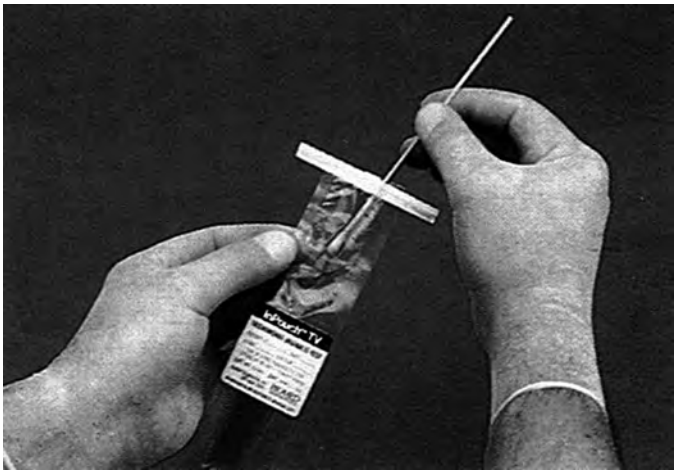


Figure 9.9.4–1 InPouch TV diagnostic system for culturing *Trichomonas vaginalis* (BioMed Diagnostics). The swab containing a specimen from the patient is inserted into the liquid medium within the plastic pouch.

V. PROCEDURE *(continued)*

6. Squeeze pouch closed, fold the top edge down, and roll three times. Fold the wire tape's end-tabs behind the pouch to lock the roll.
7. Label with patient's name. *Do not obstruct clear plastic with label.*
8. Hold specimen pouch upright at room temperature until prompt delivery to laboratory.

B. Specimen not received in InPouch TV
1. Notify submitter that laboratory procedure requires submission in InPouch TV.
2. If a specimen is received in saline within 30 min of collection, do a wet mount.
3. If a swab is received within 30 min of collection, inoculate an InPouch TV.

C. Microscopic evaluation immediately upon receipt
1. Concentrate the cellular material by standing the pouch vertically for at least 15 min prior to microscopic evaluation. The trichomonads will concentrate at the bottom of the chamber.
2. Place the upper chamber of the pouch on the raised platform of the open plastic microscope clip, positioning the pouch so that the lower portion of the upper chamber is a few millimeters above the lower border of the clip. Close and lock the clip over the pouch.
3. View the pouch from the upper (open window) side of the microscope clip. Observe with a microscope under low power (10×); use high dry power (40×) if necessary for confirmation. Focus on the liquid and not on the textured plastic of the pouch. Clue cells and yeast can also be observed.
4. When no trichomonads can be found in the direct preparation in the upper pouch chamber, the inoculum has fewer than 100 organisms or may be negative. Remove microscope clip after observation.

D. Culture and microscopic evaluation
1. Express the contents of the upper pouch chamber into the lower chamber.
2. Roll down the pouch until the tape is at the top of the label. Fold the wire tape's end tabs to lock the roll. This action helps maintain partial anaerobiasis.
3. Incubate the pouch vertically at 37°C for 18 to 24 h.
4. Prior to reading, distribute the trichomonads by gently rubbing your thumb across the viewing area or pull the pouch upward (five times) across the edge of a table for mixing.
5. Place the bottom of the lower chamber on the raised platform of the open microscope slip, and then close and lock the clip over the pouch.
6. Observe microscopically under low power (10×). The best location in the pouch to find trichomonads is slightly above the bottom edge of the pouch. Focus on the liquid and not on the textured plastic of the pouch.
7. Repeat evaluations daily for the presence of motile trichomonads for up to 5 days. Do not mistake Brownian motion of small debris particles for evidence of *Trichomonas* activity.

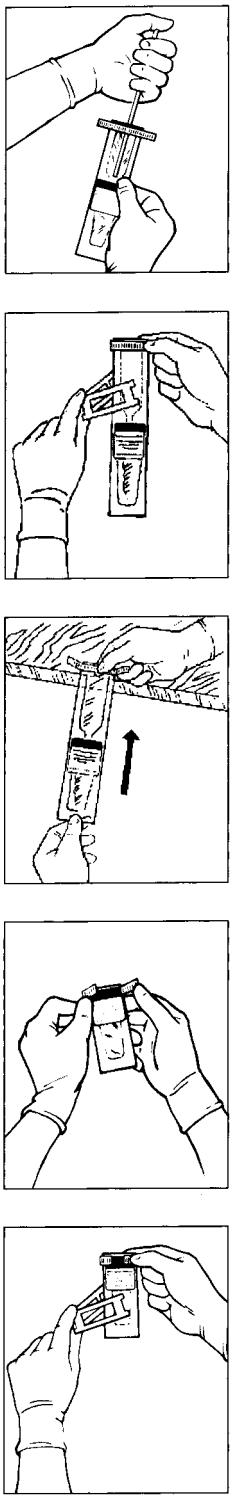


Figure 9.9.4–2 Illustration of the InPouch TV culture system for *Trichomonas vaginalis* (BioMed Diagnostics). From top to bottom: (1) introduction of the specimen into the upper chamber containing a small amount of medium; (2) application of a plastic holder for microscope viewing prior to expressing medium into the lower chamber (optional); (3) transfer of a small amount of medium in the upper chamber to the lower chamber; (4) rolling down the upper chamber and sealing it with tape; (5) plastic viewing frame used to immobilize the medium in the pouch for examination under the microscope. (Diagram courtesy of BioMed Diagnostics; reprinted from **L. S. Garcia,** *Diagnostic Medical Parasitology*, 5th ed., 2007, ASM Press, Washington, DC.)

VI. RESULTS

T. vaginalis trophozoites may be recovered and identified.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. If organisms are found prior to or at the end of 96 h, report the specimen as positive.
Example: Positive for *Trichomonas vaginalis*

B. If no trophozoites are seen after 5 days of incubation, then discard the pouch and report as negative.
Example: Negative for *Trichomonas vaginalis*

VIII. PROCEDURE NOTES

A. Specificity: for cultivation of *T. vaginalis* only. Other *Trichomonas* species will not survive and replicate at the pH and medium composition found in the InPouch TV test kit.

B. Culture: *T. vaginalis* present in low numbers will replicate within 5 h after inoculation into the InPouch system. An inoculum containing 1 to 10 organisms is sufficient to cause a positive test (1, 4).

C. Patient materials may occasionally contain nonviable organisms; it is recommended that if suspicious nonmotile objects are seen, stained smears can be prepared (Giemsa stain).

D. Use the same lot number of medium for controls and patient specimens.

IX. LIMITATIONS OF THE PROCEDURE

A. Even though cultivation is the most sensitive method for the diagnosis of trichomoniasis, it may take up to 5 days to arrive at a diagnosis. Every effort, therefore, must be made to carefully review contents of the pouch on a daily basis.

B. Do not report results of patient specimens as positive unless control cultures are positive.

C. InPouch TV medium suppresses but does not entirely eliminate the growth of yeast.

REFERENCES

1. **BioMed Diagnostics, Inc.** Technical bulletin no. 11 (document 100-045). BioMed Diagnostics, Inc., San Jose, CA.
2. **BioMed Diagnostics, Inc.** April 2007. InPouch™ TV *Trichomonas vaginalis* test package insert. Document 100-001. BioMed Diagnostics, Inc., San Jose, CA.
3. **Borchardt, K. A.** 1997. Trichomoniasis, p. 205–213. *In* K. A. Borchardt and M. A. Noble (ed.), *Sexually Transmitted Diseases.* CRC Press, Boca Raton, FL.
4. **Borchardt, K. A., and R. F. Smith.** 1991. An evaluation of an InPouch TV culture method for diagnosing *Trichomonas vaginalis* infection. *Genitourin. Med.* **67:**49–52.
5. **Borchardt, K. A., M. Z. Zhang, and H. Shing.** 1996. A comparison on the sensitivity of the InPouch, Diamond's, and Trichosel media for detection of *Trichomonas vaginalis*, abstr. 721. *Abstr. 96th Gen. Meet. Am. Soc. Microbiol. 1996.* American Society for Microbiology, Washington, DC.
6. **Borchardt, K. A., S. Al-Haraci, and N. Maida.** 1995. Prevalence of *Trichomonas vaginalis* in a male sexually transmitted disease clinic by interview, wet mount microscopy, and InPouch TV culture. *Genitourin. Med.* **71:**405–406.

9.9.5 Parasite Culture: *Leishmania* spp. and *Trypanosoma cruzi*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Leishmania spp. produce in humans a wide variety of diseases ranging from a mild, self-curing cutaneous form (Oriental sore) to the relatively severe mucocutaneous disease (espundia) to severe, fatal visceral leishmaniasis (kala-azar). *Leishmania* spp. have two stages in their life cycle: the extracellular promastigote, which occurs in the gut of the sand fly vector, and an intracellular amastigote stage, which multiplies in the macrophage of the vertebrate host. The culture form is usually the promastigote stage; however, differentiation of promastigotes into the amastigote form in cell cultures can also be achieved (1–4). In vitro cultivation of *Leishmania* greatly facilitates diagnosis in clinical infections and studies of the taxonomy, biochemistry, physiology, metabolism, immunology, and ultrastructure of the organism. Cultivation is also valuable for in vitro screening of drugs that might lead to advances in chemotherapy. Axenic cultivation of the parasite also helps in understanding the pathological process in experimental animals, which can be infected with organisms grown in culture, thereby inducing disease processes that simulate the naturally occurring disease.

Trypanosoma cruzi, which causes Chagas' disease, is a major public health problem in Latin America. In addition to the promastigote and amastigote forms, *T. cruzi* also has epimastigote and trypomastigote stages. The culture form is usually the epimastigote stage; however, differentiation of the epimastigotes into metacyclic trypomastigotes in cell-free media and amastigotes in cell cultures can also be achieved. Cultivation of the organisms from patient specimens is an important diagnostic tool. In vitro cultivation of *T. cruzi* also greatly facilitates study of the organism, provides a mechanism for drug screening, and provides a source of organisms for animal studies.

II. SPECIMENS

Observe standard precautions.

Specimens for culturing *Leishmania* spp. may consist of aspirates, scrapings, or biopsy material from skin lesions of patients with cutaneous leishmaniasis; bone marrow aspirates or, more rarely, splenic aspirates from visceral-leishmaniasis patients; or normal skin biopsy specimens, lymph node aspirates, or pieces of liver and spleen from suspected or potential wild- or domestic-animal reservoirs. Specimens for culturing *T. cruzi* may consist of the patient's blood or the gut contents of the triatomid bug.

III. MATERIALS

A. Reagents (*see* Appendix 9.9.5–1)

B. Supplies

1. Disposable sterile Pasteur and serological pipettes
2. Microscope slides (1 by 3 in. or larger)
3. Coverslips (no. 1, 22 by 22 mm or larger)
4. Volumetric flasks (1,000 ml)
5. Erlenmeyer flasks (500 ml)
6. Graduated cylinders (100 and 500 ml)
7. Sterile and nonsterile screw-cap tubes (16 by 125 mm)
8. Culture tube racks
9. Sterile cryovials or screw-cap vials (to hold 1 ml)
10. Box for vial storage in freezer
11. Büchner funnel
12. Filter paper, Whatman no. 42
13. 0.22-μm-pore-size membrane filters

III. MATERIALS *(continued)*

14. ATCC 30883 *(Leishmania mexicana)*
15. ATCC 30160 *(T. cruzi)*

C. Equipment

1. Binocular microscope with 10×, 40×, and 100× objectives; phase-contrast and/or differential interference contrast optics preferred
2. Binocular inverted microscope with 10×, 40×, and 100× objectives; phase-contrast and differential interference contrast optics are preferred (this microscope is recommended but not mandatory)
3. Oculars should be 10×. Some may prefer 5×; however, overall smaller magnification may make final organism identifications more difficult.
4. Tabletop centrifuge, preferably refrigerated (for tubes containing patient and animal specimens, e.g., blood and aspirates, or for culture tubes)
5. Water bath (range up to 80°C)
6. Freezer, −20°C
7. Magnetic stirrer and stir bar
8. pH meter

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Check all reagents and media at least once a week. The media should be free of any signs of precipitation and bacterial and/or fungal contamination.
B. The microscope(s) should be calibrated, and the original optics used for the calibration should be in place on the microscope(s). Post the calibration factors for all objectives on the microscope for easy access (*see* procedures 9.1 and 9.3.2). Although there is not universal agreement, the microscope should probably be recalibrated once each year. This recommendation should be considered with heavy use or if the microscope has been bumped or moved multiple times. If the microscope does not receive heavy use, then recalibration is not required on a yearly basis.
C. Maintain stock cultures of *Leishmania* spp.
1. Transfer stock cultures weekly.
a. Always culture stock organisms at the same time a patient specimen is inoculated into culture medium.
b. If the stock organisms multiply and remain viable during the 96 h, then report patient results.
2. Stain
a. Stain a slide prepared from stock culture in parallel with the patient slide.
b. Staining results are acceptable when the control organisms stain well.
D. Known positive microscope slides, Kodachrome (2- by 2-in.) projection slides, and photographs (reference books) should be available at the workstation. Images are also available online on various Web sites.
E. Record all QC results.

V. PROCEDURE

Observe standard precautions.

A. *Wear gloves when performing this procedure.*
B. Inoculation of culture medium
1. Remove tubes containing culture medium (one of NNN medium, modified NNN medium, or Tobie's medium and one of Schneider's medium for leishmaniasis and one of NNN medium or Tobie's medium and one of LIT medium for *T. cruzi*) from 4°C, add fetal bovine serum and antibiotics if required, and incubate at 20 to 23°C for 1 to 2 h.
2. Inoculate the specimen (aspirate, scraping, or biopsy material from skin lesions from cutaneous-leishmaniasis patients; bone marrow aspirates or splenic aspirates from visceral-leishmaniasis patients; or normal skin biopsy specimens, lymph node aspirates, or pieces of liver and spleen from suspected or potential wild- or domestic-animal reservoirs) into the culture tubes. For Chagas' disease, inoculate a few drops of buffy coat into the culture tubes.

V. PROCEDURE *(continued)*

3. Add 0.5 ml of overlay (either saline or other overlay, depending on medium).
4. Incubate the tubes at 20 to 24°C.
5. Once every 2 to 3 days, remove a drop of medium and examine it under the low power (100×) of a microscope, preferably one equipped with phase-contrast optics.
6. If promastigotes are seen, then inoculate a couple of drops of the medium into fresh culture tubes. Add a couple of drops of 0.85% NaCl or the overlay solution (depending on the culture medium used) to the old tube.
7. If visible contamination occurs, add antimicrobials to the overlay (to contain 200 U of penicillin and 200 μg of streptomycin per ml).
8. Incubate the tubes containing the patient's specimen for at least 2 weeks.
9. If no organisms are seen even after 2 weeks of incubation, then examine several drops of fluid under the microscope for promastigotes.

VI. RESULTS

A. *Leishmania* spp. may be recovered and identified.
B. *T. cruzi* may be recovered and identified.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

A. If organisms are found prior to or at the end of 2 weeks, report as positive.
Examples: Positive for *Leishmania* spp., positive for *Trypanosoma cruzi*
B. If no organisms are seen even after 2 weeks of incubation, then discard the tubes and report as negative.
Examples: Negative for *Leishmania* spp., negative for *Trypanosoma cruzi*
C. Identification of the species is generally made on the basis of the clinical symptoms, geographic origin, culture characteristics, isoenzyme profile, and DNA buoyant density.

VIII. PROCEDURE NOTES

A. Ensure that the skin surrounding the ulcer is thoroughly cleaned and swabbed with 70% alcohol (sterile saline is not acceptable as a cleansing agent) and allowed to dry before the sample is removed. Also ensure that alcohol does not get into the ulcerated area or broken skin.
B. Patient skin may be simply touched to the wall of the tube to release the amastigotes. If skin from an animal reservoir is used, the specimen must be macerated or triturated.
C. A punch biopsy taken from the advancing margin of the lesion is often recommended.
D. It is imperative that only a few drops of bone marrow juice or spleen aspirate be inoculated into tubes. Inoculate several tubes with a few drops each rather than a single tube with a large volume (1 to 2 ml), as the serum in the specimen may contain leishmanicidal or inhibitory factors that will prevent the growth of organisms.
E. Alternatively, bone marrow juice may be centrifuged for 10 min at 250 × *g*, and the sediment may be washed in 0.85% NaCl by centrifugation and then inoculated into culture tubes.
F. Buffy coat from the blood sample rather than whole blood should be inoculated.
G. Because leishmaniae are fastidious organisms and all isolates may not grow in any one medium, it is imperative that at least two media be used; for example, use NNN or modified Tobie's medium and Schneider's *Drosophila* medium.
H. It is advisable to use two different media such as LIT and NNN media for the initial isolation of *T. cruzi*. Once growth is established, use the medium in which best growth is obtained for subculture. According to James Sullivan, LIT me-

VIII. PROCEDURE NOTES *(continued)*

dium used as an overlay on Tobie's slants is excellent for isolation and diagnosis. The major culture form is the epimastigote; occasionally, however, trypomastigotes and amastigotes may also be seen. Make sure that the defibrinated rabbit blood is fresh. In any case, it should not be more than 10 days old. It should be aseptically collected and stored at 4°C until used.

I. Although blood collected using EDTA anticoagulant can be used for routine stock culture subcultures, it may not be quite as effective as defibrinated blood in isolating organisms from patient specimens. However, if defibrinated blood is not available, blood collected using EDTA anticoagulant can be used.

IX. LIMITATIONS OF THE PROCEDURE

Cultivating the organism from suspected materials provides a definitive diagnosis, but it may take 3 to 7 days. Every effort, therefore, must be made to microscopically examine wet smears and/or stained smears so that appropriate therapy can be instituted without delay if findings are positive.

REFERENCES

1. **Evans, D. A.** 1978. Kinetoplastida, p. 55–88. *In* A. E. R. Taylor and J. R. Baker (ed.), *Methods of Cultivating Parasites In Vitro.* Academic Press, Orlando, FL.
2. **Evans, D. A.** 1987. *Leishmania,* p. 52–75. *In* A. E. R. Taylor and J. R. Baker (ed.), *In Vitro Methods for Parasite Cultivation.* Academic Press, Orlando, FL.
3. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology,* 5th ed. ASM Press, Washington, DC.
4. **Schuster, F. L., and J. J. Sullivan.** 2002. Cultivation of clinically significant hemoflagellates. *Clin. Microbiol. Rev.* **15:**374–389.

APPENDIX 9.9.5–1

Include QC information on reagent container and in QC records.

Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

A. NNN medium (leishmaniasis or Chagas' disease)

Bacto Agar (Difco) ……1.4 g
sodium chloride (NaCl) ……0.6 g
double-distilled water …… 90.0 ml

1. Mix the NaCl and agar in the distilled water in a 500-ml flask.
2. Heat the mixture until the agar melts.
3. Autoclave at 121°C for 15 min.
4. Cool to about 50°C.
5. Add 10 ml of aseptically collected defibrinated rabbit blood.
6. Dispense 4 ml into sterile screw-cap culture tubes (16 by 125 mm).
7. Place the tubes at a 10° angle (shallow slant position) until the agar sets.
8. Immediately transfer the tubes into test tube stands, and let stand in an upright position at 4°C so that the bottom portion of the slants will be covered with the water of condensation. Rapid cooling increases the water of condensation.
9. Label as NNN medium with the preparation date and an expiration date of 3 weeks from the date of preparation.
10. Store at 4°C.

B. NNN medium, Offutt's modification (leishmaniasis)

blood agar base (Difco) ……8.0 g
double-distilled water ……200.0 ml

1. Heat until the agar is dissolved in the distilled water in a 500-ml flask.
2. Autoclave at 121°C for 15 min.
3. Cool to about 50°C.
4. Add 15 ml of aseptically collected defibrinated rabbit blood.
5. Dispense 4 ml into sterile screw-cap culture tubes (16 by 125 mm).
6. Place the tubes at a 10° angle (shallow slant position) until the agar sets.

APPENDIX 9.9.5–1 *(continued)*

7. Immediately transfer the tubes into test tube stands, and let stand in an upright position at 4°C so that the bottom portion of the slants is covered with the water of condensation. Rapid cooling increases the water of condensation.
8. Label as Offutt's medium with the preparation date and an expiration date of 3 weeks from the date of preparation.
9. Store at 4°C.

C. Overlay solution (to be used with NNN or NNN modified medium)

sodium chloride (NaCl) ……4.5 g
double-distilled water ……500.0 ml

1. Autoclave at 121°C.
2. Dispense 4 ml aseptically into sterile culture tubes (16 by 125 mm).
3. Label as 0.9% NaCl with the preparation date and an expiration date 3 weeks from the date of preparation.
4. Store at 4°C.

D. Evan's modified Tobie's medium (leishmaniasis or Chagas' disease)

beef extract (Oxoid Lab-Lemco L29) ……0.3 g
bacteriological peptone (Oxoid L37) …….0.5 g
sodium chloride (NaCl) ……0.8 g
agar (Oxoid purified) ……2.0 g
double-distilled water ……100.0 ml

1. Mix all the ingredients in the distilled water in a large beaker by using a magnetic stirrer.
2. Heat the mixture until the agar melts.
3. Dispense 5 ml into screw-cap culture tubes (16 by 125 mm).
4. Autoclave at 121°C for 15 min.
5. Cool to about 50°C.
6. Add 1.2 ml of aseptically collected defibrinated horse blood.
7. Hold the tubes in an upright position in the palms of your hands, and roll the tubes gently to mix the blood and agar well.
8. Place the tubes at a 10° angle (shallow slant position) until the agar sets.
9. Immediately transfer the tubes into test tube stands, and let stand in an upright position at 4°C so that the bottom portion of the slants will be covered with the water of condensation. Rapid cooling increases the water of condensation.
10. Label as Evan's modified Tobie's medium with the preparation date and an expiration date 3 weeks from the date of preparation.
11. Store at 4°C.

E. Overlay solution (to be used with Tobie's medium)

potassium chloride (KCl) ……0.4 g
sodium phosphate, dibasic
($Na_2HPO_4 \cdot 12H_2O$) …… 0.06 g
potassium phosphate, monobasic
(KH_2PO_4) …… 0.06 g
calcium chloride ($CaCl_2 \cdot 2H_2O$) ……0.185 g
magnesium sulfate ($MgSO_4 \cdot 7H_2O$) …….0.1 g
magnesium chloride ($MgCl_2 \cdot 6H_2O$) …….0.1 g
sodium chloride (NaCl) ……8.0 g
L-proline ……1.0 g
phenol red ……0.5 ml
double-distilled water ……1,000.0 ml

1. Place 750 ml of distilled water in a 1-liter beaker, and add the above ingredients one at a time in the order given until dissolved. Use a magnetic stirrer.
2. Adjust the pH to 7.2 by adding slowly, while stirring, solid Tris.
3. Bring the volume to 1,000 ml with distilled water.
4. Dispense 100 ml into a number of screw-cap flasks or bottles.
5. Autoclave at 121°C for 15 min.

APPENDIX 9.9.5–1 *(continued)*

6. Label as overlay solution with the preparation date and an expiration date of 1 month.
7. Store at 4°C.

F. Yaeger's liver infusion tryptose (LIT) medium (for Chagas' disease)

liver infusion (Difco)	35.0 g
tryptose (Difco)	5.0 g
sodium chloride (NaCl)	4.0 g
potassium chloride (KCl)	0.4 g
sodium phosphate, dibasic ($Na_2HPO_4 \cdot 12H_2O$)	8.0 g
glucose	2.0 g
hemin (stock solution)	4.0 ml
double-distilled water to	1,000.0 ml

1. Add all the ingredients to the distilled water, and mix well by using a magnetic stirrer until dissolved. Heat if necessary to dissolve all the ingredients.
2. Using a Whatman no. 42 filter paper in a Büchner funnel, filter with suction. Do this filtration once more, using new filter paper.
3. Adjust pH to 7.2 with 1 N NaOH or 1 N HCl.
4. Sterilize by filtration using a 0.22-μm-pore-size membrane filter.
5. Dispense 4.5 ml into each tube.
6. Label as LIT medium with the date of preparation and an expiration date of 1 month.

G. Hemin stock solution

hemin	100.0 mg
triethanolamine	10.0 ml
sterile double-distilled water	10.0 ml

1. Mix triethanolamine with water, add the mixture to tube containing hemin, shake well, and let dissolve.
2. Complete medium
   Just before inoculation, add 0.5 ml of inactivated fetal bovine serum and 0.25 ml of antimicrobial solution. Final concentrations of the antimicrobials are 100 U of penicillin, 100 μg of streptomycin, and 0.2 μg of amphotericin B per ml.

H. Schneider's *Drosophila* medium (leishmaniasis)

This medium was originally designed for cultivation of insect tissue culture cells. Hendricks used this medium containing 30% inactivated fetal bovine serum to isolate and grow etiologic agents of cutaneous leishmaniasis. This medium can be purchased from GIBCO, New York, NY.

I. Stock antimicrobial solution (to be used with all media)

sodium penicillin G	1,000,000 U
streptomycin sulfate	1,000,000 μg
amphotericin B	2,000 μg
sterile double-distilled water	50.0 ml

1. Mix the above thoroughly. Concentrations of stock solution are

penicillin	20,000 U/ml
streptomycin	20,000 μg/ml
amphotericin B	40 μg/ml

2. Dispense 1 ml of the antimicrobial mixture into sterile screw-cap vials or sterile cryovials.
3. Label as antimicrobial solution with the date of preparation and an expiration date of 1 year.
4. Store at −20°C in cryoboxes.

J. Fetal bovine serum

K. Rabbit blood, fresh, defibrinated

L. Horse blood

M. 1 N NaOH

N. 1 N HCl

9.10.1 Appendix 9.10.1–1—Identification Aids: Artifacts

I. PRINCIPLE

Laboratorians must differentiate extraneous materials present in a specimen from actual parasites.

II. STOOL ARTIFACTS

A. Sources

Gross and microscopic examination of stool may be complicated by the presence of artifacts resembling parasitic trophozoites, cysts, eggs, larvae, and adult worms (Table 9.10.1–A1). Many such artifacts arise from the large array of vegetable and meat products ingested every day by humans. Cells of human enteric origin may also mimic pathogenic or commensal protozoa in their appearance. Spurious infections with human or nonhuman parasites are known to occur following ingestion of contaminated or infected meats. The use of improper collection techniques offers another mechanism by which specimens are contaminated with extraneous organisms (3, 4).

B. Protozoa

1. Amebae

Improper collection and preservation of feces may result in contamination with free-living amebae from soil or water. Most species have large cytoplasmic contractile vacuoles, thick cyst walls, and nuclei with large karyosomes. Inflammatory cells are often present in a variety of infectious and noninfectious enteric syndromes and include PMNs, eosinophils, lymphocytes, and macrophages. Careful evaluation should be made to prevent these cells being mistaken as intestinal parasites, especially *Entamoeba histolytica* or the *E. histolytica/E. dispar* group. Report any occurrence of inflammatory cells, and make a quantitative assessment. Amebae also must be differentiated from intestinal epithelial cells (squamous or columnar), yeasts, plant cells, and another protozoan, *Blastocystis hominis.*

2. Flagellates

Free-living aquatic flagellates may be recovered from feces contaminated with water or saline and are difficult to differentiate from enteric flagellates. Stool samples contaminated with urine may also contain *Trichomonas vaginalis* and urine samples contaminated with stool may contain *Pentatrichomonas hominis.*

3. Ciliates

As with amebae and flagellates, free-living ciliates are also commonly found in standing water and may contaminate improperly collected stool specimens. Some species appear similar to *Balantidium coli* and may require differentiation by an expert.

Table 9.10.1–A1 Clinical specimens: summary of artifacts resembling parasites

Source and type of artifact	Resemblance
Stool	
Cells of host origin	
PMNs	*Entamoeba histolytica/E. dispar* cysts
Macrophages	*E. histolytica/E. dispar* trophozoites
Columnar epithelial cells	Amebic trophozoites
Squamous epithelial cells	Amebic trophozoites
Coprozoic (free-living protozoa)	
Amebae	Amebic trophozoites or cysts
Flagellates	Any of the intestinal flagellates
Ciliates	*Balantidium coli* trophozoites
Yeasts	Protozoan cysts (particularly *Endolimax nana*)
Fungal conidia	Helminth eggs
Plant cells	Protozoan cysts, helminth eggs
Plant hairs	Nematode larvae
Pollen grains	Helminth eggs (particularly *Ascaris* or *Taenia* eggs)
Diatoms	Helminth eggs
Starch granules, fat globules, air bubbles, mucus	Protozoan cysts
Ingested mite eggs	Helminth eggs
Ingested plant nematode eggs, larvae	Helminth eggs, nematode larvae
Blood	
Platelets	Malaria, *Babesia* spp.
Stain precipitate	Malaria, *Babesia* spp.
Stain contaminants	
Yeasts	Fungemia, parasitemia
Bacteria	Bacteremia
Plant fibers	Microfilariae
Body fluid	
Detached ciliary tufts	Ciliate or flagellate protozoa

II. STOOL ARTIFACTS *(continued)*

4. Coccidia

The identification of *Cryptosporidium* spp. poses significant challenges because of the smallness of the organism and the lack of specificity of the commonly used acid-fast stains *(certain other organisms, including some yeasts, may stain positive).* Use of an immunofluorescence method increases specificity significantly and may be used as either a primary or confirmatory method. Certain pollen grains are known to mimic *Isospora belli* or *Sarcocystis* sp. oocysts and should be differentiated on the basis of size.

5. Microsporidia

The identification of microsporidia also presents challenges because of the small size of the spores and the lack of specificity of the commonly used modified trichrome stains (other organisms, including bacteria and small yeasts, often stain pink/reddish pink, as do the microsporidial spores). Optical brightening agents (calcofluor white) are helpful but also nonspecific; microsporidial spores, as well as small yeasts, will fluoresce. Unfortunately, no specific immunoassay reagents are commercially available for the identification of the microsporidia.

II. STOOL ARTIFACTS
(continued)

C. Helminths

1. Adult worms

Many types of partially digested vegetable or fruit fibers are similar in appearance to adult nematodes or tapeworm proglottids. This is especially common with individuals whose enteric transit time has been decreased following administration of a cathartic. Adult free-living nematodes may be recovered from stool specimens contaminated with soil or water. Specimens in question should be preserved in formalin and forwarded for identification.

2. Helminth eggs

A large variety of plant cells, algae, pollen grains, and fungal conidia are routinely seen in feces and may resemble *Ascaris lumbricoides, Taenia* spp., *Clonorchis sinensis,* and other helminth eggs. Vegetables contaminated with mites or infected with plant nematodes are a ready source of eggs similar in size and shape to pathogenic forms. With the ingestion of meat products from mammals, fish, birds, or other hosts, coincidental ingestion of a wide variety of helminths and their eggs may occur and result in spurious infections. Organisms with which such infections have been reported have included *Fasciola hepatica, Dicrocoelium dendriticum,* and *Capillaria hepatica,* among others (1). True infections may be ruled out with subsequent stool examinations.

3. Helminth larvae

Many kinds of plant or root hairs show a superficial resemblance in size and shape to nematode larvae. However, most appear as clear, refractile structures that lack both symmetry and identifiable internal organs. Unlike plant hairs, true larvae retain iodine when stained.

III. BLOOD ARTIFACTS

Artifacts present in thick and thin blood films may be mistaken for parasites and result in inappropriate or unnecessary treatment of the patient. These artifacts are usually of two types: (i) normal cellular elements, such as platelets, which may mimic malarial parasites when superimposed on erythrocytes, and (ii) contaminants from the staining process, such as yeast cells, bacteria, stain precipitate, or cellulose fibers, which may mimic true fungemia, bacteremia, or parasitemia with malaria or microfilariae.

IV. BODY FLUID ARTIFACTS

The appearance of detached ciliary tufts (ciliocytophthoria) in a variety of body fluids (especially peritoneal and amniotic fluids) has been recognized for many years. Such tufts are the luminal remnants of ciliated epithelium that occur as part of the normal cellular turnover in a variety of organs (respiratory tract and sinuses, ventricles of the brain, central canal of the spinal cord, and epithelia of the male and female reproductive tracts). In wet preparations, these ciliated tufts are 10 to 15 μm in diameter, exhibit rhythmic motion, and may easily be confused with ciliated or flagellated protozoa, including *Trichomonas, B. coli, Giardia lamblia,* and *Chilomastix mesnili.* However, on close examination of direct wet or permanent stained preparations, there is little internal structure reminiscent of such organisms (2).

V. CORRECTIVE ACTION

Misidentification of artifacts as parasites may be minimized by providing appropriate training of the parasitologist, performing preservation and staining procedures according to established protocols, stressing the importance of correct collection procedures to patients or staff, and strictly adhering to established morphological criteria when performing the microscopic examinations.

REFERENCES

1. **Ash, L. R., and T. C. Orihel.** 1984. *Atlas of Human Parasitology*, 2nd ed. ASCP Press, Chicago, IL.
2. **Ashfag-Drewett, R., C. Allen, and R. L. Harrison.** 1990. Detached ciliary tufts: comparison with intestinal protozoa and a review of the literature. *Am. J. Clin. Pathol.* **93:**541–545.
3. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.
4. **Garcia, L. S.** 2009. *Practical Guide to Diagnostic Parasitology*, 2nd ed. ASM Press, Washington, DC.

9.10.2

Appendix 9.10.2–1—Information Tables

Table 9.10.2–A1 Body sites and specimen collection

Site	Specimen option	Collection method[a]
Blood	Smears of whole blood	Fresh thick and thin films (1st choice)
	Anticoagulated blood	Anticoagulant (2nd choice) EDTA (1st choice) Heparin (2nd choice)
Bone marrow	Aspirate	Sterile
Central nervous system	Spinal fluid	Sterile
Cutaneous ulcers	Aspirates from below surface	Sterile plus air-dried smears
	Biopsy specimen	Sterile, nonsterile to histopathology (formalin acceptable)
Eye	Biopsy specimen	Sterile (in saline)
	Scrapings	Sterile (in saline)
	Contact lens	Sterile (in saline)
	Lens solution	Sterile
Intestinal tract	Fresh stool	0.5-pt (ca. 0.237-liter) waxed container
	Preserved stool	5 or 10% formalin, MIF, SAF, Schaudinn's, PVA
	Sigmoidoscopy material	Fresh, PVA or Schaudinn's smears
	Duodenal contents	Entero-Test or aspirates
	Anal impression smear	Cellulose tape (pinworm examination)
	Adult worm or worm segments	Saline, 70% alcohol
Liver, spleen	Aspirates	Sterile, collected in four separate aliquots (liver)
	Biopsy specimen	Sterile, nonsterile to histopathology (formalin acceptable)

(continued)

Table 9.10.2–A1 Body sites and specimen collection *(continued)*

Site	Specimen option	Collection method[a]
Lung	Sputum	True sputum (not saliva)
	Induced sputum	No preservative (10% formalin if time delay)
	Bronchoalveolar lavage fluid	Sterile
	Transbronchial aspirate	Air-dried smears
	Tracheobronchial aspirate	Air-dried smears
	Brush biopsy specimen	Air-dried smears
	Open lung biopsy specimen	Air-dried smears
	Aspirate	Sterile
Muscle	Biopsy specimen	Fresh, squash preparation
		Nonsterile to histopathology (formalin acceptable)
Skin	Scrapings	Aseptic, smear or vial
	Skin snip	No preservative
	Biopsy specimen	Sterile (in saline)
		Nonsterile to histopathology
Urogenital system	Vaginal discharge	Saline swab, transport swab (no charcoal), culture medium
		Air-dried smear for FA
	Urethral discharge	Air-dried smear for FA
	Prostatic secretions	Air-dried smear for FA
	Urine	Unpreserved spot specimen or 24-h unpreserved specimen
		Midday urine

[a] MIF, merthiolate-iodine-formalin; SAF, sodium acetate-acetic acid-formalin; PVA, polyvinyl alcohol; FA, fluorescent-antibody assay.

Table 9.10.2–A2 Body sites and possible parasites recovered (trophozoites, cysts, oocysts, spores, adults, larvae, eggs, amastigotes, and trypomastigotes)[a]

Site(s)	Parasites
Blood	
RBCs	*Plasmodium* spp.
	Babesia spp.
WBCs	*Leishmania donovani*
	Toxoplasma gondii
Whole blood/plasma	*Trypanosoma* spp.
	Microfilariae
Bone marrow	*Leishmania donovani*
	Trypanosoma cruzi
	Plasmodium spp.
Central nervous system	*Taenia solium* (cysticerci)
	Echinococcus spp.
	Naegleria fowleri
	Acanthamoeba and *Hartmanella* spp.
	Balamuthia mandrillaris
	Sappinia diploidea
	Toxoplasma gondii
	Microsporidia
	Trypanosoma spp.
Cutaneous ulcers	*Leishmania* spp.
	Acanthamoeba spp.
	Entamoeba histolytica
Eyes	*Acanthamoeba* spp.
	Toxoplasma gondii
	Loa loa
	Microsporidia
Intestinal tract	*Entamoeba histolytica*
	Entamoeba dispar
	Entamoeba coli
	Entamoeba hartmanni
	Endolimax nana
	Iodamoeba bütschlii
	Blastocystis hominis
	Giardia lamblia
	Chilomastix mesnili
	Dientamoeba fragilis
	Pentatrichomonas hominis
	Balantidium coli
	Cryptosporidium parvum
	Cryptosporidium hominis
	Cryptosporidium spp.
Intestinal tract *(continued)*	*Cyclospora cayetanensis*
	Cystoisospora (*Isospora*) *belli*
	Enterocytozoon bieneusi
	Encephalitozoon intestinalis
	Microsporidia
	Ascaris lumbricoides
	Enterobius vermicularis
	Hookworm
	Strongyloides stercoralis
	Trichuris trichiura
	Hymenolepis nana
	Hymenolepis diminuta
	Taenia saginata
	Taenia solium
	Diphyllobothrium latum
	Clonorchis sinensis (*Opisthorchis*)
	Metagonimus yokogawai
	Heterophyes heterophyes
Liver, spleen	*Echinococcus* spp.
	Entamoeba histolytica
	Leishmania donovani
	Microsporidia
Lungs	*Cryptosporidium* spp.[b]
	Echinococcus spp.
	Paragonimus spp.
	Microsporidia
Muscles	*Taenia solium* (cysticerci)
	Trichinella spp.
	Onchocerca volvulus (nodules)
	Trypanosoma cruzi
	Microsporidia
Skin	*Leishmania* spp.
	Onchocerca volvulus
	Microfilariae
Urogenital system	*Trichomonas vaginalis*
	Schistosoma spp.
	Microsporidia
	Microfilariae

[a] This table does not include every possible parasite that could be found in a particular body site. However, the most likely organisms have been listed. Modified from Garcia, L. S. 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.

[b] Disseminated in severely immunosuppressed individuals.

Table 9.10.2–A3 Body site, specimen, and recommended stain(s)[a]

Body site	Specimen(s)	Recommended stain for suspect organism	Comments
Blood	Whole or anticoagulated blood	Giemsa for all blood parasites Hematoxylin-based stain for microfilariae (sheathed)	Most drawings and organism descriptions of blood parasites were originally based on Giemsa-stained blood films. Although Wright's stain (or Wright-Giemsa combination stain) will work, stippling in malaria will normally not be visible and organism colors will not match descriptions. However, if other stains (those listed above) are used in addition to some of the "quick" blood stains, organisms should be visible on blood films.
Bone marrow	Aspirate	Giemsa for all blood parasites	See comments for blood
Central nervous system	Spinal fluid, brain biopsy specimen	Giemsa for trypanosomes, *Toxoplasma gondii* Giemsa, trichrome, or calcofluor for amebae (*Naegleria*, *Acanthamoeba*, and *Balamuthia* spp.) (cysts only) Giemsa, acid-fast PAS, modified trichrome, calcofluor, and silver methenamine for microsporidia Hematoxylin and eosin (routine histology) for larval cestodes	If CSF is received (with no suspect organism suggested), Giemsa is the best choice, but calcofluor is recommended as a second stain. If brain biopsy material is received (particularly from an immunocompromised patient), EM studies may be required to identify microsporidia to the genus or species level.
Cutaneous ulcer	Aspirate, biopsy specimen	Giemsa for leishmaniae Hematoxylin and eosin (routine histology) for *Acanthamoeba* spp., *Entamoeba histolytica*	Most likely causative agents would be leishmaniae, all of which would stain with Giemsa. Hematoxylin and eosin (routine histology) could also be used to identify these organisms.
Eye	Biopsy specimen, scrapings, contact lens, lens solution	Calcofluor for amebae (*Acanthamoeba* spp.) (cysts only) Giemsa for amebae (trophozoites, cysts) Hematoxylin and eosin (routine histology) for cysticerci, *Loa loa*, *Toxoplasma gondii* Silver methenamine, PAS, acid-fast stains, calcofluor, and EM studies for microsporidia	Some free-living amebae (most commonly *Acanthamoeba* spp.) have been implicated as a cause of keratitis. Although calcofluor will stain cyst walls, it will not stain trophozoites. Therefore, in suspected cases of amebic keratitis, use both stains. Hematoxylin and eosin (routine histology) can be used to detect and confirm cysticercosis. Adult *Loa loa* worm, when removed from the eye, can be stained with hematoxylin-based stain (Delafield's) or can be stained and examined via routine histology. *Toxoplasma* infection could be diagnosed by using routine histology and/or serology results. Confirmation of microsporidia to the genus and species levels may require EM studies.
Intestinal tract	Stool, sigmoidoscopy material, duodenal contents	Trichrome or iron hematoxylin for intestinal protozoa Modified trichrome and calcofluor for microsporidia Modified acid-fast stain for *Cryptosporidium* spp. and *Cyclospora cayetanensis* Immunoassay reagents (EIA, FA, cartridge format) for *Entamoeba histolytica/E. dispar*, *Entamoeba histolytica*, *Giardia lamblia*, *Cryptosporidium* spp., and microsporidia (experimental)	Although trichrome or iron hematoxylin stains can be used on almost all specimens from the intestinal tract, actual worm segments (tapeworm proglottids) can be stained with special stains. However, after routine dehydration with alcohols and xylenes (or xylene substitutes), the branched uterine structure will be visible, allowing identification of proglottids to the species level. Immunoassay detection kits are also available for the identification of *Giardia lamblia*, *Entamoeba histolytica/E. dispar*, *Entamoeba histolytica*, and *Cryptosporidium* spp. Confirmation of microsporidia to the genus or species level may require EM studies.

Table 9.10.2–A3 *(continued)*

Body site	Specimen(s)	Recommended stain for suspect organism	Comments
Intestinal tract *(continued)*	Anal impression smear	No stain, cellulose tape, or egg collection device	Four to six consecutive negative tapes are required to rule out infection
	Adult worm or worm segments	Carmine stains (rarely used)	Proglottids can usually be identified to the species level without using tissue stains
	Biopsy specimen	Hematoxylin and eosin (routine histology) for *Entamoeba histolytica* (also PAS), *Cryptosporidium* spp., *Cyclospora cayetanensis, Cystoisospora* (*Isospora*) *belli, Giardia lamblia,* and microsporidia	Special stains may be helpful in the identification of microsporidia: tissue Gram stains, silver stains, PAS, and Giemsa stain
Liver, spleen	Aspirates Biopsy specimen	Giemsa for leishmaniae Hematoxylin and eosin (routine histology)	Aspirates and/or touch preparations from biopsy material can be routinely stained with Giemsa stain. This will allow identification of leishmaniae. Definite risks are associated with spleen aspirates and/or biopsy material. Other parasites, such as larval cestodes, trematodes, amebae, or microsporidia can be seen and identified by routine histological staining.
Lung	Sputum, induced sputum, bronchoalveolar lavage fluid, transbronchial aspirate, tracheobronchial aspirate, brush biopsy specimen, open lung biopsy specimen	Modified acid-fast stains for *Cryptosporidium* spp. Hematoxylin and eosin (routine histology) for *Strongyloides stercoralis, Paragonimus* spp., amebae Silver methenamine stain, PAS, acid-fast, modified trichrome, tissue Gram stains, and EM studies for microsporidia	Routine histology procedures would allow identification of any helminths or helminth eggs in the lung.
Muscle	Biopsy specimen	Hematoxylin and eosin (routine histology) for *Trichinella spiralis,* cysticerci Silver methenamine, PAS, acid-fast and tissue Gram stains and EM studies for microsporidia	If *Trypanosoma cruzi* is present in the striated muscle, it can be identified from routine histological preparations. Confirmation of microsporidia to the genus or species level may require EM studies.
Skin	Aspirates Skin snip, scrapings, biopsy specimen	See Cutaneous Ulcer above. Hematoxylin and eosin (routine histology) for *Onchocerca volvulus, Dipetalonema streptocerca*	Any of these parasites can be identified by using routine histological procedures and stains.
Urogenital system	Vaginal discharge Urethral discharge, prostatic secretions Urine Biopsy specimen	Giemsa Immunoassay reagents (FA) for *Trichomonas vaginalis* Delafield's hematoxylin for microfilariae Hematoxylin and eosin (routine histology) for *Schistosoma haematobium,* microfilariae Silver methenamine stain, PAS, acid-fast, modified trichrome, tissue Gram stains, and EM studies for microsporidia	Although *Trichomonas vaginalis* is probably the most common parasite identified, there are others to consider, the most recently implicated organisms being in the microsporidia. Microfilariae can also be recovered and stained.

[a] PAS, periodic acid-Schiff; EM, electron microscopy; FA, fluorescent antibody.

Table 9.10.2–A4 Examination of tissues and body fluids

Suspect causative agent(s)	Disease(s)	Appropriate test(s)	Positive result
Protozoa			
Naegleria fowleri	Primary amebic meningoencephalitis	1. Wet examination of CSF (not in counting chamber) 2. Stained preparation of CSF sediment	Trophozoites present and identified
Acanthamoeba spp.	Amebic keratitis, chronic meningoencephalitis	1. Culture or stained smears 2. Calcofluor (cysts only) 3. Biopsy or routine histology	Trophozoites and/or cysts present and identified
Balamuthia mandrillaris	Chronic meningoencephalitis (granulomatous amebic encephalitis)	1. Calcofluor (cysts only) 2. Biopsy for routine histology	Trophozoites and/or cysts present and identified
Entamoeba histolytica	Amebiasis	Biopsy or routine histology	Trophozoites present and identified
Giardia lamblia	Giardiasis	1. Duodenal aspirate 2. Duodenal biopsy or routine histology 3. Entero-Test capsule 4. Immunoassays	Trophozoites and/or cysts present and identified
Leishmania spp. (cutaneous lesions)	Cutaneous leishmaniasis	1. Material from under bed of ulcer a. Smear b. Culture c. Animal inoculation 2. Punch biopsy a. Routine histology b. Squash preparation c. Culture d. Animal inoculation	Amastigotes recovered in macrophages of skin or from animal inoculation; other stages recovered in culture
Leishmania spp. (mucocutaneous lesions)	Mucocutaneous leishmaniasis	As for cutaneous leishmaniasis	Amastigotes recovered in macrophages of skin and mucous membranes or from animal inoculation; other stages recovered in culture
Leishmania spp. (visceral)	Visceral leishmaniasis (kala-azar)	1. Buffy coat a. Stain b. Culture c. Animal inoculation 2. Bone marrow a. Stain b. Culture c. Animal inoculation 3. Liver or spleen biopsy with routine histology a. Stain b. Culture c. Animal inoculation	Amastigotes recovered in cells of reticuloendothelial system
Toxoplasma gondii	Toxoplasmosis	1. Lymph node biopsy a. Routine histology b. Tissue culture isolation c. Animal inoculation 2. Serology	Identification of organisms plus appropriate serological test results
Cryptosporidium spp.	Cryptosporidiosis	1. Duodenal scraping 2. Duodenal biopsy a. Stain b. Routine histology 3. Punch biopsy a. Routine histology b. Squash preparation 4. Sputum 5. Immunoassays	Identification of organisms in microvillus border or other tissues (lung and gall bladder have also been involved); routine stains or monoclonal antibody reagents to identify oocysts in stool

Table 9.10.2–A4 *(continued)*

Suspect causative agent(s)	Disease(s)	Appropriate test(s)	Positive result
Microsporidia *Nosema* spp. *Encephalitozoon* spp. *Enterocytozoon* spp. *Pleistophora* spp. *Trachipleistophora* spp. *Anncaliia* spp. *Microsporidium* spp. *Vittaforma corneae*	Microsporidiosis	Routine histology; modified trichrome, tissue Gram stains, silver, periodic acid-Schiff, and Giemsa stains recommended (spores); animal inoculation not recommended/latent infections Electron microscopy may be necessary for confirmation.	These organisms (spores) have been found as insect or other animal parasites; route of infection is probably ingestion. Human cases involve muscle, CSF (AIDS); other body sites have also been documented.
Helminths			
Larvae (*Ascaris* and *Strongyloides* spp.)	"Pneumonia"	Sputum, wet preparation	This is an incidental finding but has been reported in severe infections.
Eggs (*Paragonimus* spp.)	Paragonimiasis	Sputum, wet preparation	Eggs will be coughed up and appear as "iron filings"; eggs could also be found in stool.
Hooklets (*Echinococcus* spp.)	Hydatid disease	Sputum, wet preparation	Rare finding, but hooklets can be found when the hydatid cyst is in the lung.
Onchocerca volvulus *Mansonella streptocerca*	Onchocerciasis	Skin	Skin snips examined in saline; microfilariae may be present.
Schistosoma spp.	Schistosomiasis	1. Rectal valve biopsy 2. Bladder biopsy	Eggs present and identified

Table 9.10.2–A5 Protozoa of the intestinal tract and urogenital system: key characteristics[a]

Organism	Trophozoite or tissue stage	Cyst or other stage in specimen	Comments
Amebae			
Entamoeba histolytica (pathogenic)	Cytoplasm clean; presence of RBCs is diagnostic, but cytoplasm may also contain some ingested bacteria; peripheral nuclear chromatin evenly distributed, with central, compact karyosome	Mature cyst contains four nuclei; chromatoidal bars have smooth, rounded ends; immature cyst usually contains one enlarged nucleus (precyst)	Considered pathogenic; report to Public Health; trophozoites can be confused with macrophages and cysts can be confused with WBCs in the stool
Entamoeba dispar (nonpathogenic)	Morphology identical to that of *E. histolytica* (confirmed by presence of RBCs in cytoplasm). If no RBCs, zymodeme analysis[b] or immunoassay is necessary to confirm species designation.	Mature cyst has morphology identical to that of *E. histolytica.*	Nonpathogenic: morphology resembles that of *E. histolytica;* these organisms will continue to be signed out as *E. histolytica/E. dispar* and reported to Public Health. Immunoassay reagents are now available to differentiate pathogenic *E. histolytica* and nonpathogenic *E. dispar;* some laboratories may decide to use these reagents on a routine basis, depending on positivity rate and cost.
Entamoeba histolytica/ E. dispar			Correct way to report, unless immunoassay is used to identify *E. histolytica* or trophozoites are seen with ingested RBCs *(E. histolytica)*
Entamoeba hartmanni (nonpathogenic)	Looks identical to *E. histolytica* but smaller (<12 μm); RBCs will not be ingested	Mature cyst contains four nuclei but often stops at two; chromatoidal bars often present and look like those in *E. histolytica* (size, <10 μm); immature cyst (precyst) may mimic *E. histolytica* but will be <10 μm	Shrinkage occurs on permanent stain (especially in cyst form). *E. histolytica* may actually be below the 12- and 10-μm cutoff limits; it could be as much as 1.5 μm below the limits quoted for wet preparation measurements. Entire space (including shrinkage) must be measured.
Entamoeba coli (nonpathogenic)	Cytoplasm dirty, may contain ingested bacteria or debris; peripheral nuclear chromatin unevenly distributed, with a large, eccentric karyosome	Mature cyst contains eight nuclei, may see more; chromatoidal bars (if present) tend to have sharp, pointed ends	If a smear is too thick or thin and if stain is too dark or light, then *E. histolytica/E. dispar* and *E. coli* are often confused; much overlap in morphology. Assess smear thickness before identifying organisms to the species level.
Endolimax nana (nonpathogenic)	Cytoplasm clean, not diagnostic, great deal of nuclear variation, may even be some peripheral nuclear chromatin. Normally, only karyosomes are visible.	Cyst is round to oval, with four nuclear karyosomes visible	More nuclear variation in this ameba than in any others; can be confused with *Dientamoeba fragilis* and/or *E. hartmanni*
Iodamoeba bütschlii (nonpathogenic)	Cytoplasm contains much debris; organisms usually larger than *E. nana* but may look similar; large karyosome	Cyst contains single nucleus (may be "basket nucleus") with bits of nuclear chromatin arranged on nuclear membrane (karyosome is the basket, bits of chromatin are the handle); large glycogen vacuole	Glycogen vacuole stains brown with addition of iodine in wet preparation; "basket nucleus" more common in cyst but can be seen in trophozoite; vacuole may be so large that the cyst collapses on itself

Table 9.10.2–A5 *(continued)*

Organism	Trophozoite or tissue stage	Cyst or other stage in specimen	Comments
Flagellates			
Giardia lamblia (pathogenic)	Trophozoites teardrop shaped from front, like a curved spoon from side; contain nuclei, linear axonemes, and curved median bodies	Cyst is round to oval; contains multiple nuclei, axonemes, and median bodies	Organisms live in duodenum, and multiple stools may be negative; may have to use additional techniques (immunoassay, aspirate, Entero-Test). Organism numbers may have little relevance to presence of symptoms.
Chilomastix mesnili (nonpathogenic)	Trophozoites teardrop shaped; cytostome must be visible for identification	Cyst is lemon shaped, with one nucleus and curved fibril called shepherd's crook	Cyst can be identified much easier than trophozoite form. Trophozoite will look like other small flagellates.
Dientamoeba fragilis (pathogenic)	Cytoplasm contains debris; may contain one or two nuclei (chromatin often fragmented into four dots)	No known cyst form	Tremendous size and shape range on a single smear; trophozoites with one nucleus can resemble *E. nana*
Trichomonas vaginalis (pathogenic)	Supporting rod (axostyle) present; undulating membrane comes halfway down the organism; small dots may be seen in cytoplasm along axostyle	No known cyst form	Recovered from genitourinary system; often diagnosed at bedside with wet preparation (motility)
Pentatrichomonas hominis (nonpathogenic)	Supporting rod (axostyle) present; undulating membrane comes all the way down the organism; small dots may be seen in cytoplasm along axostyle	No known cyst form	Recovered in stool; trophozoites may resemble other small flagellate trophozoites
Ciliate			
Balantidium coli (pathogenic)	Very large trophozoites (50–100 μm long) covered with cilia; large bean-shaped nucleus	Morphology not significant except for large, bean-shaped nucleus	Rarely seen in United States; causes severe diarrhea with large fluid loss; will be seen in proficiency testing specimens
Coccidia			
Cryptosporidium spp. (pathogenic)	Seen in intestinal mucosa (edge of brush border), gall bladder, and lung biopsy specimens	Oocysts seen in stool and/or sputum; organisms acid fast, 4–6 μm long; hard to find if few in number	Chronic infection in compromised host (internal autoinfective cycle), self-cure in immunocompetent host; numbers of oocysts correlate with stool consistency; can cause severe, watery diarrhea; oocysts immediately infective when passed
Cyclospora cayetanensis (pathogenic)	Experience with this organism is not extensive; organism may be difficult to identify in tissue; since patients are immunocompetent, biopsy specimens will probably rarely be required or requested	Oocysts seen in stool; approximately 8–10 μm in size; are unsporulated and thus difficult to recognize as coccidia; will mimic *C. parvum* (4–6 μm) on modified acid-fast-stained smears.	Oocysts have been seen in stool but were not recognized as coccidia until recently. To date, most infections are associated with immunocompetent individuals, but they may also be seen in immunosuppressed patients; organism may be associated with traveler's diarrhea and has been linked to the ingestion of imported food (raspberries, mesclun, basil)

(continued)

Table 9.10.2–A5 Protozoa of the intestinal tract and urogenital system: key characteristics[a] *(continued)*

Organism	Trophozoite or tissue stage	Cyst or other stage in specimen	Comments
Coccidia *(continued)*			
Cystoisospora (*Isospora*) *belli* (pathogenic)	Seen in intestinal mucosal cells and in biopsy specimens; less common than *C. parvum*	Oocysts seen in stool; organisms acid fast; best technique is concentration, not permanent stained smear	Thought to be the only *Isospora* sp. that infects humans; oocysts not immediately infective when passed; oocysts will be missed if PVA-preserved specimens are concentrated
Microsporidia *Nosema* spp. *Encephalitozoon* spp. *Pleistophora* spp. *Trachipleistophora* spp. *Anncaliia* spp. *Enterocytozoon* spp. "*Microsporidium*" spp. *Vittaforma comeae*	Developing stages sometimes difficult to identify; spores can be identified by size, shape, and presence of polar tubules	Depending on the genus involved, spores could be identified in stool or urine using the modified trichrome stain, calcofluor, or immunoassay reagents (experimental).	Spores are generally quite small (1–2.0 µm for *Enterocytozoon* spp.) and can easily be confused with other organisms or artifacts (particularly in stool). Infections tend to be present in immunosuppressed patients. It is unclear whether all genera listed here can disseminate to all parts of the body (gastrointestinal tract, urogenital system); however, the list is complete in terms of all body sites.

[a] PVA, polyvinyl alcohol; EM, electron microscopy.

[b] Zymodeme analysis is based on the identification of various isoenzymes found in cultured isolates of *Entamoeba histolytica/E. dispar*. These zymodemes (given separate numbers) tend to separate into two groups: those from symptomatic patients, some of whom have extraintestinal amebiasis, tend to group with the zymodeme isolates characteristic of the pathogen *E. histolytica*, while those from asymptomatic carriers tend to group with the zymodeme isolates characteristic of the nonpathogen *E. dispar*.

Table 9.10.2–A6 Tissue protozoa: characteristics

Species	Shape and size	Other features[a]
Toxoplasma gondii		
Trophozoites (tachyzoites)	Crescent shaped; 4–6 μm long by 2–3 μm wide	Found in peritoneal fluid of experimentally infected mice; intracellular forms somewhat smaller and not usually seen in humans. May be isolated in tissue culture, particularly from CSF. Diagnosis is most frequently based on clinical history and serological evidence (acute- and convalescent-phase sera).
Cysts (bradyzoites)	Generally spherical; 200 μm to 1 mm in diam	Occur in many body tissues (approx 30–50% of the U.S. population have these organisms in tissues, indicating past infection). Many infections are asymptomatic. Infections in the compromised host are very serious and involve the CNS. In these patients, particularly those with AIDS, diagnostic serological titers may be very difficult to demonstrate. *Note*: Organisms identified in histological preparations or isolated in animal or tissue culture systems may or may not be causative agents of symptoms.
Cryptosporidium spp.	Oocyst usually round, 4–6 μm, each mature oocyst containing sporozoites (infective on passage)	Oocyst usually diagnostic stage in stool, sputum, and possibly other body specimens. Various other stages in life cycle can be seen in biopsy specimens taken from GI tract (brush border of epithelial cell-intestinal tract) and other tissues (lung, gall bladder). Several modified acid-fast stains have been used successfully. Direct detection methods using immunoassay reagents are also available. *Note*: Infection in the immunocompetent host is self-limiting; however, in immunodeficient patients (AIDS), infection is chronic because of an autoinfective capability in the life cycle. The number of oocysts usually correlates with symptoms (watery diarrhea = many oocysts in specimen). The more normal the stool, the more difficult it is to find oocysts. Risk groups include animal handlers, travelers, immunocompromised individuals, children in day care centers, and those who come in contact with these individuals. Since oocysts are immediately infective, nosocomial transmission has been documented.
Cyclospora cayetanensis	Oocyst usually round, 8–10 μm; each oocyst is immature on passage; no internal morphology visible; oocysts appear as "wrinkled" cellophane	Oocyst is the diagnostic stage in stool. Various other stages in the life cycle can be seen in biopsy specimens taken from the GI tract (within epithelial cells and intestinal tract). The morphology is similar to that of *Cystoisospora* (*Isospora*) *belli.* A number of modified acid-fast stains have been used successfully to demonstrate the oocysts (quite acid-fast variable). Detection methods involving immunoassay reagents are under development.
Cystoisospora (*Isospora*) *belli*	Ellipsoidal oocyst; usual range, 20–30 μm long by 10–19 μm wide; sporocysts rarely seen broken out of oocysts but measure 9–11 μm	Mature oocyst contains two sporocysts with four sporozoites each; usual diagnostic stage in feces is immature oocyst containing spherical mass of protoplasm (diarrhetic stool). Developing stages can be recovered from intestinal biopsy specimens. Oocysts are also acid fast and can be detected during acid-fast staining of stool for *Cryptosporidium* spp. Oocysts are often detected in concentration sediment (wet preparation).

(continued)

Table 9.10.2–A6 Tissue protozoa: characteristics *(continued)*

Species	Shape and size	Other features[a]
Microsporidia *Nosema* spp. *Encephalitozoon* spp. *Pleistophora* spp. *Trachipleistophora* spp. *Anncaliia* spp. *Enterocytozoon* spp. "*Microsporidium*" spp. *Vittaforma corneae*	Spores are extremely small and have been recovered from all body sites, including the eye.	These organisms have been found as insect or other animal parasites; the route of infection may be ingestion, inhalation, or direct inoculation (eye). Histology results vary (spores are acid fast); PAS, silver, tissue Gram, and Giemsa stains are recommended for spores. Animal inoculation is not recommended; laboratory animals may carry occult infection; electron microscopy may be necessary for confirmation and identification to the genus and species levels. Although difficult to diagnose, infections have been found in a large number of AIDS patients (*Enterocytozoon bieneusi*, *Encephalitozoon intestinalis* in the intestinal tract, *Pleistophora* spp. in muscle, and various other microsporidia in other tissues, including the CNS). To date, it is still somewhat difficult to diagnose this infection by examining stool specimens prepared with optical brightening agents (calcofluor) or routine stains (modified trichrome, acid-fast trichrome stains). Diagnostic immunoassay reagents are under development but are not yet commercially available.
Sarcocystis hominis, *S. suihominis*, *S. bovihominis*	Thin-walled oocyst contains two mature sporocysts, each containing four sporozoites; frequently thin oocyst wall ruptures; ovoidal sporocysts are 9–16 μm long by 7.5–12 μm wide	Thin-walled oocyst or ovoidal sporocysts occur in stool. Compromised host may show fever, severe diarrhea, abdominal pain, and weight loss, although the number of patients has been small. Infections occur from ingestion of uncooked pork or beef. Life cycle occurs within intestinal cells, with eventual production of sporocysts in stool.
Sarcocystis "*lindemanni*"	Shapes and sizes of skeletal and cardiac muscle sarcocysts vary considerably.	When humans accidentally ingest oocysts from other animal stool sources, sarcocysts that develop in human muscle apparently do little if any harm. These can be identified by routine histological methods.

[a] CNS, central nervous system; BAL, bronchoalveolar lavage; GI, gastrointestinal; FA, fluorescent antibody; PAS, periodic acid-Schiff.

Table 9.10.2–A7 Helminths: key characteristics[a]

Helminth	Diagnostic stage	Comments
Nematodes (roundworms)		
Ascaris lumbricoides (pathogenic)	Egg: both fertilized (oval to round with thick, mammillated-tuberculated shell) and unfertilized (tends to be more oval-elongate, with bumpy shell exaggerated) can be found in stool. Adult worms: 10–12 in., found in stool. Rarely (in severe infections), migrating larvae can be found in sputum.	Unfertilized eggs will not float in flotation concentration method; adult worms tend to migrate when irritated (anesthesia, high fever), so check patients from areas of endemicity for infection prior to elective surgery.
Trichuris trichiura (whipworm) (pathogenic)	Egg: barrel shaped, with two clear, polar plugs; adult worm rarely seen; quantitate eggs (rare, few, etc.), since light infections may not be treated.	Dual infections with *Ascaris* may be seen (both infections acquired from egg ingestion in contaminated soil); in severe infections, rectal prolapse may occur in children or bloody diarrhea can be mistaken for amebiasis (bloody diarrhea usually not seen in United States).
Enterobius vermicularis (pinworm)	Egg: football shaped, with one flattened side Adult worm: about 3/8 in. long, white with pointed tail; female migrates from anus and deposits eggs on perianal skin.	May cause symptoms (itching) in some patients; test of choice is tape preparation; six consecutive tapes necessary to rule out infection; symptomatic patient often treated without actual confirmation of infection; eggs become infective within a few hours.
Ancylostoma duodenale (Old World hookworm), *Necator americanus* (New World hookworm) (pathogenic)	Egg: eggs of both identical; oval, with broadly rounded ends, thin shell, clear space between shell and developing embryo (8–16 ball stage) Adult worms: rarely seen in clinical specimens	May cause symptoms in some patients (blood loss anemia on differential smear in heavy infections). If stool remains unpreserved for several hours or days, eggs may continue to develop and hatch; rhabditiform larvae may resemble those of *Strongyloides stercoralis.*
Strongyloides stercoralis (pathogenic)	Rhabditiform larvae (noninfective) usually found in stool (short buccal cavity or capsule with large, genital primordial packet of cells ["short and sexy"]); in very heavy infections, larvae occasionally found in sputum and/or filariform (infective) larvae found in stool (slit in tail)	May see unexplained eosinophilia, abdominal pain, unexplained episodes of sepsis and/or meningitis, pneumonia (migrating larvae) in compromised patient. Potential for internal autoinfection can maintain low-level infections for many years (patient will be asymptomatic, with elevated eosinophilia); hyperinfection can occur in compromised patient (leading to disseminated stronglyoidiasis and death); agar plate culture is the most sensitive diagnostic method; many infections are low level, and larvae are difficult to recover.
Ancylostoma braziliensis (dog-cat hookworm) (pathogenic)	Humans are accidental hosts; larvae wander through outer layer of skin, creating tracks (severe itching, eosinophilia); no practical microbiological diagnostic tests	Cause of cutaneous larva migrans; typical setup for infection: dogs and cats defecate in sandbox, and hookworm eggs hatch and penetrate human skin in contact with infected sand or soil (children playing in sandbox).
Toxocara cati or *Toxocara caninum* (dog-cat ascarid) (pathogenic)	Humans are accidental hosts; ingestion of dog or cat ascarid eggs from contaminated soil; larvae wander through deep tissues (including eye); can be mistaken for cancer of the eye; serology helpful for confirmation; eosinophilia	Cause of visceral larva migrans and ocular larva migrans; requests for laboratory services often originate in ophthalmology clinic.
Cestodes (tapeworms)		
Taenia saginata (beef tapeworm)	Scolex (four suckers, no hooklets), gravid proglottids (>12 branches on single side) are diagnostic; eggs indicate *Taenia* spp. only (thick, striated shell containing six-hooked embryo or oncosphere); worm usually approx 12 ft long	Adult worm can cause symptoms in some individuals; acquired from ingestion of raw or poorly cooked beef; usually only single worm/patient; individual proglottids may crawl from anus; proglottids can be injected with India ink to show uterine branches for identification.

(continued)

Table 9.10.2–A7 Helminths: key characteristics[a] *(continued)*

Helminth	Diagnostic stage	Comments
Cestodes (tapeworms) *(continued)*		
Taenia solium (pork tapeworm)	Scolex (four suckers with hooklets), gravid proglottids (<12 branches on single side) are diagnostic; eggs indicate *Taenia* spp. only (thick, striated shell, containing six-hooked embryo or oncosphere), worm usually approx 12 ft long	Adult worm can cause GI complaints in some individuals; cysticercosis (accidental ingestion of eggs) can cause severe symptoms in the CNS; acquired from ingestion of raw or poorly cooked pork; usually only single worm/patient; occasionally two to three proglottids (hooked together) are passed; proglottids can be injected with India ink to show uterine branches for identification; cysticerci are normally small and contained within enclosing membrane; occasionally they may develop as racemose type, where worm tissue grows in the body like a metastatic cancer.
Diphyllobothrium latum (broad fish tapeworm)	Scolex (lateral sucking grooves), gravid proglottid (wider than long, reproductive structures in center "rosette"); eggs operculated	Can cause GI complaints in some individuals; acquired from ingestion of raw or poorly cooked freshwater fish; life cycle has two intermediate hosts (copepod, fish); worm may be 30 ft long; associated with vitamin B_{12} deficiency in genetically susceptible groups (Scandinavians)
Hymenolepis nana (dwarf tapeworm)	Adult worm not normally seen; egg round to oval, thin shell, containing six-hooked embryo or oncosphere with polar filaments lying between embryo and egg shell	Can cause GI complaints in some individuals; acquired from ingestion of eggs (only life cycle where the intermediate host, the grain beetle, can be bypassed); life cycle of egg to larval form to adult can be completed in human; most common tapeworm in the world
Hymenolepis diminuta (rat tapeworm)	Adult worm not normally seen; egg round to oval, thin shell, containing six-hooked embryo or oncosphere with *no* polar filaments lying between embryo and egg shell	Uncommon; egg can be confused with that of *H. nana;* eggs will be submitted in proficiency testing specimens and must be differentiated from those of *H. nana.*
Echinococcus granulosus (pathogenic)	Adult worm found only in the carnivore (dog); hydatid cysts develop (primarily in liver) when humans accidentally ingest eggs from dog tapeworms; cyst contains daughter cysts and many scolices; examine fluid aspirated from cyst at surgery.	Humans are accidental intermediate hosts; normal life cycle is in sheep and dog, with hydatid cysts developing in liver, lung, etc., of sheep. Human may be unaware of infection unless fluid leaks from cyst (can trigger an anaphylactic reaction) or pain is felt from cyst location.
Echinococcus multilocularis (pathogenic)	Adult worm found only in the carnivore (fox, wolf); hydatid cysts develop (primarily in liver) when humans accidentally ingest eggs from carnivore tapeworms; cyst grows like a metastatic cancer with no limiting membrane.	Humans are accidental intermediate hosts; prognosis poor; surgical removal of tapeworm tissue very difficult; found in Canada, Alaska, and, less frequently, in the northern United States, although it is becoming more common in the United States, where the geographic range is moving further south
Trematodes (flukes)		
Fasciolopsis buski (giant intestinal fluke)	Eggs found in stool; very large and operculated (morphology like that of *Fasciola hepatica* eggs)	Symptoms depend on worm burden; acquired from ingestion of plant material (water chestnuts) on which metacercariae have encysted; worms hermaphroditic
Fasciola hepatica (sheep liver fluke)	Eggs found in stool; cannot be differentiated from those of *F. buski*	Symptoms depend on worm burden; acquired from ingestion of plant material (watercress) on which metacercariae have encysted; worms hermaphroditic
Opisthorchis (Clonorchis) sinensis (Chinese liver fluke)	Eggs found in stool; very small (<35 μm); operculated, with shoulders into which operculum fits	Symptoms depend on worm burden; acquired from ingestion of raw fish; eggs can be missed unless 400× power is used for examination; eggs can resemble those of *Metagonimus yokogawai* and *Heterophyes heterophyes* (small intestinal flukes); worms hermaphroditic
Paragonimus westermani *Paragonimus* spp. (lung flukes)	Eggs coughed up in sputum (brownish "iron filings" = egg packets); can be recovered in sputum or stool (if swallowed); operculated, with shoulders into which operculum fits	Symptoms depend on worm burden and egg deposition; acquired from ingestion of raw crabs; eggs can be confused with those of *D. latum;* infections seen in Orient; infections with *Paragonimus mexicanus* found in Central and South America; worms hermaphroditic but often cross-fertilize with another worm if present

Table 9.10.2–A7 *(continued)*

Helminth	Diagnostic stage	Comments
Trematodes (flukes) *(continued)*		
Schistosoma mansoni (blood fluke) (pathogenic)	Eggs recovered in stool (large lateral spine); collect specimens with no preservatives (to indicate egg viability); worms in veins of large intestine	Acquired from skin penetration of single cercariae from freshwater snail; pathology caused by body's immune response to eggs in tissues; adult worms in veins cause no problems; adult worms are separate sexes.
Schistosoma haematobium (blood fluke) (pathogenic)	Eggs recovered in urine (large terminal spine); collect specimens with no preservatives (to indicate egg viability); worms in veins of bladder	Acquired from skin penetration of single cercariae from freshwater snail; pathology as with *S. mansoni;* collect 24-h and spot urine samples; chronic infection associated bladder cancer; adult worms are separate sexes.
Schistosoma japonicum (blood fluke) (pathogenic)	Eggs recovered in stool (very small lateral spine); collect specimens with no preservatives (to indicate egg viability); worms in veins of small intestine	Acquired from skin penetration of multiple cercariae from freshwater snail; pathology as with *S. mansoni;* infection usually most severe of the three because of original-loading infective dose of cercariae from freshwater snail (multiple cercariae stick together); pathology associated with egg production, which is greatest in *S. japonicum* infections

[a] GI, gastrointestinal; CNS, central nervous system.

Table 9.10.2–A8 Parasites found in blood: characteristics[a]

Protozoa	Diagnostic stage	Comments
Malaria		
Plasmodium vivax (benign tertian malaria)	Ameboid rings; Schüffner's dots, beginning in older rings (appear later than those in *P. ovale*); all stages seen in peripheral blood; mature schizont contains 16–18 merozoites.	Infects young cells; 48-h cycle; large geographic range; tends to have true relapse from residual liver stages, enlarged RBCs
Plasmodium ovale (ovale malaria)	Nonameboid rings; Schüffner's dots, beginning in young rings (appear earlier than those in *P. vivax*); all stages seen in peripheral blood; mature schizont contains 8–10 merozoites; RBCs may be oval and have fimbriated edges.	Infects young cells; 48-h cycle; narrow geographic range; tends to have true relapse from residual liver stages, enlarged RBCs
Plasmodium malariae (quartan malaria)	Thick rings; no stippling; all stages seen in peripheral blood; "band forms" and "rosette"-shaped mature schizont; lots of malarial pigment	Infects old cells; 72-h cycle; narrow geographic range; associated with recrudescence and nephrotic syndrome, no true relapse, normal or small RBCs
Plasmodium falciparum (malignant tertian malaria)	Multiple rings; appliqué-accolé forms; no stippling (rare Mauer's clefts); rings and crescent-shaped gametocytes seen in peripheral blood (no other developing stages; rare exception, mature schizont)	Infects all cells, 36- to 48-h cycle; large geographic range; no true relapse; most pathogenic of four species; plugged capillaries can cause severe symptoms and sequelae (cerebral malaria, RBC lysis, etc).
Plasmodium knowlesi	Ring forms have multiple nuclei; delicate rings (mimic *P. falciparum*); late trophozoites tend to form bands; schizonts and gametocytes similar to *P. vivax*; some parasites consistent with *P. malariae* (parasitemia, 0.03%, band forms); stippling present but probably not easily recognized	Infects all cells, 24-h cycle. Geographic range included Thailand, Myanmar, peninsular Malaysia, Malaysian Borneo, Palawan Island in the Philippines, Vietnam; can be as pathogenic as *P. falciparum*—should be considered a medical emergency
Babesia spp.	Ring forms only (resemble *P. falciparum* rings); seen in splenectomized patients; endemic in United States (no travel history necessary); if present, "Maltese cross" configuration diagnostic, but it is not always seen	Tick-borne infection associated with Nantucket Island; infection mimics malaria; ring forms more pleomorphic than those in malaria; more rings/cell (usually) than in malaria; endemic in several areas within the United States; organisms occasionally seen outside RBCs (unlike malaria merozoites)
Trypanosomes		
Trypanosoma brucei gambiense (West African sleeping sickness)	Trypomastigotes long and slender, with typical undulating membrane; lymph nodes and blood can be sampled; microhematocrit tube concentration helpful; examine spinal fluid in later stages of infection.	Tsetse fly vector; tends to be chronic infection, exhibiting real symptoms of sleeping sickness
Trypanosoma brucei rhodesiense (East African sleeping sickness)	Trypomastigotes long and slender, with typical undulating membrane; lymph nodes or blood can be sampled; microhematocrit tube concentration helpful; examine spinal fluid in later stages of infection.	Tsetse fly vector; tends to be more severe, short-lived infection (particularly in children); patient may expire before progressive symptoms of sleeping sickness appear.
Trypanosoma cruzi (Chagas' disease; American trypanosomiasis)	Trypomastigotes short, stumpy, often curved in C shape; sample blood early in infection; trypomastigotes enter striated muscle (heart, GI tract) and transform into amastigote form.	Reduviid bug ("kissing bug") vector; chronic in adults, severe in young children; great morbidity associated with cardiac failure and loss of muscle contractility in heart and GI tract

Table 9.10.2–A8 *(continued)*

Protozoa	Diagnostic stage	Comments
Trypanosomes *(continued)*		
Leishmania spp. (cutaneous) (not actually a blood parasite but presented for comparison with *L. donovani*)	Amastigotes found in skin macrophages; intracellular forms containing nucleus and kinetoplast are diagnostic.	Not actually blood parasite but presented for comparison with *Leishmania donovani;* sand fly vector; organisms recovered from site of lesion only; stain specimens or culture in NNN and/or Schneider's medium; animal inoculation (hamster) rarely used
Leishmania braziliensis (mucocutaneous) (not actually a blood parasite but presented for comparison with *L. donovani*)	Amastigotes found in macrophages of skin and mucous membranes; intracellular forms containing nucleus and kinetoplast are diagnostic.	Not actually blood parasite but presented for comparison with *L. donovani;* sand fly vector; organisms recovered from site of lesion only; stain specimens or culture in NNN and/or Schneider's medium; animal inoculation (hamster) rarely used
Leishmania donovani (visceral)	Amastigotes found throughout reticuloendothelial system; spleen, liver, bone marrow, etc.; intracellular forms containing nucleus and kinetoplast are diagnostic.	Sand fly vector; organisms recovered from buffy coat (rarely found), bone marrow aspirate, spleen or liver puncture (rarely performed); stain specimens or culture in NNN and/or Schneider's medium; animal inoculation (hamster) rarely used; cause of kala-azar
Helminths		
Wuchereria bancrofti	Microfilaria sheathed, clear space at end of tail; nocturnal periodicity; elephantiasis seen in chronic infections	Pathogenicity due to adult worms; mosquito vector; microfilariae recovered in blood (membrane filtration, Knott concentrate, thick films); hematoxylin stains sheath; sheath does not stain well with Giemsa, but the Innenkörper (inner body) stains a pink color
Brugia malayi	Microfilaria sheathed, subterminal and terminal nuclei at end of tail; nocturnal periodicity; elephantiasis seen in chronic infections	Pathogenicity due to adult worms; mosquito vector; microfilariae recovered in blood (membrane filtration, Knott concentrate, thick films); hematoxylin stains sheath; sheath tends to stain pink with Giemsa
Loa loa (African eye worm)	Microfilaria sheathed, nuclei continuous to tip of tail; diurnal periodicity; adult worm may cross conjunctiva of the eye.	Pathogenicity due to adult worms; mango fly vector; history of Calabar swellings, worms in the eye; microfilariae difficult to recover from blood; hematoxylin stains sheath
Mansonella spp.	Microfilaria unsheathed, nuclei may or may not extend to tip of tail (depending on species); nonperiodic; symptoms usually absent or mild	Pathogenicity mild and due to adult worms; midge or blackfly vector; microfilariae recovered in blood (membrane filtration, Knott concentrate, thick films)
Mansonella streptocerca	Microfilaria unsheathed, nuclei extend to tip of tail; when immobile, curved like shepherd's crook; adults in dermal tissues	Pathogenicity mild and due to adult worms and/or microfilariae; midge vector; microfilariae found in skin snips; microfilarial tails split rather than blunt
Onchocerca volvulus	Microfilaria unsheathed, nuclei do not extend to tip of tail; adults in nodules	Pathogenicity due to microfilariae; blackfly vector; microfilariae found in skin snips; microfilariae migrate to optic nerve; cause of river blindness

[a] GI, gastrointestinal.

Table 9.10.2–A9 Parasitic infections: clinical findings in healthy and compromised hosts

Organism(s)	Clinical findings in:	
	Healthy host	Compromised host[a]
Entamoeba histolytica	Asymptomatic to chronic-acute colitis; extraintestinal disease may occur (primary site: right upper lobe of liver).	Diminished immune capacity may lead to extraintestinal disease.
Free-living amebae	Patients tend to have eye infections with *Acanthamoeba* spp.; linked to poor eye care	Primary amebic meningoencephalitis caused by *Naegleria fowleri*, granulomatous amebic encephalitis caused by *Acanthamoeba* spp. and *Balamuthia*; severe cutaneous infections in compromised patients (*Acanthamoeba* spp.)
Giardia lamblia	Asymptomatic to malabsorption syndrome	Certain immunodeficiencies tend to predispose an individual to infection.
Blastocystis hominis	Asymptomatic to symptomatic, often depending on number of organisms present	Not enough information to tell whether actual differences occur in compromised patients; infections may be more difficult to eradicate in AIDS patients
Toxoplasma gondii	Approximately 50% of individuals have antibody and organisms in tissue but are asymptomatic.	Disease in compromised host tends to involve CNS, with various neurological symptoms.
Cryptosporidium spp.	Self-limiting infection with diarrhea and abdominal pain	Due to autoinfective nature of life cycle, will not be self-limiting, may produce fluid loss of over 10 liters/day, and may show multisystem involvement; no known totally effective therapy
Cyclospora cayetanensis	Self-limiting infection with diarrhea (3–4 days), with relapses common	Diarrhea may persist for 12 wk or more; biliary disease has also been reported for this group, particularly for those with AIDS.
Cystoisospora (*Isospora*) *belli*	Self-limiting infection with mild diarrhea or no symptoms	May lead to severe diarrhea, abdominal pain, and possible death (rare case reports); diagnosis may occasionally be missed due to nonrecognition of oocyst stage; will not be seen when concentrated from PVA fixative
Sarcocystis spp.	Self-limiting infection with diarrhea or mild symptoms	Symptoms may be more severe and last longer.
Microsporidia (*Nosema, Annncaliia, Vittaforma, Encephalitozoon, Enterocytozoon, Pleistophora, Trachipleistophora,* "*Microsporidium*" spp.)	Less known about these infections in the healthy host; serological evidence suggests that infections may be more common than recognized.	Can infect various parts of the body; diagnosis often depends on histological examination of tissues; routine examination of clinical specimens (stool, urine, etc.) is becoming more common; organisms can cause death.
Leishmania spp.	Asymptomatic to mild disease	More serious manifestations of visceral leishmaniasis; some cutaneous species will manifest visceral disease; difficult to treat and manage; definite coinfection with AIDS
Strongyloides stercoralis	Asymptomatic to mild abdominal complaints; can remain latent for many years due to low-level infection maintained by internal autoinfective life cycle	Can result in disseminated disease (hyperinfection syndrome due to autoinfective nature of life cycle); abdominal pain, pneumonitis, sepsis-meningitis with gram-negative bacilli, eosinophilia

Table 9.10.2–A9 *(continued)*

Organism(s)	Clinical findings in:	
	Healthy host	Compromised host[a]
Crusted (Norwegian) scabies *(Sarcoptes scabiei)*	Infections can range from asymptomatic to causing moderate itching.	Severe infection with reduced itching response; hundreds of thousands of mites on body; infection very easily transferred to others; secondary infection very common

[a] CNS, central nervous system; PVA, polyvinyl alcohol; EM, electron microscopy.

FECAL IMMUNOASSAYS FOR INTESTINAL PROTOZOA

Fecal immunoassays for antigen detection are generally simple to perform and allow a large number of tests to be performed at one time, thereby reducing overall costs. A major disadvantage of antigen detection in stool specimens is that the method can detect only one or two pathogens at a time. The routine ova-and-parasite (O&P) examination must be performed in order to detect other parasitic pathogens. The current commercially available antigen tests have excellent sensitivity and specificity compared with routine microscopy.

Current formats include the ELISA, the fluorescence assay (FA), and the rapid membrane flow immunochromatographic cartridges. Sensitivity and specificity are comparable among the various formats and diagnostic kits currently available. The selection of any particular immunoassay format will often depend on the work flow options within the laboratory; different laboratories may select different formats for the rapid tests available. The methods are different, but the results are comparable.

For diagnosis of amebiasis, antigen-based fecal immunoassays have several significant advantages over other methods currently used: (i) some of the assays can differentiate the true pathogen *E. histolytica* from the nonpathogen *Entamoeba dispar;* (ii) they have excellent sensitivity and specificity; (iii) they are readily usable by most laboratory personnel; and (iv) the assays have potential use in situations such as waterborne outbreaks. Because there are distinct genetic differences between *E. dispar* and *E. histolytica,* commercial kits have been developed to detect their presence and differentiate them in clinical samples. However, current antigen detection tests require the examination of fresh or frozen (not preserved) stool specimens, while many laboratories have switched to stool collection methods using various preservatives.

A number of commercially available immunoassay kits are available for *Cryptosporidium* spp. and are more sensitive and specific than routine microscopic examination of smears stained by modified acid-fast staining. Stool specimens may be fresh, frozen, or fixed; however, polyvinyl alcohol (PVA)-fixed specimens are unacceptable for use in fecal immunoassays.

For *Giardia lamblia,* antigen detection tests are reliable and more sensitive and specific than the routine O&P examination. Commercial immunoassay kits are readily available. Users have to evaluate which kit format will be most useful for their own laboratories. Some of the kits may not detect both trophozoites and cysts of *G. lamblia* but may be selective for only one life cycle stage; most antibodies used for testing are designed to detect the cyst form. Because there are some shared antigens between trophozoites and cysts, occasionally positive reactions may be obtained in specimens primarily containing trophozoites. However, this situation could also lead to a false-negative result.

Although not currently available commercially, several reagents are in various developmental phases. These include antigen detection kits for *Dientamoeba fragilis, Blastocystis hominis, Cyclospora cayetanensis,* and various species of the microsporidia.

Some comments that may be helpful in performing various immunoassay formats are provided in Table 9.10.2–A10 to assist in test performance and/or result interpretation. *It is very important to read the kit information sheet before use.* Currently, fecal immunoassays are available for *Giardia lamblia,* the *Entamoeba histolytica/E. dispar* group, *Entamoeba histolytica,* and *Cryptosporidium* spp. Based on the published literature, fecal immunoassays are more sensitive and specific than the routine O&P examination; this is particularly true for *Giardia lamblia.* However, unlike the O&P examination, which facilitates the recovery of many different parasites, the fecal immunoassays are limited to one or two organisms only. The fecal immunoassays are also more sensitive than the special stains (modified acid-fast stains) for the coccidia (*Cryptosporidium* spp.).

Fresh specimens can be stored at 2 to 8°C and should be tested within 48 h, or they should be frozen at −20 to −70°C (freezing is not acceptable for the FA method—the freeze-thaw cycle will damage organisms). Stool specimens preserved in 10% formalin, merthiolate-formalin, or sodium acetate-acetic acid-formalin fixatives may be refrigerated (2 to 8°C) or stored at room temperature (20 to 25°C) and should be tested within 2 months. Stool specimens submitted in Cary Blair transport medium (or equivalent) should be refrigerated or frozen and tested within 1 week after collection. Fecal specimens that have been preserved in fixatives containing PVA are not acceptable for testing.

Table 9.10.2–A10 Fecal antigen detection method options

EIAs	Fluorescence assays	Rapid cartridge assays
EIAs (antigen detection, no centrifugation recommended); the antigen will be found in the top fluid layer of the stool collection vial. Remember to thoroughly rinse the wells according to the instructions; do not eliminate any of the rinse steps. Make sure each well receives the total number of rinses required. Make sure the stream of buffer goes directly into the wells. Use a wash bottle with a small opening, so you have to squeeze the bottle to get the fluid to squirt directly into the wells. When the directions tell you to "slap" the tray down onto some paper towels to remove the last rinse fluid, make sure you slap it several times. Do not be too gentle; the cups will not fall out of the holder. Prior to adding the last reagents, the wells should be empty of rinse buffer (not dry, but empty of excess fluid). Note: If you shake the specimen vial prior to testing, allow the vial contents to settle out for several minutes. Adding too much particulate stool to the wells will interfere with testing.	Fluorescence (visual identification [microscopy] of the organisms): since you will be looking for the actual organisms (cysts of *Giardia* and/or oocysts of *Cryptosporidium*), this test should be performed on centrifuged stool sediment (500 × *g* for 10 min) to increase the sensitivity. Remember to thin out the smear; make sure the slides are thoroughly dry before adding reagents. The slides can be placed in a 35°C incubator for about 30 min to an hour to make sure they are dry prior to processing. If the material on the wells is too thick, it may not dry thoroughly and may fall off the glass. It is better to let the slides dry longer rather than too short a time. A heat block is *not* recommended for this purpose. Gently rinse the reagents from the wells; do not squirt directly into the wells, but allow the rinse fluid to flow over the wells. Not all clinical specimens will provide the 3+ to 4+ fluorescence that is often seen in the positive control. Also, from time to time, you may see fluorescing bacteria and/or some yeasts in certain patient specimens. This is not that common, but the shapes can be distinguished from *Giardia* cysts and/or *Cryptosporidium* oocysts. Rarely, one might see the faint outlines of a *Giardia* trophozoite (they do not fluoresce as brightly as the cysts). The intensity of the fluorescence may vary, depending on the filters. If the fluorescent microscope dual-filter system is used, it demonstrates both the yellow-green fluorescence and the red-orange counterstain, and both *Giardia* and *Cryptosporidium* may not appear quite as bright as when using the yellow-green filter only. Both approaches are acceptable and may reflect personal laboratory preferences. However, remember that when the single FITC[a] (yellow-green) filter is used alone, some artifact materials may also appear to fluoresce more brightly, while the artifact material might not be seen when both filters (FITC and counterstain) are used. Artifact material may fluoresce a dull color without the bright outlines around the *Giardia* cysts and *Cryptosporidium* oocysts that can be seen using both filter systems. *Both filters = less fluorescence intensity, fewer visible artifacts. Single FITC filter = brighter fluorescence, more visible artifacts.* Make sure to examine the edges of the wells. Sometimes in a light infection, the edges may contain organisms, while in the middle of the well, the organisms may be a bit more difficult to detect (thick area).	Lateral-flow cartridges (antigen detection, no centrifugation recommended); the antigen will be found in the top fluid layer of the stool collection vial. If the stool is too thick, the addition of reagents will not thin it out enough. If the specimen poured into the well remains too thick, the fluid will not flow up the membrane. If your specimens arrive in fixative and there is no fluid at the top of the vial overlaying the stool, this means the vial may have been overfilled with stool. These specimens will have to be diluted with the appropriate diluent before testing. It is always important to see the control line indicated as positive all the way across the membrane, not just at the edges. A positive test result may be much lighter than the control line; this is normal. At the cutoff time to read the result, any color at all visible in the test area should be interpreted as a positive. Do not read/interpret the results after the time indicated in the directions; you may get a false-positive result. **NOTE:** If you shake the specimen vial prior to testing, allow the vial contents to settle out for several minutes. Adding too much particulate stool to the wells will interfere with testing.

[a] FITC, fluorescein isothiocyanate.

SUPPLEMENTAL READING

Garcia, L. S., and R. Y. Shimizu. 1997. Evaluation of nine immunoassay kits (enzyme immunoassay and direct fluorescence) for detection of *Giardia lamblia* and *Cryptosporidium parvum* in human fecal specimens. *J. Clin. Microbiol.* **35:**1526–1529.

Garcia, L. S., and R. Y. Shimizu. 2000. Detection of *Giardia lamblia* and *Cryptosporidium parvum* antigens in human fecal specimens using the ColorPAC combination rapid solid-phase qualitative immunochromatographic assay. *J. Clin. Microbiol.* **38:**1267–1268.

Hanson, K. L., and C. P. Cartwright. 2001. Use of an enzyme immunoassay does not eliminate the need to analyze multiple stool specimens for sensitive detection of *Giardia lamblia. J. Clin. Microbiol.* **39:**474–477.

Isenberg, H. D. (ed.). 2004. *Clinical Microbiology Procedures Handbook,* 2nd ed., vol. 2, p. 9.0.1–9.10.8.3. ASM Press, Washington, DC.

Wilson, M., and P. M. Schantz. 2000. Parasitic immunodiagnosis, p. 1117–1122. *In* G. T. Strickland (ed.), *Hunter's Tropical Medicine and Emerging Infectious Diseases,* 8th ed. W. B. Saunders Co., Philadelphia, PA.

9.10.3 Appendix 9.10.3–1—Common Problems in Organism Identification

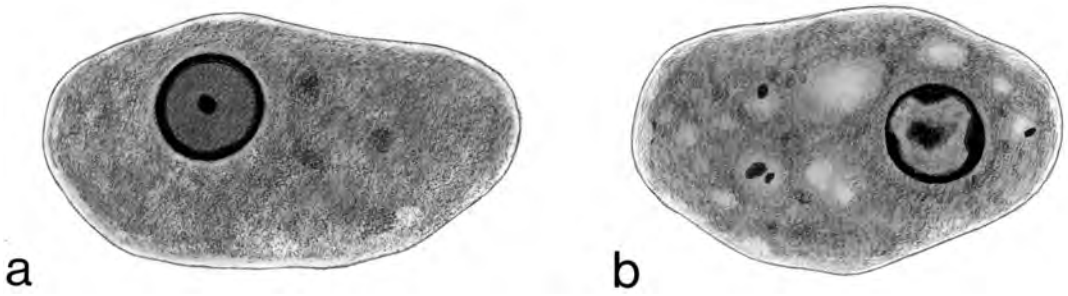


Figure 9.10.3–A1 (a) *Entamoeba histolytica/E. dispar* trophozoite. Note the evenly arranged nuclear chromatin, central compact karyosome, and relatively "clean" cytoplasm. (b) *Entamoeba coli* trophozoite. Note the unevenly arranged nuclear chromatin, eccentric karyosome, and "messy" cytoplasm. These characteristics are very representative of the two organisms. (Illustration by Sharon Belkin.)

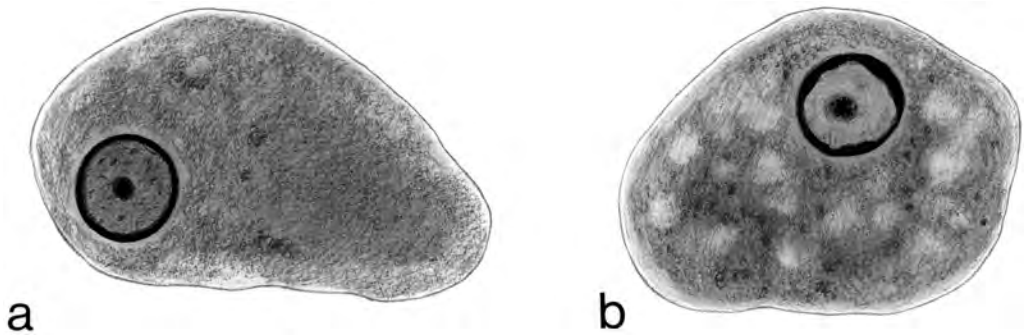


Figure 9.10.3–A2 (a) *Entamoeba histolytica/E. dispar* trophozoite. Note the evenly arranged nuclear chromatin, central compact karyosome and "clean" cytoplasm. (b) *Entamoeba coli* trophozoite. Note that the nuclear chromatin appears to be evenly arranged, the karyosome is central (but more diffuse), and the cytoplasm is "messy," with numerous vacuoles and ingested debris. The nuclei of these two organisms tend to resemble one another (very common finding in routine clinical specimens). (Illustration by Sharon Belkin.)

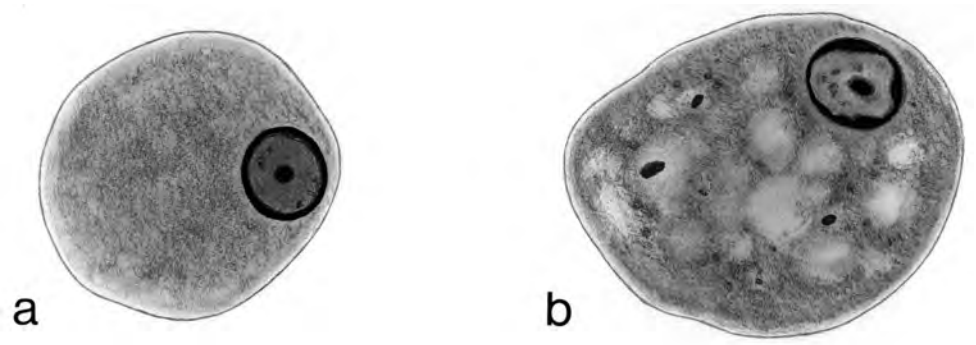


Figure 9.10.3–A3 (a) *Entamoeba histolytica/E. dispar* trophozoite. Again, note the typical morphology (evenly arranged nuclear chromatin, central compact karyosome, and relatively "clean" cytoplasm). (b) *Entamoeba coli* trophozoite. Although the nuclear chromatin is eccentric, note that the karyosome seems to be compact and central. However, note the various vacuoles containing ingested debris. These organisms show some characteristics that are very similar (very typical in clinical specimens). (Illustration by Sharon Belkin).

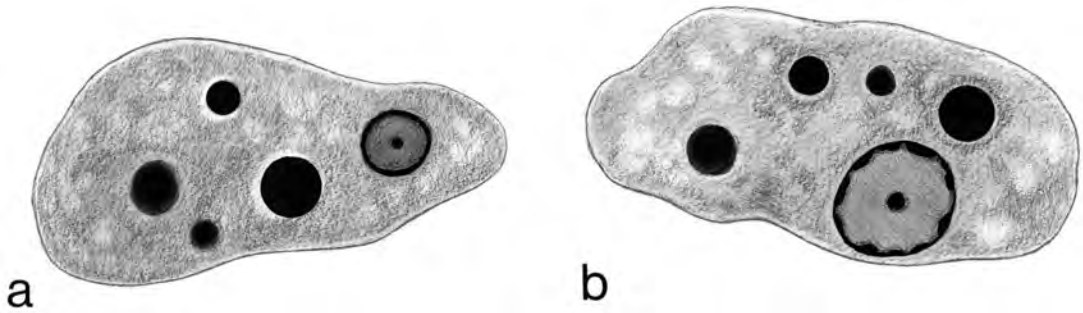


Figure 9.10.3–A4 (a) *Entamoeba histolytica* trophozoite. Note the evenly arranged nuclear chromatin, central compact karyosome, and RBCs in the cytoplasm. (b) Human macrophage. The key difference between the macrophage nucleus and that of *E. histolytica* is the size. Usually, the ratio of nucleus to cytoplasm in a macrophage is approximately 1:6 or 1:8, while the true organism has a nucleus/cytoplasm ratio of approximately 1:10 or 1:12. The macrophage also contains ingested RBCs. In cases of diarrhea or dysentery, trophozoites of *E. histolytica* and macrophages can often be confused, occasionally leading to a false-positive diagnosis of amebiasis when no parasites are present. Both the actual trophozoite and the macrophage may also be seen without ingested RBCs, and they can mimic one another. (Illustration by Sharon Belkin.)

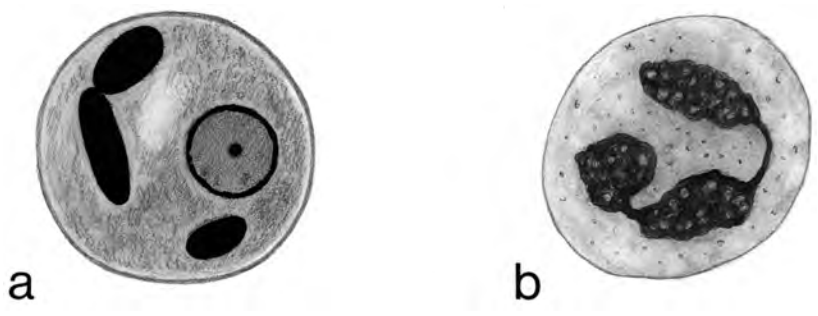


Figure 9.10.3–A5 (a) *Entamoeba histolytica/E. dispar* precyst. Note the enlarged nucleus (prior to division) with evenly arranged nuclear chromatin and central compact karyosome. Chromatoidal bars (rounded ends, with smooth edges) are also present in the cytoplasm. (b) PMN. The nucleus is somewhat lobed (normal morphology) and represents a PMN that has not been in the gut very long. Occasionally, the positioning of the chromatoidal bars and the lobed nucleus of the PMN can mimic one another. The chromatoidal bars will stain more intensely, but shapes can overlap, as seen here. (Illustration by Sharon Belkin.)

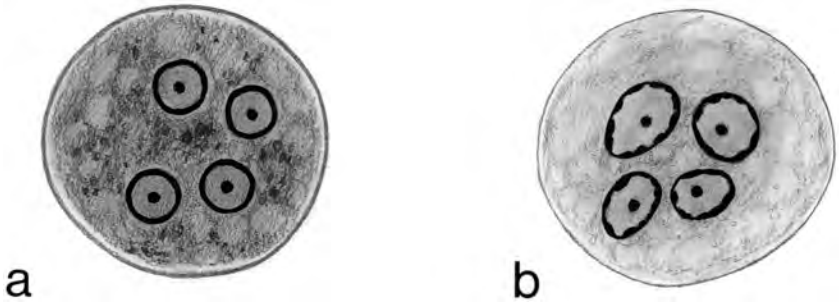


Figure 9.10.3–A6 (a) *Entamoeba histolytica/E. dispar* cyst. Note that the four nuclei are very consistent in size and shape. (b) PMN. Note that the normal lobed nucleus has now broken into four fragments, which mimic four nuclei with peripheral chromatin and central karyosomes. When PMNs have been in the gut for some time and have begun to disintegrate, the nuclear morphology can mimic that seen in an *E. histolytica/E. dispar* cyst. However, human cells are often seen in the stool in cases of diarrhea; with rapid passage of the gastrointestinal tract contents, there will not be time for amebic cysts to form. Therefore, in cases of diarrhea and/or dysentery, if "organisms" are seen that resemble the cell in panel b, think first of PMNs, not *E. histolytica/E. dispar* cysts. (Illustration by Sharon Belkin.)

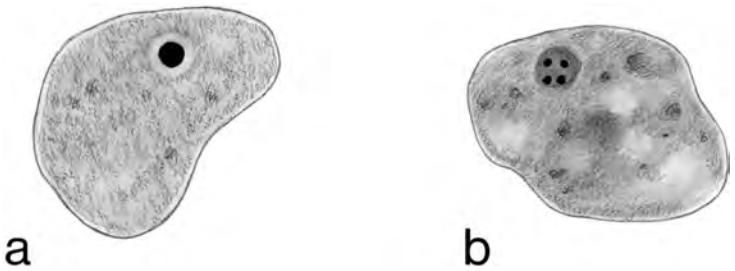


Figure 9.10.3–A7 (a) *Endolimax nana* trophozoite. This organism is characterized by a large karyosome with no peripheral chromatin, although there are normally many nuclear variations seen in any positive specimen. (b) *Dientamoeba fragilis* trophozoite. Normally, the nuclear chromatin is fragmented into several dots (often a "tetrad" arrangement). The cytoplasm is normally more "junky" than that seen in *E. nana.* If the morphology is typical, as in these two illustrations, then differentiating between these two organisms is not that difficult. However, the morphologies of the two will often be very similar. (Illustration by Sharon Belkin.)

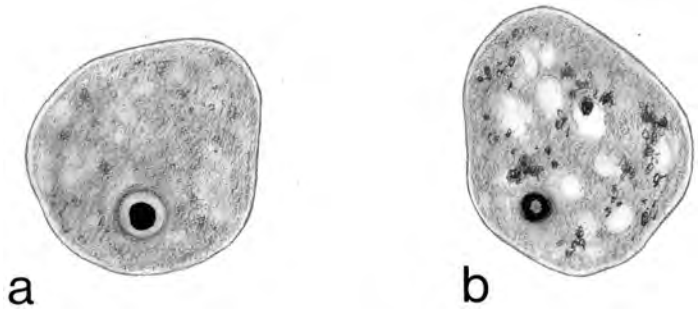


Figure 9.10.3–A8 (a) *Endolimax nana* trophozoite. Notice that the karyosome is large and surrounded by a "halo," with very little, if any, chromatin on the nuclear membrane. (b) *Dientamoeba fragilis* trophozoite. In this organism, the karyosome is beginning to fragment, and there is a slight clearing in the center of the nuclear chromatin. If the nuclear chromatin has not become fragmented, *D. fragilis* trophozoites can very easily mimic *E. nana* trophozoites. This could lead to a report indicating that no pathogens were present, when, in fact, *D. fragilis* is considered a definite cause of symptoms. (Illustration by Sharon Belkin.)

Figure 9.10.3–A9 (a) *Endolimax nana* trophozoite. Note the large karyosome surrounded by a clear space. The cytoplasm is relatively "clean." (b) *Iodamoeba bütschlii.* Although the karyosome is similar to that of *E. nana*, note that the cytoplasm in *I. bütschlii* is much more heavily vacuolated and contains ingested debris. Often, these two trophozoites cannot be differentiated. However, the differences in the cytoplasm are often helpful. There will be a definite size overlap between the two genera. (Illustration by Sharon Belkin.)

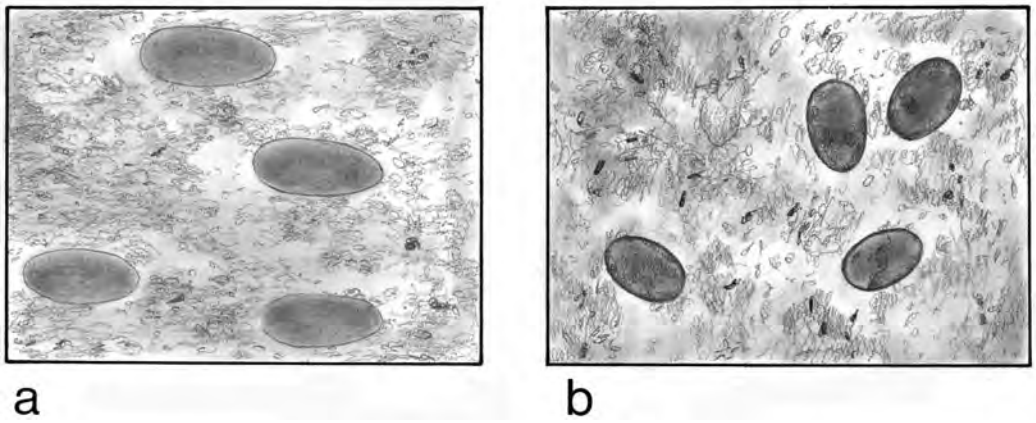


Figure 9.10.3–A10 (a) RBCs on a stained fecal smear. Note that the cells are very pleomorphic but tend to be positioned in the direction the stool was spread onto the slide. (b) Yeast cells on a stained fecal smear. These cells tend to remain oval and are not aligned in any particular way on the smear. These differences are important when the differential identification is between *Entamoeba histolytica* containing RBCs and *Entamoeba coli* containing ingested yeast cells. If RBCs or yeast cells are identified in the cytoplasm of an organism, they must also be visible in the background of the stained fecal smear. (Illustration by Sharon Belkin.)

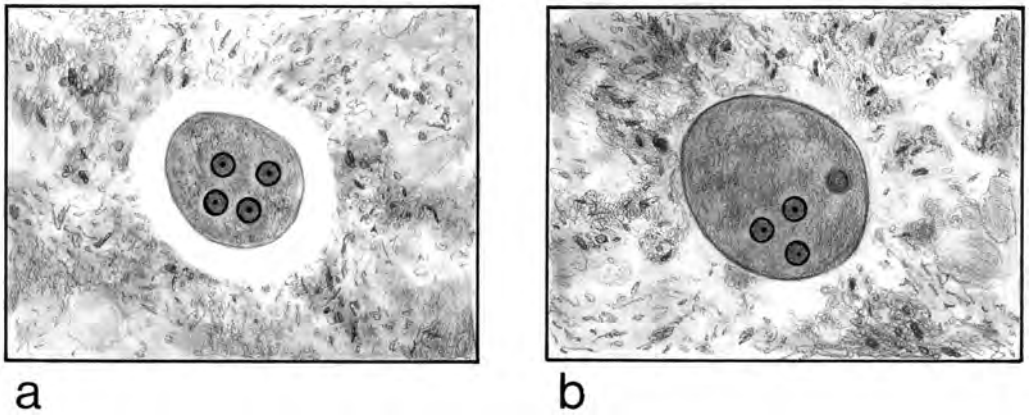


Figure 9.10.3–A11 (a) *Entamoeba histolytica/E. dispar* cyst. Note the shrinkage due to dehydrating agents in the staining process. (b) *E. histolytica/E. dispar* cyst. In this case, the cyst exhibits no shrinkage. Only three of the four nuclei are in focus. Normally, this type of shrinkage is seen with protozoan cysts and is particularly important when a species is measured and identified as either *E. histolytica/E. dispar* or *Entamoeba hartmanni.* The whole area, including the halo, must be measured prior to species identification. If just the cyst is measured, the organism would be identified as *E. hartmanni* (nonpathogenic) rather than *E. histolytica/E. dispar* (possibly pathogenic). (Illustration by Sharon Belkin.)

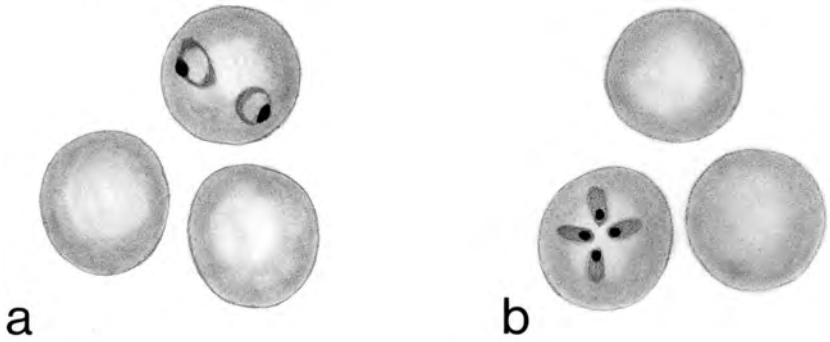


Figure 9.10.3–A12 (a) *Plasmodium falciparum* rings. Note the two rings in the RBC. Multiple rings per cell are more typical of *P. falciparum* than of the other species of human malaria. (b) *Babesia* rings. In one of the RBCs are four small *Babesia* rings. This particular arrangement is called the Maltese cross and is diagnostic for *Babesia* spp. However, the Maltese cross configuration is not always present. *Babesia* infections can be confused with cases of *P. falciparum* malaria, primarily because multiple rings can be seen in the RBCs. Another difference involves ring morphology. *Babesia* rings are often of various sizes and tend to be very pleomorphic, while those of *P. falciparum* tend to be more consistent in size and shape. (Illustration by Sharon Belkin.)

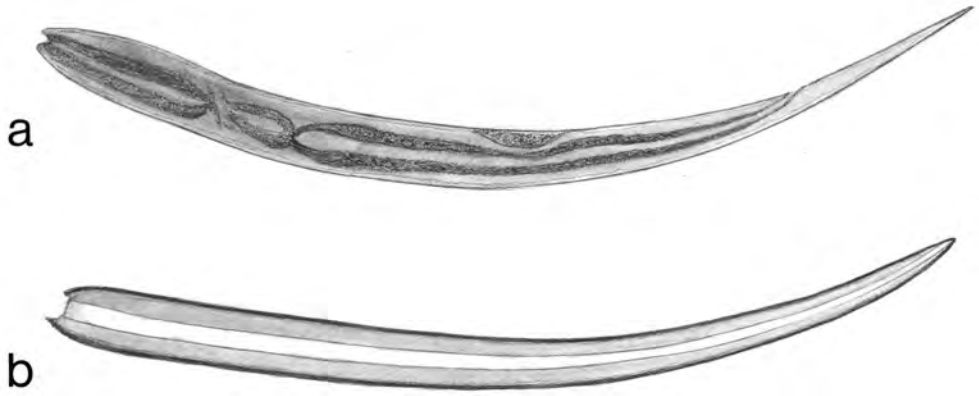


Figure 9.10.3–A13 (a) *Strongyloides stercoralis* rhabditiform larva. Note the short buccal capsule (mouth opening) and the internal structure, including the genital primordial packet of cells. (b) Root hair (plant material). Note that there is no specific internal structure and the end is ragged (where it was broken off from the main plant). Often plant material mimics some of the human parasites. This comparison is one of the best examples. These artifacts are occasionally submitted as proficiency testing specimens. (Illustration by Sharon Belkin.)

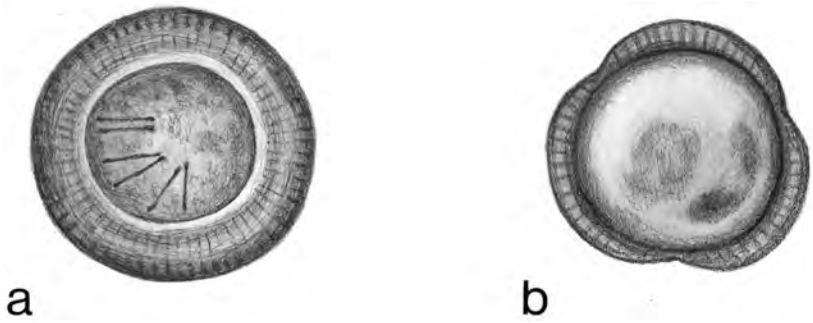


Figure 9.10.3–A14 (a) *Taenia* egg. This egg has been described as having a thick, radially striated shell containing a six-hooked embryo (oncosphere). (b) Pollen grain. Note that this trilobed pollen grain has a similar type of "shell" and, if turned the right way, could resemble a *Taenia* egg. This represents another confusion between a helminth egg and a plant material artifact. When examining fecal specimens in a wet preparation, tap on the coverslip to get objects to move around. As they move, you can see more morphological detail. (Illustration by Sharon Belkin.)

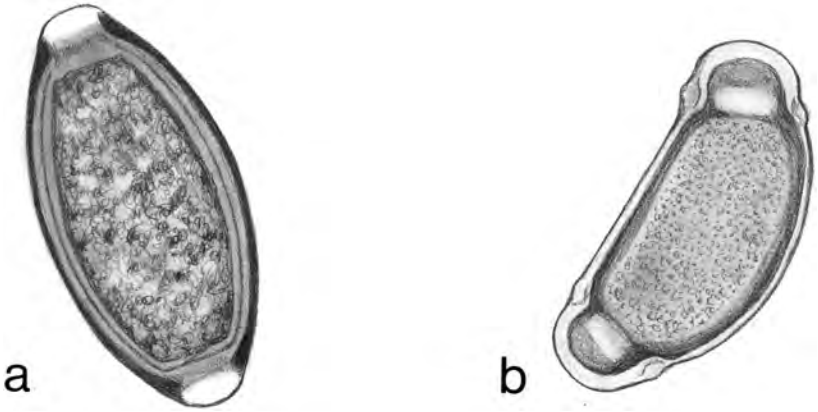


Figure 9.10.3–A15 (a) *Trichuris trichiura* egg. This egg is typical and is characterized by the barrel shape with thick shell and two polar plugs. (b) Bee pollen. This artifact certainly mimics the actual *T. trichiura* egg. However, note that the actual shape is somewhat distorted. This is an excellent example of a parasite "look-alike" and could be confusing. (Illustration by Sharon Belkin.)

9.10.4

Appendix 9.10.4–1—Quality Control Recording Sheets

Forms on pages 9.10.4.2 to 9.10.4.5 are for use in QC testing of cultures and reagents.

DIAGNOSTIC PARASITOLOGY QC (REAGENTS)—EXAMPLE

DIAGNOSTIC PARASITOLOGY QC (REAGENTS)—EXAMPLE

1. Reagent Name:_____

2. QC Requirements (frequency):_____

3. Acceptable Criteria:_____

Negative Control:_____

Positive Control:_____

Date	Lot No.	Exp. Date	QC Organism	Tech	Results (A/NA)[1]	Comments Corrective Action

Comments:_____

Date	Lot No.	Exp. Date	QC Organism	Tech	Results (A/NA)	Comments Corrective Action

Comments:_____

Date	Lot No.	Exp. Date	QC Organism	Tech	Results (A/NA)	Comments Corrective Action

Comments:_____

Date	Lot No.	Exp. Date	QC Organism	Tech	Results (A/NA)	Comments Corrective Action

Comments:_____

[1]A = Acceptable, NA = Not Acceptable

DIAGNOSTIC PARASITOLOGY QC (REAGENTS)
EXAMPLE FOR MULTIPLE REAGENTS

Manufacturer/Product: _____

QC Frequency: _____

Acceptable (A): _____

Not Acceptable (NA): _____

Reagent	Lot #	Exp. Date

QC Results	Date	Tech	QC Results	Date	Tech

DIAGNOSTIC PARASITOLOGY QC (CULTURE) EXAMPLE OF A WORKSHEET

DIAGNOSTIC PARASITOLOGY QC (CULTURE)
EXAMPLE OF A WORKSHEET

1. Patient's Name/Organism trying to isolate:_____

2. Medium Number 1:_____

   Medium Number 2:_____

3. ATCC Control Strain (Indicate Organism Name and ATCC Number):_____

4: Frequency: QC strain must be set up every time patient specimen is cultured (there are no exceptions to this requirement):

Medium 1:_____

Date	Medium Lot No.	Exp. Date	Tech	A[1]	NA[2]	Date Read	Comments[3] Corrective Action

Comments:_____

Medium 2:_____

Date	Medium Lot No.	Exp. Date	Tech	A[1]	NA[2]	Date Read	Comments[3] Corrective Action

Comments:_____

[1]Acceptable: QC organism growth or motile trophozoites can be detected microscopically in sample of QC medium (inoculated with ATCC strain).

[2]Not Acceptable: QC organism growth or motile trophozoites cannot be detected microscopically in sample of QC medium (inoculated with ATCC strain).

[3]Follow-up: in the event the QC culture is negative, the control strain should be resubbed to fresh media.

EQUIPMENT MAINTENANCE

Equipment Description:_____
(Include Inventory Number)

Serial Number:_____ Model Number:_____ Purchase Date:_____

Equipment Location:_____

Maintenance Requirements and Time Frames:
(List specific requirements, time frames below - use table for recording actual maintenance.)

1._____

2._____

3._____

4._____

Date and Initials	Maintenance (List # of Maintenance Requirements)	Comments/Corrective Action

Remember to attach all relevant paperwork to this sheet (repair invoices, replacement part invoices, etc.).

9.10.5

Appendix 9.10.5–1—Flowcharts for Diagnostic Procedures

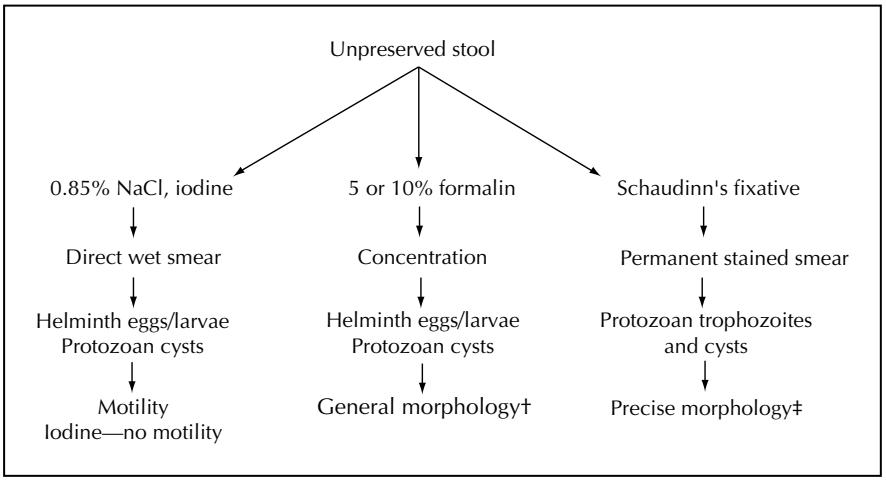


Figure 9.10.5–A1 Procedure for processing fresh stool for the ova and parasite examination. Special stains will be necessary for *Cryptosporidium* and *Cyclospora* (modified acid-fast) and the microsporidia (modified trichrome, calcofluor). Immunoassay kits are now available for some of these organisms. If the permanent staining method (iron hematoxylin) contains a carbol fuchsin step, the coccidia will stain pink. Symbols: †, some protozoa may not be identified using the wet examination only; ‡, protozoa (primarily trophozoites) can be identified and cysts can be confirmed.

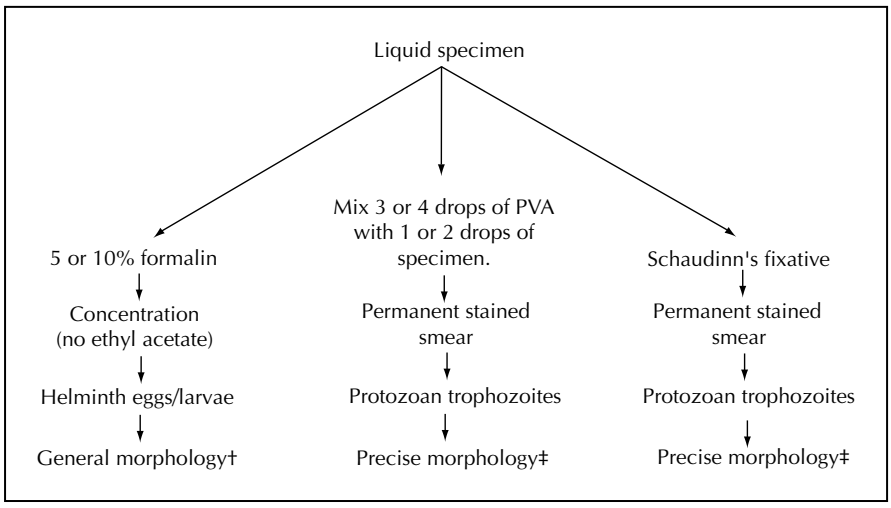


Figure 9.10.5–A2 Procedure for processing liquid specimens for the ova and parasite examination. Polyvinyl alcohol (PVA) and specimen will be mixed together on the slide, allowed to air dry, and then stained (fixation is sufficient for liquid specimen but not for formed stool). Symbols: †, some protozoa may not be identified from the concentration procedure; ‡, protozoa (trophozoites can be identified).

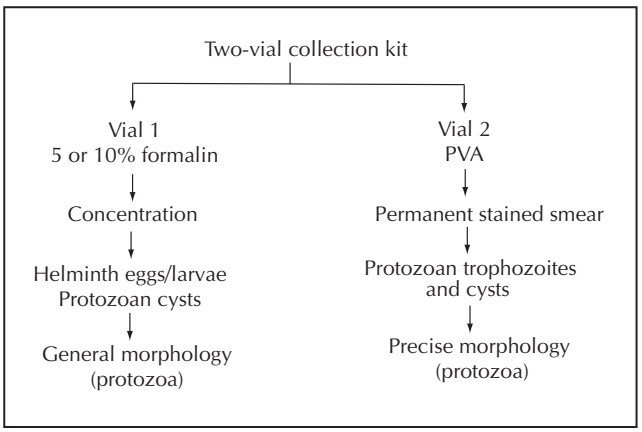


Figure 9.10.5–A3 Procedure for processing preserved stool for the ova and parasite examination. Fixatives and effects: mercuric chloride, best polyvinyl alcohol (PVA) (trichrome, iron hematoxylin); zinc, current best substitute (trichrome, hematoxylin probably okay); copper sulfate, fair substitute (trichrome, iron hematoxylin: both fair to poor); sodium acetate-acetic acid-formalin, good substitute for PVA fixative (iron hematoxylin is best, and trichrome is okay).

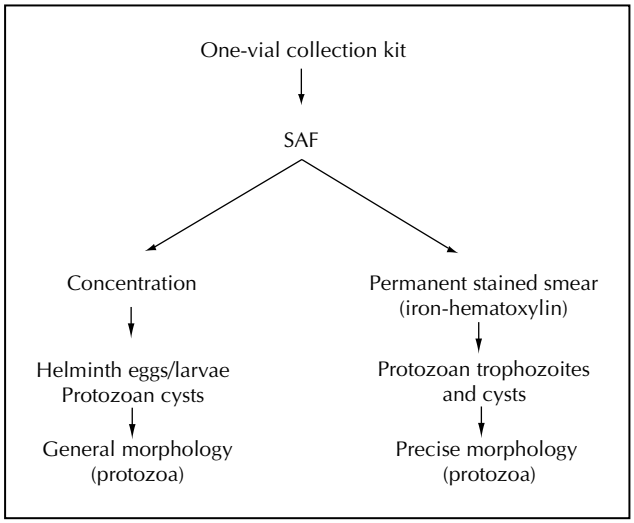


Figure 9.10.5–A4 Procedure for processing sodium acetate-acetic acid-formalin (SAF)-preserved stool for the ova and parasite examination. SAF can also be used with EIA, fluorescent-antibody, and cartridge immunoassay kits and the modified trichrome stain for microsporidia.

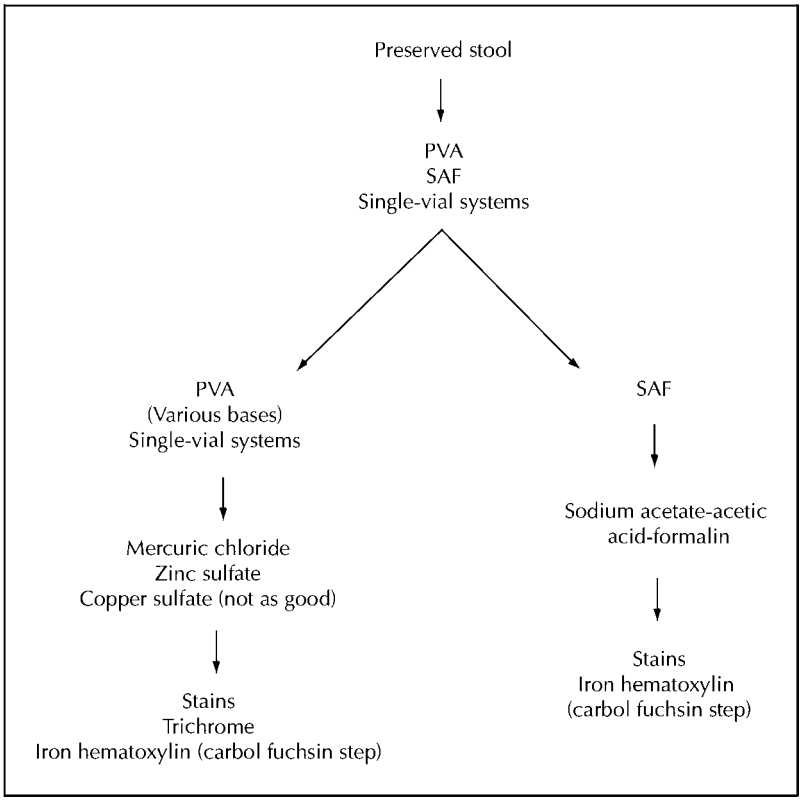


Figure 9.10.5–A5 Use of various fixatives and their recommended stains. PVA, polyvinyl alcohol; SAF, sodium acetate-acetic acid-formalin. Five or 10% formalin, SAF, and *some* of the single-vial systems can also be used with immunoassay kits and the modified trichrome stains for microsporidia.

9.10.6 Appendix 9.10.6–1—Commercial Supplies and Suppliers

See Tables 9.10.6–A1 to 9.10.6–A7 on the following pages. (Company names may not reflect recent mergers and subsequent name changes.)

Table 9.10.6–A1 Sources of commercial reagents and supplies[a]

Reagent or supply	AJP Scientific	ALPHA-TEC	Becton Dickinson	Bio-Spec	City Chemical Corp.	Evergreen Scientific	Hardy Diagnostics	Harleco
Specimen collection kit(s)								
Formalin, PVA		×		×		×	×	
PVA, modified (copper base)		×					×	
PVA, modified (zinc base)		×				×	×	
MIF		×					×	
SAF		×					×	
Pinworm paddles			×			×	×	
Universal fixative								
Preservatives (bulk)								
Schaudinn's fixative solution		× + −		× + −			× +	
PVA fixative solution	×	×		×			×	
PVA powder[b]				×				
MIF solution	×	×					×	
SAF solution		×						
Concentration systems								
Formalin-ethyl acetate		×				×	×	
Zinc sulfate (sp gr, 1.18 or 1.20)		×		×			×	
Stains								
Trichrome, solution	× (W)	× (W)		× (W)			× (W)	
Trichrome, modified[c]							×	
Trichrome, dye powders								
Chromotrope 2R								×
Fast green FCF								×
Light green SF yellowish								×
Hematoxylin, solution				×			×	
Hematoxylin, powder								×
Chlorazol black E, powder					×			
Giemsa, solution	×			×			×	×
Giemsa, powder								×
Carbol fuchsin, Kinyoun's	×	×		×			×	×
Modified acid fast with DMSO		×						
Auramine-rhodamine								
Acridine orange								
Toluidine blue O								
Polychrome IV		×						
Miscellaneous								
Lugol's iodine, diluted 1:5	×	×		×			×	×
Dobell & O'Connor's iodine		×					×	
Triton X-100		×					×	
Eosin-saline solution (1%)				×				
Mayer's albumin		×					×	
Control slides or suspensions		×					×	

[a] PVA, polyvinyl alcohol; MIF, merthiolate-iodine-formalin; SAF, sodium acetate-acetic acid-formalin; W, Wheatley; G, Gomori; DMSO, dimethyl sulfoxide; +, with acetic acid; −, without acetic acid.

[b] Use grade with high hydrolysis and low viscosity for parasite studies.

[c] Used for the identification of microsporidial spores in stool or other specimens.

Mallinkrodt Baker	Medical Chemical Corp.	Meridian Diagnostics	MML Diagnostic	PML Microbiologicals	PolySciences, Inc.	Remel	Sigma Aldrich	Rowley Biochemical	Scientific Device Laboratory	Thermo Fisher Scientific	Volu-Sol
	×	×	×	×		×					
	×	×	×	×		×					×
	×	×		×		×					
	×	×		×		×					
	×	×	×	×		×				×	
									×		
	×										
	× +	× +				× −		× −			× −
	×	×		×		×			×		×
×										×	
	×	×									
	×			×							
	×	×									
×	×								×		
	× (W)	× (W)		×		× (W)		× (W, G)	× (W)		× (W, G)
	×	×				×			×		×
×								×			
×								×		×	
×								×		×	
	×			×				×			×
×								×		×	
×					×			×			
×	×							×		×	×
×								×		×	
	×			×		×		×		×	×
				×	×						×
					(auramine-rhodamine)						
				×		×					×
	×										
	×										
	×	×		×		×		×			×
						×			×	×	
×	×	×		×		×	×		×		
	×									×	
						×					
		×		×	×	×			×		

Table 9.10.6–A2 Addresses of suppliers listed in Table 9.10.6–A1

AJP Scientific, Inc.
P.O. Box 1589
Clifton, NJ 07015
(800) 922-0223
(201) 472-7200
Products also available through various distributors

ALPHA-TEC Systems, Inc.
P.O. Box 5435
Vancouver, WA 98668
(800) 221-6058
(360) 260-2779
Fax: (360) 260-3277
http://www.alphatecsystem.com
Products also available through various distributors

Becton Dickinson
1 Becton Dr.
Franklin Lakes, NJ 07417
(800) 255-6334
http://www.bd.com

Bio-Spec, Inc.
179 Mason Circle, Suite A
Concord, CA 94520-1213
(415) 689-0771
Products also available through VWR Scientific

City Chemical Corp.
32 West 22nd St.
New York, NY 10011
(212) 929-2723
Products also available through Amfak Drug or Bergen-Brunswick

Evergreen Scientific
2300 East 49th St.
P.O. Box 58248
Los Angeles, CA 90058-0248
(800) 421-6261
(800) 372-7300 in California
(213) 583-1331
Fax: (213) 581-2503
http://www.evergreensci.com
Products also available through various distributors

Hardy Diagnostics
1430 West McCoy Ln.
Santa Maria, CA 93455
(800) 266-2222
Fax: (805) 346-2760
http://www.hardydiagnostics.com
Products also available through various distributors

Harleco (Voigt Global Distribution)
P.O. Box 412762
Kansas City, MO 64141-2762
(877) 484-3552
http://www.vgdusa.com

Mallinkrodt Baker
222 Red School Ln.
Phillipsburg, NJ 08865
(908) 859-2151
Fax: (908) 859-9318
http://www.mallbaker.com
Products also available through American Scientific Products or VWR

Medical Chemical Corp.
19430 Van Ness Ave.
Torrance, CA 90501
(800) 424-9394
Fax: (310) 787-4464
http://www.med-chem.com
Products also available through various distributors

Meridian Bioscience, Inc.
3741 River Hills Dr.
Cincinnati, OH 45244
(800) 543-1980
(513) 271-3700
Fax: (513) 271-0124
http://www.meridianbioscience.com
Products available only through Baxter MicroScan and Scientific Products Division, CMA, and various other regional distributors

MML Diagnostic Packaging
P.O. Box 458
Troutdale, OR 97060
(503) 666-8398
(800) 826-7186
Products available through various distributors

PML Microbiologicals
P.O. Box 459
Tualatin, OR 97062
(800) 547-0659
(503) 639-1500 in Oregon
Fax: (800) 765-4415
http://www.pmlmicro.com
Products also available through other distributors

Table 9.10.6–A2 *(continued)*

Polysciences, Inc.
400 Valley Rd.
Warrington, PA 18976
(800) 523-2575
(215) 343-6484
Fax: (215) 343-0214
http://www.polysciences.com
Products available only through company

Remel
12076 Santa Fe Dr.
Lenexa, KS 66215
(800) 255-6730
Fax: (800) 477-5781
http://www.remelinc.com
Products available through Rupp & Bowman and certain other distributors

Rowley Biochemical Institute
U.S. Route 1
Rowley, MA 01969
(508) 948-2067
Fax: (508) 948-2206
http://www.rowleybio.com
Products available only through company

Scientific Device Laboratory, Inc.
411 E. Jarvis Ave.
Des Plaines, IL 60018
(847) 803-9495
Fax: (847) 803-8251
http://www.scientificdevice.com
Products also available through various distributors

Thermo Fisher Scientific
81 Wyman St.
Waltham, MA 02454
(800) 678-5599
Fax: (781) 622-1207
http://www.fishersci.com

Volu-Sol, Inc. (a division of Biomune, Inc.)
5095 West 2100 South
Salt Lake City, UT 84120
(800) 821-2495
(801) 974-9474
Fax: (800) 860-4317
http://www.volusol.com
Products also available through various distributors
(Laboratory Specialists International, http://www.labspec.com)

Table 9.10.6–A3 Sources of available reagents for immunodetection of parasitic organisms or antigens[a]

Company	*Cryptosporidium* spp. EIA	*Cryptosporidium-Giardia lamblia* combination DFA or EIA	*Entamoeba histolytica* EIA	*E. histolytica/ E. dispar* group EIA	*Giardia lamblia* EIA	*Cryptosporidium-Giardia* cartridge	*Cryptosporidium-Giardia–E. histolytica/ E. dispar* group cartridge	*Trichomonas vaginalis* (various methods)
Antibodies Inc.					×			
Biosite Diagnostics, Inc.							× (Triage parasite panel)	
CeLLabs								
Chemicon International, Inc.								× (DFA)
Genzyme Diagnostics (Becton Dickinson)						×		
Integrated Diagnostics								× (latex agglutination; dual latex agglutination with *Candida*)
Inverness Medical				×	×			
Meridian Bioscience, Inc.	×	× (DFA)			×	×		×
Medical Chemical Corp.	×	× (DFA)		×	×	×		
MicroProbe Corp. (Becton Dickinson)								× (DNA probe; Affirm VP III) *(Trichomonas, Gardnerella, Candida)*
Polysciences, Inc.								
Remel	×	×		×	×	×		
TechLab, Inc.	×	× (DFA)	×	×	×			

[a] Any procedure for the *Entamoeba histolytica/E. dispar* group or *E. histolytica* requires the use of fresh or fresh, frozen stools. All other tests (with the exception of direct fluorescent antibody [DFA] for the *Cryptosporidium-Giardia* combination) can be used with fresh, frozen, or preserved stools (formalinized base, not PVA [some single-vial systems without PVA may be acceptable; consult the manufacturer]). Since the combination *Cryptosporidium-Giardia* tests (DFA) are based on visual recognition of the fluorescing oocysts and/or cysts, the specimens must not be frozen if fresh stools are used for testing.

Table 9.10.6–A4 Addresses of suppliers listed in Table 9.10.6–A3

Antibodies, Inc.
P.O. Box 1560
Davis, CA 95617-1560
(800) 824-8540
Fax: (530) 758-6307
E-mail: antiinc@aol.com

Biosite Diagnostics, Inc.
9975 Summer Ridge Rd.
San Diego, CA 92121
(858) 455-4808
Fax: (858) 455-4815
http://www.biosite.com

CeLLabs
P.O. Box 421
Brookvale, NSW, 2100
Australia
http://www.cellabs.com.au

Chemicon International, Inc.
28835 Single Oak Dr.
Temecula, CA 92590
(800) 437-7500
Fax: (800) 437-7502
E-mail: custserv@chemicon.com, tech-serv@chemicon.com
http://www.chemicon.com

Genzyme Diagnostics
500 Kendall St.
Cambridge, MA 02142
http://www.genzyme.com

Integrated Diagnostics (PanBio InDx, Inc.)
1756 Sulphur Springs Rd.
Baltimore, MD 21227
(410) 737-8500
Fax: (410) 536-1212
E-mail: indx2@erols.com

Inverness Medical
P.O. Box 1001
Cranbury, NJ 08512
(800) 257-9525
Fax: (800) 532-0295
http://www.invernessmedicalpd.com

Medical Chemical Corp.
19430 Van Ness Ave.
Torrance, CA 90501
(800) 424-9394
Fax: (310) 787-4464
http://www.med-chem.com

Meridian Bioscience, Inc.
3471 River Hills Dr.
Cincinnati, OH 45244
(513) 271-3700
Fax: (513) 271-0124
http://www.meridianbioscience.com

MicroProbe Corp.
(Becton Dickinson Affirm VPIII)
1725 220th St. NE
Bothell, WA 98021
(Trichomonas, Gardnerella, Candida)
BD (201) 847-6800
http://www.bd.com

Polysciences, Inc.
400 Valley Rd.
Warrington, PA 18976
(800) 523-2575
Fax: (215) 343-0214
http://www.polysciences.com

Remel
12076 Santa Fe Dr.
Lenexa, KS 66215
(800) 255-6730
Fax: (800) 477-5781
http://remelinc.com

TechLab, Inc.
2001 Kraft Dr.
Blacksburg, VA 24060-6358
(800) 832-4522
(540) 953-1664
Fax: (540) 953-1665
http://www.techlab.com

Table 9.10.6–A5 Commercial suppliers of diagnostic parasitology products[a]

Abbott Laboratories
Diagnostics Division
100 Abbott Park Rd.
Abbott Park, IL 60064-3500
(847) 937-6100
http://www.abbott.com
Trypanosoma cruzi (Chagas' disease) EIA, *Toxoplasma* (EIA)

ALPHA-TEC Systems, Inc.
P.O. Box 5435
Vancouver, WA 98668
(800) 221-6058
(360) 260-2779
Fax: (360) 260-3277
http://www.alphatechsystems.com
Control slides, reagents, stains (including polychrome IV), collection systems, concentration systems, QC suspensions

Amrad ICT
13 Rodborough Rd.
French Forest, NSW
2086, Australia
(612) 9453-4411
Fax: (612) 9453-4411
http://www.amrad.com.au
Antigen detection (rapid or dipstick) for filariasis, malaria, and *P. falciparum* malaria

Antibodies Inc.[c]
P.O. Box 1560
Davis, CA 95617
(800) 824-8540
(916) 758-4400 in California
Fax: (530) 758-6307
E-mail: antiinc@aol.com
Giardia (EIA)

Bayer
511 Benedict Ave.
Tarrytown, NE
(800) 242-2787
http://www.bayer.com
Toxoplasma (EIA)

Beckman Coulter
4300 N. Harbor Blvd.
Fullerton, CA 92834
(800) 233-4685
Fax: (800) 643-4366
http://www.beckman.com
Toxoplasma (EIA)

Becton Dickinson Advanced Diagnostics
2350 Qume Dr.
San Jose, CA 95131-1087
(800) 223-8226
Fax: (800) 954-2347
http://www.bdfacs.com
Malaria P.f. ParaSight F dipstick, QBC acridine orange tube

Biokit USA, Inc.
113 Hartwell Ave.
Lexington, MA 02173
(800) 926-2253
Fax: (617) 861-4065
Toxoplasma (LA)

BioMed Diagnostics
1430 Koll Circle, Suite 101
San Jose, CA 95112
(800) 964-6466
InPouch TV (visual identification and culture system for *Trichomonas vaginalis*)

bioMérieux Vitek
595 Anglum Dr.
Hazelwood, MO 63042
(800) 638-4835
http://www.biomerieux.com
Toxoplasma (EIA)

Biosite Diagnostics, Inc.
9975 Summer Ridge Rd.
San Diego, CA 92121
(858) 455-4808
Fax: (858) 455-4815
http://www.biosite.com
Triage Parasite Panel-Rapid *(Cryptosporidium, Giardia, Entamoeba histolytica/E. dispar)*

Table 9.10.6–A5 *(continued)*

Biotecx Labs
6023 S. Loop East
Houston, TX 77033
(800) 535-6286
Fax: (713) 643-3743
Toxoplasma (EIA)

Biotools
Av. General Peron
2 E-28020 Madrid, Spain
(341) 571-1660
Fax: (341) 571-1232
Malaria (PCR, DNA)

Chemicon International, Inc.
28835 Single Oak Dr.
Temecula, CA 92590
(800) 437-7500
Fax: (800) 437-7502
E-mail: custserv@chemicon.com, techserv@chemicon.com
Trichomonas vaginalis (DFA) antigen detection

Dade Behring, Inc.
20400 Mariani Ave.
Cupertino, CA 95014
(408) 239-2000
or
Dade Behring, Inc.
1375 Shore Bird Way
Mountain View, CA 94053
(650) 567-6000
http://www.dadebehring.com
Toxoplasma (EIA)

Diagnostic Products Corp.
5700 W. 96th St.
Los Angeles, CA 90045
(800) 444-5757
Fax: (800) 234-4872
http://www.dpcweb.com
Toxoplasma (EIA)

Diagnostic Technology, Inc.
240 Vanderbuilt Motor Parkway
Hauppauge, NY 11788
(516) 582-4949
Fax: (516) 582-4694
http://www.dpcweb.com
Toxoplasma (EIA, IgG, IgM)

Diamedix Corporation
2140 N. Miami Ave.
Miami, FL 33127
(800) 327-4565
Fax: (305) 324-2395
Toxoplasma (EIA, IgG, IgM), *Toxoplasma* (EIA, IgM Capture—approval pending)

DiaSorin
1990 Industrial Blvd.
P.O. Box 285
Stillwater, MN 55082
(800) 328-1482
Fax: (612) 779-7847
http://www.diasorin.com
Toxoplasma (EIA)

DiaSys Corporation
49 Leavenworth St.
Waterbury, CT 06702
http://www.diasyscorp.com
Semiautomated stool concentrate examination system

Eastman Kodak Co.
Rochester, NY 14650
(800) 225-5352
(716) 458-4014
Products available only through American Scientific Products, Fisher Scientific, VWR, and company
PVA powder, Giemsa powder

Empyrean Diagnostics, Inc.
2761 Marine Way
Mountain View, CA 94043
(415) 960-0516
Fax: (415) 960-0515
Visual identification and culture system for *Trichomonas vaginalis*

Evergreen Scientific
2300 East 49th St.
Los Angeles, CA 90058
(800) 421-6261
(213) 583-1331
Fax: (213) 581-2503
http://www.evergreensci.com
Collection system, concentration system

Flow Inc.
6127 SW. Corbett
Portland, OR 97201
(503) 246-2710
Fax: (503) 245-7666
E-mail: MIKEatFLOW@aol.com
http://www.malariatest.com
OptiMAL[b]

Genzyme Diagnostics
500 Kendall St.
Cambridge, MA 02142
ColorPac, immunochromatographic rapid immunoassay for *Cryptosporidium-Giardia*

(continued)

Table 9.10.6–A5 Commercial suppliers of diagnostic parasitology products[a] *(continued)*

Gull Laboratories, Inc.
1011 East Murray Holladay Rd.
Salt Lake, City, UT 84117
(800) 448-4855
Fax: (801) 265-9268
http://www.gullabs.com
Toxoplasma (EIA, IFA), *Trypanosoma cruzi* (EIA), formalin suspensions (parasites), parasite slides (stained and unstained)

Hardy Diagnostics
1430 West McCoy Ln.
Santa Maria, CA 93455
(800) 266-2222
Fax: (805) 346-2760
http://www.hardydiagnostics.com
Stains, reagents, collection system, concentration systems, control slides

HDC Corp.
2109 O'Toole Ave., Suite M
San Jose, CA 95131
(408) 954-1909
Fax: (408) 954-0340
http://www.hdccorp.com
Entero-Test capsules (adult and pediatric) (method of sampling upper gastrointestinal tract)

Hemagen Diagnostics
34-40 Bear Hill Rd.
Waltham, MA 02154
(800) 436-2436
Fax: (781) 890-3748
http://www.hemagen.com
Chagas' disease (EIA), *Toxoplasma* (EIA, IFA)

Immunetics
63 Rogers St.
Cambridge, MA 02142
(617) 492-5416
Fax: (617) 868-7879
http://www.immunetics.com
Babesia (IB),[b] Chagas' disease (IB),[b] cysticercosis, (IB),[b] echinococcosis (IB),[b] *Leishmania* (IB)[b]

INOVA
10180 Scripps Ranch Rd.
San Diego, CA 92131
(800) 545-9495
Fax: (619) 586-9911
http://www.inovadx.com
Toxoplasma (EIA)

Integrated Diagnostics Inc.
(PanBio InDx, Inc.)
1756 Sulphur Springs Rd.
Baltimore, MD 21227
(410) 737-8500
Fax: (410) 536-1212
E-mail: indx2@erols.com
http://www.indxdi.com
Trichomonas (LA) antigen detection

Interfacial Dynamics Corp.
17300 SW Upper Boones Ferry Rd., Suite 120
Portland, OR 97224
(503) 256-0076
(800) 323-4810
Fax: (503) 255-0989
E-mail: idclatex@teleport.com
Uniform-sized polystyrene microspheres (can be used to check microscope calibrations)

Intracel
2005 NW Sammamish Rd., Suite 107
Issaquah, WA 98027
(800) 227-8357
E-mail: info@intracel.com
http://www.intracel.com
Toxoplasma (EIA)

IVD Research Inc.
5909 Sea Lion Place, Suite D
Carlsbad, CA 92008
(760) 929-7744
Fax: (760) 431-7759
http://www.ivdresearch.com, http://www.safepath.com
(SafePath offers numerous assays for veterinary, environmental, and food safety applications)
Amebiasis (EIA), cysticercosis (EIA), echinococcosis (EIA), *Toxocara* (EIA), trichinosis (EIA), *Toxoplasma* (EIA), additional antigen and antibody reagents (microbiology and parasitology)

KMI Diagnostics Inc.
818 51st Ave., NE, Suite 101
Minneapolis, MN 55421
(612) 572-9354
Fax: (612) 586-0748
http://www.kmidiagnostics.com
Toxoplasma (IFA)

Table 9.10.6–A5 *(continued)*

Medical Chemical Corp.
19430 Van Ness Ave.
Torrance, CA 90501
(800) 424-9394
Fax: (310) 787-4464
http://www.med-chem.com
Reagents, modified trichrome stain for microsporidia, other stains, collection system (including Universal Fixative), concentration system, *Giardia, Cryptosporidium* immunoassays (EIA, DFA, rapid cartridge), parasitology website (current information, photographs, commonly asked questions, etc.)

Meridian Bioscience, Inc.
3741 River Hills Dr.
Cincinnati, OH 45244
(800) 543-1980
(513) 271-3700
Fax: (513) 271-0124
http://www.meridianbioscience.com
Stains, reagents, collection system, concentration system, *Cryptosporidium* spp. (EIA, FA), *Giardia* sp. (EIA, FA), *Cryptosporidium/Giardia* rapid cartridge

MicroProbe Corporation
(Becton Dickinson Affirm VPIII)
1725 220th St. NE
Bothell, WA 98021
(201) 847-6800
http://www.bertec.com.tw
(*Trichomonas, Gardnerella, Candida*)
ColorPAC Rapid (*Cryptosporidium*/*Giardia*)

MML Diagnostic Packaging
P.O. Box 458
Troutdale, OR 97060
(503) 666-8398
(800) 826-7186
Collection system

PML Microbiologicals
P.O. Box 459
Tualatin, OR 97062
(800) 547-0659
(503) 639-1500 in Oregon
Fax: (800) 765-4415
http://www.pmlmicro.com
Stains, reagents, collection system, concentration system, control slides

Polysciences, Inc.
400 Valley Rd.
Warrington, PA 18976
(800) 523-2575
Fax: (215) 343-0214
http://www.polysciences.com
Pneumocystis (DFA) antigen detection

Remel
12076 Santa Fe Dr.
Lenexa, KS 66215
(800) 255-6730
Fax: (800) 477-5781
http://www.remelinc.com
Stains, reagents, collection system, concentration system, control slides

Sanofi Diagnostics Pasteur
1000 Lake Hazeltine Dr.
Chaska, MN 55318
(800) 666-5111
Fax: (612) 368-1110
Toxoplasma (EIA, IgG, IgM)
Pneumocystis (IFA)

Scientific Device Laboratory, Inc.
411 E. Jarvis Ave.
Des Plaines, IL 60018
(847) 803-9495
Fax: (847) 803-8251
http://www.scientificdevice.com
Stains, stain kit, modified trichrome stain for microsporidia, fixative (formalin free), formalin-fixed protozoa and helminth eggs and larvae, control slides (*Cryptosporidium, Isospora*, microsporidia, and *Pneumocystis*), stained slides

Shield Diagnostics
The Technology Park
Dundee DD2 ISW, United Kingdom
44-1382-561000
Fax: 44-1382-561056
Pneumocystis (DFA) antigen detection, *Cryptosporidium* (DFA)[b]

Sigma
545 South Ewing Ave.
St. Louis, MO 63013
(800) 325-3424
Fax: (314) 286-7813
http://www.sigma.sial.com
Amebiasis (EIA), *Toxoplasma* (EIA)

Stellar Bio Systems
9075 Guilford Rd.
Columbia, MD 21046
(301) 381-8550
Toxoplasma (IFA)

(continued)

Table 9.10.6–A5 Commercial suppliers of diagnostic parasitology products[a] *(continued)*

TechLab, Inc.
1861 Pratt Dr., Suite 1030
Corporate Research Center
Blacksburg, VA 24060-6364
(800) 832-4522
Fax: (540) 231-3942
E-mail: techlab@techlabinc.com
http://www.techlabinc.com
EIA antigen detection (amebiasis, *Entamoeba histolytica/E. dispar* group, *Entamoeba histolytica, Cryptosporidium, Cryptosporidium* [DFA], *Giardia*)

Volu-Sol, Inc. (a division of Biomune, Inc.)
5095 West 2100 South
Salt Lake City, UT 84120
(800) 821-2495
(801) 974-9474
Fax: (800) 860-4317
http://www.labspec.com
Parasitology starter kit, stains, reagents, control slides

VWR Scientific, Inc.
P.O. Box 7900
San Francisco, CA 94120
(415) 468-7150 in Northern California
(213) 921-0821 in Southern California
Rest of United States, use either number
Stains, reagents, collection system

Wampole Lab
P.O. Box 1001
Cranbury, NJ 08512
(800) 257-9525
Fax: (800) 532-0295
http://www.wampolelabs.com
EIA antigen detection (amebiasis, *Entamoeba histolytica*/*E. dispar* group, *Giardia*)

[a] Much of the updated immunology testing information provided by Marianna Wilson (CDC). Abbreviations: DFA, direct fluorescent antibody; IB, immunoblot; IFA, indirect fluorescent antibody; IgG and IgM, immunoglobulins G and M; LA, latex agglutination; Rapid, rapid immunochromatographic. Company names may not reflect recent mergers and subsequent name changes.

[b] Not Food and Drug Administration cleared for in vitro diagnostic use.

[c] No updated information received since publication of the previous edition of this book.

Table 9.10.6–A6 Sources of parasitologic specimens (catalogs of available materials and price lists available from the companies and person listed)

Ann Arbor Biological Center
6780 Jackson Rd.
Ann Arbor, MI 48103

Carolina Biological Supply Co.
2700 York Rd.
Burlington, NC 27215
(800) 334-5551
(919) 584-0381
Fax: (919) 584-3399

Meridian Bioscience, Inc.
3471 River Hills Dr.
Cincinnati, OH 45244
(800) 543-1980, Ext. 335
Fax: (513) 272-5271
http://www.meridianbioscience.com

Scientific Device Laboratory, Inc.
(800) 448-4855
411 E. Jarvis Ave.
Des Plaines, IL 60018
(708) 803-9495
Fax: (708) 803-8251

Triarch, Inc.
N8028 Union St.
P.O. Box 98
Ripon, WI 54971
(414) 748-5125
(800) 848-0810
Fax: (414) 748-3034

Tropical Biologicals
P.O. Box 139
Guaynabo, PR 00657

TURTOX
P.O. Box 92912
Rochester, NY 14692
(800) 826-6164

Ward's Natural Science Establishment, Inc.
P.O. Box 92912
Rochester, NY 14692
(800) 962-2660

Table 9.10.6–A7 Sources of Kodachrome slides (35 mm, 2 by 2 in.)

Armed Forces Institute of Pathology
Dept. ID/Parasitic Disease Pathology
6825 16th St., NW
Bldg. 54, Room 4015
Washington, DC 20306-6000

American Society of Clinical Pathologists
ASCP Press
2100 West Harrison St.
Chicago, IL 60612-3798
(312) 738-4890
Human Parasitology, teaching slide set (Ash and Orihel, 1990)
Supplement to Human Parasitology, teaching slide set (in preparation)
Parasites in Human Tissues, teaching slide set (Orihel and Ash, 1996)

Clinical Diagnostic Parasitology
Visual Teaching Aids (set of 100 2- by 2-in. slides)
Diagnostic Protozoa, Helminths, and Blood Parasites
(Lynne S. Garcia)
512 12th St.
Santa Monica, CA 90402

W. H. Curtin and Co.
P.O. Box 1546
Houston, TX 77001

TURTOX
P.O. Box 92912
Rochester, NY 14692

Dr. Herman Zaiman
(A Pictorial Presentation of Parasites)
P.O. Box 543
Valley City, ND 58072

9.10.7

Appendix 9.10.7–1—Current OSHA Regulations on the Use of Formaldehyde

OSHA requires all workers to be protected from dangerous levels of vapors and dust. Formaldehyde vapor is the air contaminant most likely to exceed the regulatory threshold in the clinical laboratory. Current OSHA regulations require vapor levels not to exceed 0.75 ppm (measured as a time-weighted average [TWA]) and 2.0 ppm (measured as a 15-min [short-term] exposure). *OSHA requires monitoring for formaldehyde vapor wherever formaldehyde is used in the workplace. The laboratory must have evidence at the time of inspection that formaldehyde vapor levels have been measured, and both 8-h and 15-min exposures must have been documented (1, 3, 4).*

If each measurement is below the permissible exposure limit and the 8-h measurement is below 0.5 ppm, no further monitoring is required providing the laboratory procedures remain constant. Changes in the laboratory that would require repeat monitoring include the following: large increase in the overall number of specimens processed each day (for example, from 20 to >100), change in location of reagents from inside a fume hood to outside the fume hood (on the open laboratory bench), or introduction of new procedures that require the use of formalin. If either the 0.5-ppm 8-h TWA or the 2.0-ppm 15-min level is exceeded, monitoring must be repeated every 6 months. If either the 0.75-ppm 8-h TWA or the 2.0-ppm 15-min level is exceeded (unlikely in a clinical laboratory setting), employees are required to wear respirators. Accidental skin contact with aqueous formaldehyde must be prevented by the use of proper clothing and equipment (gloves, laboratory coats).

The amendments of 1992 add medical removal protection provisions to supplement the existing medical surveillance requirements for employees suffering significant eye, nose, or throat irritation and for those experiencing dermal irritation or sensitization from occupational exposure to formaldehyde. In addition, these amendments establish specific hazard labeling requirements for all forms of formaldehyde, including mixtures and solutions composed of at least 0.1% formaldehyde in excess of 0.1 ppm. Additional hazard labeling, including a warning label that formaldehyde presents a potential cancer hazard, is required where formaldehyde levels, under reasonably foreseeable conditions of use, may exceed 0.5 ppm. The final amendments also provide for annual training of all employees exposed to formaldehyde at levels of 0.1 ppm or higher.

◩ **NOTE:** Monitoring badges are not sensitive enough to correctly measure the 15-min exposure level. Contact the occupational health and safety office within your institution for monitoring options. Usually, the accepted method involves monitoring airflow in the specific area(s) within the laboratory where formaldehyde vapors are found.

Chemical Hygiene Plan

OSHA also requires each laboratory to develop a comprehensive, written chemical hygiene plan (CHP). Regardless of type of risk, volume, or concentration, every hazardous chemical in the laboratory must be included in the CHP. The plan should include storage requirements, handling procedures, location of OSHA-approved material, safety data sheets, and the medical procedures that are to be followed should exposure occur. The CHP must specify the clinical signs and symptoms of the environmental conditions (such as a spill) that would give the employer reason to believe exposure had occurred. When such conditions exist, the CHP should indicate the appropriate medical attention required (2).

REFERENCES

The following regulations appeared in the *Federal Register* on Friday, 4 December 1987 (vol. 52, no. 233: Occupational Exposure to Formaldehyde).

1. *Code of Federal Regulations* (29CFR1910. 1200 and 29CFR1910.1926)
2. *Code of Federal Regulations* (29CFR1910. 1450)
3. *Code of Federal Regulations* (29CFR1910. 1048) Update 27 May 1992.
4. **Garcia, L. S.** 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, DC.

9.10.8

Appendix 9.10.8–1—CPT Codes (Parasitology)

Current Procedural Terminology (CPT) comprises a list of descriptive terms and identifying codes for reporting medical services and procedures performed by physicians/laboratorians. The CPT list is published by the American Medical Association (AMA) (website, http://www.ama-assn.org/cpt). The most recent version is CPT 2003. The purposes of the CPT codes are to (i) provide a uniform language that will accurately describe medical, surgical, and diagnostic services and (ii) provide an effective means for reliable nationwide communication among physicians, patients, and third parties. The CPT list is the most widely accepted nomenclature for the reporting of physician/laboratory procedures and services under government and private health insurance programs.

CPT comparisons are used for claims processing and the development of guidelines for medical care review. They also provide a useful basis for local, regional, and national utilization comparisons. The CPT code format is a five-digit code; one of the six sections is Pathology and Laboratory Medicine.

The following information should allow you to code the diagnostic parasitology procedures correctly for billing purposes. In addition, if a procedure requires the use of multiple codes, you should have this information in written form within your protocol manuals, preferably the appendix or another separate section so changes can be easily made without requiring changes in every individual protocol.

CPT codes for diagnostic parasitology

Procedure name	New CPT[a]	Comments
Agar plate culture (*Strongyloides*)	87081 + 87210	Includes culture and wet mount examination
Baermann concentration (plus examination)	87015 + 87210	Includes concentration and wet mount examination
Cyclospora examination (modified AFB[b])	87207	Modified acid-fast stain
Cryptosporidium examination (modified AFB)	87207	Modified acid-fast stain
Cryptosporidium examination (FA)	87015 + 87272	Immunoassay, includes concentration
Cryptosporidium examination (EIA)	87328	Immunoassay
Cryptosporidium/Giardia examination (FA)	87015 + 87272 + 87269	Immunoassay, includes concentration
Cryptosporidium/Giardia examination (EIA)	87328 (negative) 87328 (positive) + 1 more 87328 and 1 87329	Screen that detects both *Giardia* and *Cryptosporidium* but does not differentiate between the two. If positive (and individual antigens are subsequently tested), then a total of 2 87328 and 87329 procedures is appropriate; if negative, then a single 87328.
Cryptosporidium/Giardia examination (cartridge)	87328	Possibility of two answers; can bill for both; immunoassay (immunochromatographic format)
Cryptosporidium/Giardia/Entamoeba histolytica-E. dispar group examination (cartridge)	87328	Possibility of three answers, but only one test (limit billing to once); immunoassay (EIA immunoassay format)
Ectoparasite identification (macroscopic) (arthropod)	87168	Macroscopic exam
Ectoparasite identification (wet mount) (arthropod)	87210	Wet mount examination
Ectoparasite identification (skin, scabies)	87220	
Fat, stool (qualitative)	82705	
Giardia examination (FA)	87015 + 87269	Immunoassay, includes concentration
Giardia examination (EIA)	87329	Immunoassay

CPT codes for diagnostic parasitology *(continued)*

Procedure name	New CPT[a]	Comments
Harada-Mori concentration (plus examination)	87015 + 87210	Includes concentration and wet mount examination
Helminth egg hatching (concentration, wet mounts)	87177	Includes concentration and wet mount examination
Cystoisospora (*Isospora*) examination (modified AFB only)	87207	Without concentration. 87015 + 87207 would be an option if this is an orderable test, which includes concentration and modified AFB stain.
Microsporidia, special stain (modified trichrome)	87015 + 87207	Modified trichrome on concentration sediment; includes concentration and modified trichrome stain
Microsporidia, fluorescent stain (calcofluor) (on concentration sediment)	87015 + 87206	Includes concentration and fluorescent stain (optical brightening agent) on sediment
Occult blood, stool	82270	
Occult blood, gastric	82271	
Ova and parasite examination	87177	Includes direct wet mount, concentration, and concentrate wet mount examinations. This *does not* include the permanent stain.
Ova and parasite examination (permanent stain) (example: trichrome)	87209	Permanent stain only
Ova and parasite examination (routine ova and parasite exam) (performed on *fresh stool*, includes direct wet mount)	87177 + 87209	Includes direct wet mount, concentration, concentrate wet mount, and permanent stained smear examinations (*Note:* There is an error in the code book under 87177 where 88312 is specified for trichrome—it should read 88313); CPT 2003 now states that 88313 should be used.
Ova and parasite examination (routine ova and parasite exam) (performed on *preserved stool*, no direct wet mount)	87015, 87210, 87209	Includes concentration, concentrate wet mount, and permanent stained smear examinations
Parasite concentration, iodine prep (wet prep only)	87015 + 87210	Includes concentration and wet mount examination (iodine mount)
Parasite concentration, saline prep 1 (wet prep only)	87015 + 87210	Includes concentration and wet mount examination (saline mount)
Saline prep 2 (wet prep only)	87210	Multiple wet mounts may have to be examined in certain circumstances: duodenal aspirates, string test mucus, urine concentrations, etc.
Saline prep 3 (wet prep only)	87210	
Saline prep 4 (wet prep only)	87210	
Saline prep 5 (wet prep only)	87210	
Parasite examination, wet prep only	87210	Wet mount examination
Parasite examination, duodenum (concentration, wet mounts)	87177	Includes direct wet mount, concentration, and concentrate wet mount examinations. This *does not* include any type of permanent stain.
Parasite examination, duodenum (permanent stain)	87209	Trichrome stain
Parasite examination, Entero-Test (wet mounts only)	87210	Entero-Test (string test, "fishing" test, capsule test): sampling of duodenal contents
Parasite examination, Entero-Test (permanent stain only)	87209	Trichrome
Parasite examination, urine (random) (concentration, wet mounts)	87015 + 87210	Includes concentration and wet mount examination; may include multiple wet mounts prepared/examined—use additional 87210 with modifier
Parasite examination, 24-h urine (concentration, wet mounts)	87015 + 87210	May be multiple wet mounts prepared/examined—use additional 87210 with modifier
Parasite concentration, blood (concentration, wet mounts)	87015 + 87210	May be multiple wet mounts prepared/examined—use additional 87210 with modifier (will include Knott and membrane concentrations using Nuclepore filters)
Parasite, calcofluor stain (direct material, no concentration)	87206	Optical brightening agent; fluorescence
Parasite concentration (miscellaneous)	87015	Concentration only
Giemsa stain, thin blood film	87207	Giemsa stain
Giemsa stain, thick blood film	87015 + 87207	Includes concentration and Giemsa stain

(continued)

CPT codes for diagnostic parasitology *(continued)*

Procedure name	New CPT[a]	Comments
Parasite culture (intestinal and blood protozoa)	87081 + 87015 + 87210 + 87207 (Giemsa) or 88313 (trichrome)	Includes culture, concentration, wet mount, permanent stain of culture sediment (intestinal and blood protozoa)
Parasite culture, presumptive, screening only (*Trichomonas*)	87081	(Pouch) (screen) (microscope exam through plastic pouch)
Parasite culture, presumptive, screening only (*Acanthamoeba*)	87081 + 87210 + 87205 (Giemsa)	Includes culture (agar plates/bacterial overlay), wet mount, permanent stain (for the trichrome stain, use 88313, not 87205)
Parasite detection (*E. histolytica*/*E. dispar* group EIA)	87336	Immunoassay for the *E. histolytica/E. dispar* group; does not differentiate the true pathogen, *E. histolytica;* immunoassay
Parasite detection (*E. histolytica* EIA)	87337	Should read *E. histolytica;* specific for the true pathogen, *E. histolytica;* immunoassay
Parasite detection (*Cryptosporidium/Giardia* EIA)	87328 (negative) 87328 (positive) + 2 more (87328)	Immunoassay that detects both *Giardia* and *Cryptosporidium*, but does not differentiate between the two. If positive, then a total of 387328 procedures is appropriate; if negative, then a single 87328.
Parasite detection, EIA (*Cryptosporidium* only)	87328	Immunoassay
Parasite detection, EIA (*Giardia* only)	87329	Immunoassay
Parasite examination, blood (thin film)	87207	Giemsa stain
Parasite examination, blood (thick film)	87015 + 87207	Includes concentration and Giemsa stain
Parasite referral in (stool for ova and parasite exam)	87177 + 87209	Includes direct wet mount, concentration, concentrate sediment, and permanent stained smear examinations
Petri dish culture/concentration (plus examination)	87015 + 87210	Includes concentration and wet mount examination
pH, stool	83986	pH, body fluid, except blood (probably the best option), previously approved by consultants
Pinworm examination	87172	Single cellophane tape/paddle/other device examination
Plasmodium rapid test—immunochromatographic	87899[c]	Infectious agent antigen detection by immunoassay with direct optical observation; not otherwise specified
Reducing substances, stool (pH)	83986	
Tissue homogenization for culture	87176	Since this may not have been specifically ordered by physician, may not be billable unless part of written routine algorithm approved by medical staff and pathology for handling tissues
Trichomonas vaginalis antigen detection with direct optical observation	87808	
Trypsin activity, stool	84488	
Worm identification (macroscopic)	87169	Example: *Ascaris* adult worm
Worm identification (wet mount)	87210	Example: *Enterobius* adult worm; small structures that resemble worms

[a] Each time a procedure requires multiple codes for billing, this information should be included in your protocol manuals, possibly in a separate section within the appendix (specifying that the procedure includes multiple coded procedures, thus the need for multiple codes; define procedure and each code required [could be in table format]). This information is similar to that required for the use of laboratory algorithm testing, in which multiple steps/procedures are required to complete a more comprehensive test or series of tests.

[b] AFB, acid-fast bacilli.

[c] Use nonspecific code due to the fact that no analyte is listed.

REFERENCE

1. **American Medical Association.** 2009. *Current Procedural Terminology (CPT) 2009.* AMA Press, Chicago, IL.

THIRD EDITION

Clinical Microbiology Procedures Handbook

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Clinical Microbiology Procedures Handbook

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(Deceased)

ASM PRESS

Washington, DC

VOLUME 3

Address editorial correspondence to ASM Press, 1752 N St., N.W., Washington, DC 20036-2904, USA

Send orders to ASM Press, P.O. Box 605, Herndon, VA 20172, USA
Phone: 800-546-2416; 703-661-1593
Fax: 703-661-1501
E-mail: books@asmusa.org
Online: http://estore.asm.org

Copyright © 2010 ASM Press
American Society for Microbiology
1752 N St., N.W.
Washington, DC 20036-2904

Library of Congress Cataloging-in-Publication Data

Clinical microbiology procedures handbook.—3rd ed. / editor in chief, third edition and 2007 update, Lynne S. Garcia.
p. ; cm.
"Editor in chief, original and second editions Henry D. Isenberg."
Includes bibliographical references and index.
ISBN-13: 978-1-55581-527-1
ISBN-10: 1-55581-527-8
1. Diagnostic microbiology—Laboratory manuals. I. Garcia, Lynne Shore. II. Isenberg, Henry D.
[DNLM: 1. Microbiological Techniques—methods—Laboratory Manuals. WQ 25 C6415 2010]
QR67.C555 2010
616.9′041—dc22

2010014355

10 9 8 7 6 5 4 3 2 1

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Printed in the United States of America

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10.1 Laboratory Diagnosis of Viral Infections: Introduction

The virology laboratory uses several diagnostic modalities, including culture, antigen and nucleic acid detection assays, cytohistopathology, and serologic methods, to aid the physician in the diagnosis of viral infections. The method of choice is influenced by several variables, including the nature of the suspected virus, the availability of test reagents, and the intended purpose of the assay (e.g., detecting active infection, assessing response to therapy). Since no single test modality can satisfy all needs, the laboratory scientist must carefully assess factors such as the patient population and setting as well as the needs and resources of the facility. Furthermore, knowledge of the natural history and pathogenesis of viral infections is essential for the optimal implementation of assays and the interpretation of results.

Maintaining active communication with other laboratories serves to enhance identification of cell culture and reagent problems and is useful in identifying sources of reference materials and training opportunities. In some cases, arrangements may be made whereby laboratories may perform the initial steps of viral culture and forward isolates to other laboratories, including state health departments, for identification. This is useful in that specimens are not compromised by storage and transport prior to inoculation and culture can be provided in settings where it remains useful but in which resources are limited.

Information provided in this section is useful not only for a full-service virology laboratory but also for those sites offering a limited test menu or dispatching specimens to off-site or reference laboratories. Human viral infections are summarized in Table 10.1–1, while zoonotic viral infections are summarized in Table 10.1–2.

Table 10.1–1 Clinical manifestations of human viral infections[a]

Virus	Respiratory	CNS and neuromuscular	Genitourinary tract	Mucocutaneous	Gastrointestinal	Ocular	Other/comments
Adenoviruses (51 types)	URI, pharyngitis, tonsillitis, pertussis syndrome, acute respiratory disease, bronchitis, pneumonia	Meningitis, encephalitis	Acute hemorrhagic cystitis, urethritis, cervicitis, genital ulcerative lesions	Exanthems, oral ulcerative lesions	Gastroenteritis	Conjunctivitis, pharyngoconjunctival fever, follicular conjunctivitis, epidemic keratoconjunctivitis, acute hemorrhagic conjunctivitis	Myocarditis; disseminated disease in immunocompromised individuals (10)
Astroviruses					Gastroenteritis		
Caliciviruses (*Norovirus, Sapovirus*)					Gastroenteritis		
Coronaviruses (12b)							
229E, OC43	URI, common cold, pneumonia				Gastroenteritis		
NL63 (9a, 19b), HK-U1 (21b)	URI, pneumonia						
SARS-CoV	SARS						
Enteroviruses[b]							
Coxsackie group A viruses	Common cold, pharyngitis, pneumonitis in infants	Aseptic meningitis, acute rhabdomyelosis, paralysis (uncommon)		Herpangina; lymphonodular pharyngitis; gingivostomatitis; hand-foot-and-mouth disease; exanthems (vesicular, petechial, maculopapular)	Infantile diarrhea, hepatitis	Acute hemorrhagic conjunctivitis	Undifferentiated febrile illness; disseminated disease in neonates and persons with agammaglobulinemia
Coxsackie group B viruses	URI, pneumonia	Meningoencephalitis, paralysis (uncommon)		Exanthems (vesicular, petechial, maculopapular, hemangioma-like)			Undifferentiated febrile illness; disseminated disease in neonates and persons with agammaglobulinemia
Echoviruses	URI	Meningitis, encephalitis, paralysis, acute rhabdomyelosis		Hand-foot-and-mouth disease		Acute hemorrhagic conjunctivitis	
Enterovirus type 68		Pneumonia, bronchiolitis					
Enterovirus type 70		Paralytic myelitis				Acute hemorrhagic conjunctivitis	
Enterovirus type 71		Meningitis, meningoencephalitis, paralysis		Hand-foot-and-mouth disease			
Polioviruses types 1–3		Meningitis, poliomyelitis, paralysis					Late-onset muscular atrophy and pain in patients previously stricken with paralytic disease (8a, 12a)

Hepatitis viruses							
HAV, HEV					Hepatitis		Fecal-oral transmission HAV previously classified as human enterovirus type 72 HEV associated with high mortality in pregnant women (11a, 21a)
HBV, HCV, HDV							Parenteral transmission via exposure to infected blood or body fluids HBV and HCV associated with hepatocellular carcinoma HDV infection occurs only by acquisition with HBV or superimposed on HBV infection. Coinfection by HBV and HDV may result in a more aggressive and fulminant disease.
Herpesviruses							
Herpes simplex virus type 1 (HHV-1) and type 2 (HHV-2)	Pharyngitis	Meningoencephalitis, encephalitis	Anogenital vesicular/ulcerative lesions, cervicitis, proctitis	Vesicular and ulcerative lesions, herpetic whitlow, gingivostomatitis, erythema multiforme	Esophagitis, hepatitis	Conjunctivitis, keratitis	Neonatal disease Genital disease and meningitis more frequently associated with type 1 rather than type 2, which is associated more frequently with oral/ocular infection and encephalitis
Varicella-zoster virus (HHV-3)	Pneumonia	Acute cerebellar ataxia, meningitis, encephalitis		Chickenpox, zoster (shingles), postherpetic neuralgia	Hepatitis		Reye's syndrome; neonatal disease may be severe; congenital disease is rare; in immuncompromised individuals, visceral disease, retinitis, keratoconjunctivitis, chorioretinitis, myelitis
Epstein-Barr virus (HHV-4)	Pharyngitis, tonsillitis				Hepatitis		Infectious mononucleosis; AIDS-associated CNS lymphoma; posttransplant lymphoproliferative disease; associated with Burkitt's lymphoma and nasopharyngeal carcinoma
CMV (HHV-5)	Neonatal pneumonitis				Hepatitis		Heterophile-negative mononucleosis; congenital CMV syndrome (long-term sequelae may occur in children who were asymptomatic at birth); opportunistic disease (pneumonia, retinitis, encephalitis, myelitis, polyradiculomyelopathy, gastritis, esophagitis, colitis, papillary stenosis, cholangitis, proctitis, adrenalitis); bone marrow graft suppression, solid-organ graft rejection

(continued)

Table 10.1–1 Clinical manifestations of human viral infections[a] *(continued)*

Virus	Respiratory	CNS and neuromuscular	Genitourinary tract	Mucocutaneous	Gastrointestinal	Ocular	Other/comments
Herpesviruses (cont.)							
HHV-6		Meningitis, meningoencephalitis in young children (12, 15)		Exanthem subitum (roseola infantum, sixth disease)			Febrile illness; opportunistic infection, particularly in bone marrow and organ transplant recipients (8, 18) and HIV-infected individuals (17); may precipitate CMV reactivation (9c)
HHV-7				Exanthem subitum (occasional) (22)			
HHV-8							Associated with Kaposi's sarcoma (7, 11, 16), multicentric Castleman's disease (19), and body cavity-based lymphomas (6)
Human papillomaviruses (>100 types)	Laryngeal papillomatosis		Condylomata, anogenital intraepithelial neoplasia (14)	Common warts, plantar warts, epidermodysplasia verruciformis			More severe or progressive in immunocompromised individuals; some types associated with cervical carcinoma (20)
Influenza virus types A, B, and C	Influenza, pneumonia, otitis media, sinusitis	Encephalopathy (infants and young children), Guillain-Barré syndrome					Carditis, Reye's syndrome Influenza virus type C has not been associated with significant endemic or epidemic disease.
Paramyxoviruses							
Human metapneumovirus (9b, 19a)	Bronchiolitis and pneumonitis, primarily in young children						
Mumps virus		Meningoencephalitis					Parotitis, orchitis, oophoritis, pancreatitis
Parainfluenza virus types 1–4	Laryngotracheobronchitis (croup), bronchiolitis, pneumonia	Meningitis (rare)					
Respiratory syncytial virus	URI, bronchiolitis, pneumonia, croup						Major cause of severe respiratory disease in infants and young children; severe disease may occur in elderly or immunocompromised individuals and those with underlying cardiopulmonary disease (9)

Rubeola (measles virus)	Pneumonia	Encephalitis, subacute sclerosing panencephalitis		Measles	Diarrhea		Otitis media; giant cell pneumonia (in absence of rash) in immunocompromised individuals
Parvoviruses							
B19		Arthralgia		Erythema infectiosum (fifth disease)			Aplastic crisis in sickle cell patients; chronic anemia; congenital disease (fetal hydrops)
Bocavirus (HBoV)	Common cold, bronchiolitis, croup, pneumonia (1)						
Polyoma viruses							BK and JC viruses are associated with latency and reactivation.
BK virus			Tubulointerstitial nephropathy; ureteric stenosis; hemorrhagic and nonhemorrhagic cystitis in renal transplant recipients				
JC virus			Progressive multifocal leukoencephalopathy in immunocompromised individuals				
Poxviruses							
Molluscum contagiosum				Molluscum		Lesions on the eyelid may result in chronic follicular conjunctivitis; lesions may also occur on the cornea and retina.	May be progressive or disseminated in individuals with AIDS
Vaccinia (cowpox)		Postvaccinal encephalitis		Vesicular/pustular exanthem; eczema vaccinatum			May become progressive or disseminated
Variola				Smallpox			
Reovirus types 1–3	URI, pharyngitis			Maculopapular exanthems	Gastroenteritis		Association with disease is not well documented; rarely isolated from clinical specimens

(continued)

Table 10.1–1 Clinical manifestations of human viral infections[a] *(continued)*

Virus	Respiratory	CNS and neuromuscular	Genitourinary tract	Mucocutaneous	Gastrointestinal	Ocular	Other/comments
Retroviruses							
HIV-1, HIV-2		Encephalomyopathy and dementia					Acute retroviral syndrome, cell-mediated immunity dysfunction, AIDS
HTLV-1, HTLV-2		HTLV-associated myopathy (HAM), tropical spastic paraparesis				Uveitis (HTLV-1)	Adult T-cell leukemia (HTLV-1)
Rhinoviruses (>100 types)	URI; lower respiratory tract disease may occur with predisposing conditions						A rhinovirus-like picornavirus has recently been identified in association with severe lower respiratory tract disease (14a)
Rotaviruses					Gastroenteritis, primarily among infants		Infection may be chronic in immunocompromised individuals
Rubella virus		Arthralgia, polyarthralgia (primarily in females)		German measles			Congenital rubella syndrome

[a] Zoonotic viral diseases not typically associated with human-to-human transmission are included in Table 10.1–2. Abbreviations: CNS, central nervous system; URI, upper respiratory infection; SARS, severe acute respiratory syndrome; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis D virus; HEV, hepatitis E virus; CMV, cytomegalovirus; HHV, human herpesvirus; HIV, human immunodeficiency virus; HTLV, human T-cell lymphotropic virus.

[b] Members of the genus *Enterovirus* are now classified among five species, including polioviruses and human enteroviruses (HEV) A, B, C, and D. HEV-A includes coxsackieviruses A2 to A8, A10, A12, A14, and A16 and enterovirus 71. HEV-B includes coxsackieviruses A9 and B1 to B6; echoviruses 1 to 7, 9, 11 to 21, 24 to 27, and 29 to 33; and enteroviruses 69 and 73. HEV-C includes coxsackieviruses A1, A11, A13, A15, A17 to A22, and A24. HEV-D includes enteroviruses 68 and 70. Coxsackievirus A23 has been shown to be echovirus type 9, echovirus type 10 has been reclassified as a reovirus, echovirus type 28 has been reclassified as rhinovirus type 1, echovirus type 34 is related to coxsackievirus A24 as a prime strain, and enterovirus 72 has been identified as HAV. Disease association has not been demonstrated for enterovirus type 69 or 73.

Table 10.1–2 Zoonotic viruses associated with human infection

Virus[a]	Respiratory	Central nervous system and neuromuscular	Mucocutaneous	Gastrointestinal	Other	Comments
Arboviruses (3)						
Arthropod-borne viruses; more than 150 viruses associated with human illness belonging to several families (*Togaviridae, Flaviviridae, Bunyaviridae, Reoviridae,* and *Rhabdoviridae)*	Pneumonitis (e.g., vesicular stomatitis virus, VEE)	Encephalitis (e.g., West Nile fever, VEE, Eastern equine encephalitis, Western equine encephalitis, St. Louis encephalitis, California encephalitis); myalgia/arthralgia (e.g., dengue, Colorado tick fever)	Maculopapular rashes associated with several, including dengue; vesicular disease (vesicular stomatitis virus)		Undifferentiated febrile illness (e.g., Colorado tick fever), HF (e.g., Rift Valley fever, yellow fever, dengue)	Transmitted by hematophagous arthropod vectors (primarily mosquitoes, but also ticks and flies)
Arenaviruses						
Several associated with HF					HF, including Brazilian HF, Argentine HF	All are associated with rodent hosts, with transmission by inhalation of aerosolized virus or direct contact with excretions or secretions; human-to-human transmission may occur (e.g., Lassa fever)
Lymphocytic choriomeningitis (LCM) virus	Flu-like illness	Meningitis, meningoencephalitis			Undifferentiated febrile illness	Widely distributed, with the house mouse (*Mus musculus*) being its natural host. Infected mice and hamsters are potential sources for animal handlers and pet owners.
Filoviruses						
Ebola virus, Marburg virus					HF	
Hantaviruses	Hantavirus pulmonary syndrome; acute respiratory distress syndrome				HF with renal syndrome (e.g., Korean HF virus)	Mice (e.g., deer mouse [*Permyscus maniculatus*]) are natural hosts. Transmission is by the aerosol route, e.g., by exposure to dried excreta.

(continued)

Table 10.1–2 Zoonotic viruses associated with human infection *(continued)*

Virus[a]	Respiratory	Central nervous system and neuromuscular	Mucocutaneous	Gastrointestinal	Other	Comments
Orthomyxoviruses (e.g., avian influenza virus, swine influenza virus)	Influenza, URI, pneumonia					
Paramyxoviruses (21)						
Newcastle disease virus	Flu-like illness				Conjunctivitis	Transmission occurs via the aerosol route from infected fowl.
Hendra, Nipah viruses	Flu-like illness	Encephalitis				Associated with exposure to infected livestock
Poxviruses (para- and orthopoxviruses associated with several animal species)			Examples include cowpox, milker's nodule (pseudocowpox), orf (pustular dermatitis), monkeypox viruses		Fever and systemic shock (monkeypox)	Secondary transmission has been shown to occur.
Rabies virus		Encephalitis				Usually fatal in unvaccinated individuals not receiving timely postexposure prophylaxis
Simian B virus (herpes B virus)		Encephalitis	Vesicular lesions			Indigenous to Old World monkeys of the genus *Macaca* such as rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) Transmission to humans occurs primarily by bites or by direct contact (5) with saliva or infected tissues; human-to-human transmission has been reported (4). The mortality rate is high in untreated individuals.

[a] Several zoonotic viruses are categorized as BSL 3 or BSL 4 agents (http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm) and/or are designated as select agents (http://www.cdc.gov.od/sap/docs/salist.pdf). Laboratory testing for these agents is not typically available in the routine diagnostic virology laboratory. Abbreviations: HF, hemorrhagic fever; VEE, Venezuelan equine encephalitis; URI, upper respiratory infection.

REFERENCES

1. **Allander, T., M. T. Tammi, M. Eriksson, A. Bjerkner, A. Tiveljung-Lindell, and B. Andersson.** 2005. Cloning of a human parvovirus by molecular screening of respiratory tract samples. *Proc. Natl. Acad. Sci. USA.* **102:** 12891–12896.
2. **Barton, L. L.** 1996. Lymphocytic choriomeningitis virus: a neglected central nervous system pathogen. *Clin. Infect. Dis.* **22:**197.
3. **Calisher, C. H.** 1994. Medically important arboviruses of the United States and Canada. *Clin. Microbiol. Rev.* **7:**89–116.
4. **Centers for Disease Control.** 1987. B virus infection in humans—Pensacola, Florida. *Morb. Mortal. Wkly. Rep.* **36:**289–290, 295–296.
5. **Centers for Disease Control and Prevention.** 1998. Fatal *Cercopithecine herpesvirus 1* (B virus) infection following a mucocutaneous exposure and interim recommendations for worker protection. *Morb. Mortal. Wkly. Rep.* **47:**1073–1076, 1083.
6. **Cesarman, E., Y. Chang, P. S. Moore, J. W. Said, and D. M. Knowles.** 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. *N. Engl. J. Med.* **332:**1186–1191.
7. **Chang, Y., E. Cesarman, M. S. Pessin, F. Lee, J. Culpepper, D. M. Knowles, and P. S. Moore.** 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* **266:**1865–1869.
8. **Cone, R. W., M. L. Huang, L. Corey, J. Zeh, R. Ashley, and R. Bowden.** 1999. Human herpesvirus 6 infections after marrow transplantation: clinical and virologic manifestations. *J. Infect. Dis.* **179:**311–318.

8a. **Dalakas, M. C., J. L. Sever, D. L. Madden, N. M. Papadopoulos, I. C. Shekarchi, P. Albrecht, and A. Krezlewicz.** 1984. Late postpoliomyelitis muscular atrophy: clinical, virologic, and immunologic studies. *Rev. Infect. Dis.* **6:**S562–S567.

9. **Falsey, A. R., and E. E. Walsh.** 2000. Respiratory syncytial virus infection in adults. *Clin. Microbiol. Rev.* **13:**371–384.

9a. **Fouchier, R. A., N. G. Hartwig, T. M. Bestebroer, B. Niemeyer, J. C. de Jong, J. H. Simon, and A. D. Osterhaus.** 2004. A previously undescribed coronavirus associated with respiratory disease in humans. *Proc. Natl. Acad. Sci. USA* **101:**6212–6216.

9b. **Hamelin, M. E., and G. Boivin.** 2005. Human metapneumovirus: a ubiquitous and long-standing respiratory pathogen. *Pediatr. Infect. Dis. J.* **24:**S203–S207.

9c. **Harma, M., K. Hockerstedt, O. Lyytikainen, and I. Lautenschlager.** 2006. HHV-6 and HHV-7 antigenemia related to CMV infection after liver transplantation. *J. Med. Virol.* **78:**800–805.

10. **Hierholzer, J. C.** 1992. Adenoviruses in the immunocompromised host. *Clin. Microbiol. Rev.* **5:**262–274.
11. **Huang, Y. Q., J. J. Li, M. H. Kaplan, B. Poiesz, E. Katabira, W. C. Zhang, D. Feiner, and A. E. Friedman-Kien.** 1995. Human herpesvirus-like nucleic acid in various forms of Kaposi's sarcoma. *Lancet* **345:**759–761.

11a. **Hussaini, S. H., S. J. Skidmore, P. Richardson, L. M. Sherratt, B. T. Cooper, and J. G. O'Grady.** 1997. Severe hepatitis E infection during pregnancy. *J. Viral Hepat.* **4:**51–54.

12. **Ishiguro, N., S. Yamada, T. Takahashi, Y. Takahashi, T. Okuno, and K. Yamanishi.** 1990. Meningo-encephalitis associated with HHV-6 related exanthem subitum. *Acta Pediatr. Scand.* **79:**987–989.

12a. **Jubelt, B., and J. C. Agre.** 2000. Characteristics and management of postpolio syndrome. *JAMA* **284:**412–414.

12b. **Kahn, J. S.** 2006. The widening scope of coronaviruses. *Curr. Opin. Pediatr.* **18:**42–47.

13. **Khan, A. S., T. G. Ksiazek, and C. J. Peters.** 1996. Hantavirus pulmonary syndrome. *Lancet* **347:**739–741.
14. **Koutsky, L. A., D. A. Galloway, and K. K. Holmes.** 1988. Epidemiology of genital human papillomavirus infection. *Epidemiol. Rev.* **10:**122–163.

14a. **Loens, K., H. Goossens, C. de Laat, H. Foolen, P. Oudshoorn, S. Pattyn, P. Sillekens, and M. Ieven.** 2006. Detection of rhinoviruses by tissue culture and two independent amplification techniques, nucleic acid sequence-based amplification and reverse transcription-PCR, in children with acute respiratory infections during a winter season. *J. Clin. Microbiol.* **44:**166–171.

15. **McCullers, J. A., F. D. Lakerman, and R. J. Whitley.** 1995. Human herpesvirus 6 is associated with focal encephalitis. *Clin. Infect. Dis.* **21:**571–576.
16. **Schalling, M., M. Ekman, E. E. Kaaya, A. Linde, and P. Biberfeld.** 1995. A role for a new herpes virus (KSHV) in different forms of Kaposi's sarcoma. *Nat. Med.* **1:**707–708.
17. **Secchiero, P., D. R. Carrigan, Y. Asano, L. Benedetti, R. W. Crowley, A. L. Komaroff, R. C. Gallo, and P. Lusso.** 1995. Detection of HHV-6 in plasma of children with primary infection and immunosuppressed patients by polymerase chain reaction. *J. Infect. Dis.* **171:**273–280.
18. **Singh, N., R. Carrigan, T. Gayowsky, and I. R. Marino.** 1997. Human herpesvirus-6 infection in liver transplant recipients: documentation of pathogenicity. *Transplantation* **64:**674–678.
19. **Soulier, J., L. Grollet, E. Oksenhendler, P. Cacoub, D. Cazals-Hatem, P. Babinet, M. F. d'Agay, J. P. Clauvel, M. Raphael, L. Degos, et al.** 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. *Blood* **86:**1276–1280.

19a. **van den Hoogen, B. G., J. C. de Jong, J. Groen, T. Kuiken, R. de Groot, R. A. Fouchier, and A. D. Osterhaus.** 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *Nat. Med.* **7:**719–724.

19b. **van der Hoek, L., K. Pyrc, M. F. Jebbink, W. Vermeulen-Oost, R. J. Berkhout, K. C. Wolthers, et al.** 2004. Identification of a new human coronavirus. *Nat. Med.* **10:**368–371.

REFERENCES *(continued)*

20. **Walboomers, J. M., M. V. Jacobs, M. M. Manos, F. X. Bosch, J. A. Kummer, K. V. Shah, P. J. Snijders, J. Peto, C. J. Meijer, and N. Munoz.** 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol.* **189:**12–19.
21. **Wang, L., B. H. Harcourt, M. Yu, A. Tamin, P. A. Rota, W. J. Bellini, and B. T. Eaton.** 2001. Molecular biology of Hendra and Nipah viruses. *Microbes Infect.* **3:**279–287.
21a. **Wang, L., and H. Zhuang.** 2004. Hepatitis E: an overview and recent advances in vaccine research. *World J. Gastroenterol.* **10:**2157–2162.
21b. **Woo, P. C. Y., S. K. P. Lau, C.-M. Chu, K.-H. Chan, H.-W. Tsoi, Y. Huang, et al.** 2005. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. *J. Virol.* **79:**884–895.
22. **Yamanishi, K., T. Okuno, K. Shiraki, M. Takahashi, T. Kondo, Y. Asano, and T. Kurata.** 1988. Identification of human herpesvirus-6 as a causal agent for exanthem subitum. *Lancet* **i:**1065–1067.

SUPPLEMENTAL READING

Hsiung, G. D., C. K. Y. Fong, and M. L. Landry. 1994. *Hsiung's Diagnostic Virology*, 4th ed. Yale University Press, New Haven, Conn.

Knipe, D. M., P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (eds.). 2001. *Fields Virology*, 4th ed. Lippincott Williams & Wilkins, Philadelphia, Pa.

Murray, P. R., E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.). 2003. *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.

Specter, S., R. L. Hodinka, and S. A. Young. 2000. *Clinical Virology Manual*, 3rd ed. American Society for Microbiology, Washington, D.C.

U.S. Department of Health and Human Services-Public Health Service. 1991. *Biosafety in Microbiological and Biomedical Laboratories*, 4th ed. U.S. Government Printing Office, Washington, D.C.

10.2 Selection, Maintenance, and Observation of Uninoculated Monolayer Cell Cultures

I. PRINCIPLE

Viruses are obligate intracellular parasites requiring metabolically active cells to support their replication. While many viruses of diagnostic interest can be cultured in readily available monolayer cell cultures (Table 10.2–1), there are several agents that can be isolated only using specialized systems (Table 10.2–2) such as organ culture (e.g., coronaviruses), leukocyte culture (e.g., the human immunodeficiency viruses and Epstein-Barr virus), or animals (e.g., rabies virus, several coxsackie A viruses, and arboviruses). In addition, there are several important viral agents for which an in vitro system has not been identified (e.g., hepatitis B and C viruses, human papillomaviruses, Norwalk virus, and parvovirus B19).

Cell cultures routinely used in the diagnostic laboratory are available commercially (Appendix 10.2–1) and consist of single layers (monolayers) of metabolically active cells which are adherent to the surface of a tube or to a coverslip contained in a flat-bottomed shell vial. Viruses exhibit a selective pattern of replication in cell cultures. The number of different cell culture types utilized should be consistent with the anticipated spectrum of isolates. This procedure provides information necessary for laboratories using commercially supplied cell cultures. For on-site preparation of cell cultures, refer to procedure 10.3.

II. MATERIALS

A. Cell culture monolayers (received or prepared at least weekly)

Viral culture laboratories generally utilize a combination of tube and shell vial cultures. Shell vials are particularly valuable for performing cytomegalovirus (CMV) (7, 15) and varicella-zoster virus (VZV) (6, 17, 24) cultures but have been applied to the rapid detection of several other viruses, including herpes simplex virus (HSV) (8), respiratory viruses (3–5, 9, 12, 14, 19, 23), and measles virus (13). Although the shell vial technique is rapid and provides good sensitivity in many cases, some laboratories have experienced lower rates of recovery of CMV from blood (10, 15) and of adenoviruses from respiratory samples (14) with shell vial cultures. See Appendix 10.2–1 for commercial cell culture sources.

B. Culture medium

Eagle's minimum essential medium in either Earle's or Hanks' balanced salt solution (EBSS or HBSS, respectively) and containing 2% heat-inactivated fetal bovine serum (FBS) is a medium suitable for the maintenance of routinely used, uninoculated and inoculated monolayer cell cultures (*see* Appendix 10.2–2).

C. Supplies

1. Racks designed to hold culture tubes in a horizontal position, with the tube necks slightly raised, or shell vials in the upright position
2. Sterile individually wrapped pipettes (5 and 10 ml) and safety pipetting devices
3. PPE
4. 1:10 Dilution of household bleach
5. Autoclavable containers

D. Equipment

1. Microscope ($\times$100)

   Shell vials can be examined by using an inverted microscope with a glass microscope slide used to support the vial. Tube cultures can be examined with a standard or inverted microscope; tubes can be held in place with a holder consisting of two parallel tracks glued to the stage.

   ◩ **NOTE:** One end of the holder should be slightly higher so that the culture medium does not flow into the caps during observation.

Table 10.2–1 Monolayer cell cultures used for viral culture

Virus[a]	Cell cultures[b]	Comments
Adenovirus types 1–39, 42, and higher	HNK, A-549	Primary or low-passage human kidney cells are preferred to continuous cell lines; isolation in PMK is variable.
Adenovirus types 40–41 (enteric)	Graham-293 (1, 21), A-549	
BK virus	MRC-5, WI-38, HLF, HNK	CPE may require several weeks to develop; can use shell vial cultures. Antibody to simian virus 40 T antigen cross-reacts with human polyomaviruses.
Coronaviruses		
NL63	Tertiary MK (rhesus) (4a)	Trypsin included in culture medium
SARS-CoV	Vero (2b, 5a, 9a), ML (5a), RhMK (5a), CMK (5a)	
CMV	MRC-5, WI-38, HLF, ML shell vials[c]	
Enteroviruses[d]	Maximum recovery achieved with a panel of 4 cell types (primary human and monkey kidney cells, BGMK, H292 RD; diploid human lung cells (e.g., MRC-5) have limited sensitivity.	RD cells support the replication of a number of group A coxsackieviruses that cannot be cultivated in other cell lines (11, 18). However, this cell line monolayer overgrows rapidly and is difficult to interpret.
HSV types 1 and 2	A-549, RK, ML, HNK, MRC-5, WI-38	Isolation in less sensitive cell lines (e.g., MRC-5) may be enhanced by using shell vial cultures. A genetically engineered baby hamster kidney cell line, which contains a reporter transgene for HSV, has been shown to be useful for detecting this virus in shell vials (16, 20).
Influenza virus types A, B, and C	PMK (rhesus), A-549, ML shell vials[c]	MDCK cells provide good sensitivity with incorporation of trypsin (1–2 μg/ml) in cell culture medium (5, 22).
Measles virus	PMK, HNK, A-549 shell vials (13)	
Metapneumovirus	Tertiary MK (rhesus) (8a, 11a, 22a), Vero (11a), LLC-MK2 (2a, 2b, 8a, 11a, 15a), HEp-2 (2b)	Trypsin dependence demonstrated (22a), including subtype-specific dependence (11a)
Mumps virus	PMK, HNK	
Parainfluenza virus types 1–4	PMK (rhesus) HNK, A-549, ML shell vials[c]	
Poxviruses (orthopoxviruses [e.g., vaccinia, monkeypox, cowpox] and parapoxviruses [e.g., orf, milker's nodule])	PMK, MRC-5, WI-38, HNK	
Respiratory syncytial virus	HEp-2, H292, A-549	PMK and MRC-5 are also useful (2) but do not develop characteristic CPE.
Reovirus types 1–3	PMK, PHK	Not commonly isolated
Rhinoviruses (>100 types)	MRC-5, WI-38, PMK	
Rubella virus	AGMK	
VZV	MRC-5, WI-38, A-549, CV-1	Shell vials recommended

[a] Several viruses (e.g., SARS-CoV, variola virus, highly pathogenic avian influenza virus) that are categorized as BSL 3 or BSL 4 agents can infect commonly used cell cultures. Do not perform viral culture in a BSL 2 setting on specimens collected from individuals suspected of having such infections. Contact your Laboratory Response Network (LRN) reference laboratory or local public health department for testing information regarding these agents. Information for sentinel laboratories can be obtained at http://www.cdc.gov and http://www.asm.org.

[b] Primary or low-passage cell cultures: AGMK, African green monkey kidney; CMK, cynomolgus monkey kidney; HNK, human neonatal kidney; PMK, monkey kidney; RK, rabbit kidney. Semicontinuous (diploid) cell cultures: HLF, MRC-5, WI-38, human embryonic lung fibroblasts. Continuous cell lines: A-549, human epidermoid lung carcinoma; BGMK, buffalo green monkey kidney; CV-1, African green monkey kidney; Graham-293, adenovirus-transformed human kidney; H292, human pulmonary epidermoid carcinoma; HEp-2, human laryngeal carcinoma; LLC-MK2, rhesus monkey kidney; MDCK, Madin-Darby canine kidney; ML, mink lung; RD, rhabdomyosarcoma; Vero, African green monkey kidney.

[c] Viral antigen can be detected in the absence of viral replication.

[d] Members of the genus *Enterovirus* are now classified among five species, including polioviruses and human enteroviruses (HEV) A, B, C, and D. HEV-A includes coxsackieviruses A2 to A8, A10, A12, A14, and A16 and enterovirus 71. HEV-B includes coxsackieviruses A9 and B1 to B6; echoviruses 1 to 7, 9, 11 to 21, 24 to 27, and 29 to 33; and enteroviruses 69 and 73. HEV-C includes coxsackieviruses A1, A11, A13, A15, A17 to A22, and A24. HEV-D includes enteroviruses 68 and 70. Several coxsackie A viruses cannot be cultivated in cell culture.

Table 10.2–2 Viral culture systems and availability

Culture system	Culture availability	Representative viruses
Monolayer cell cultures	Widely available, with a few exceptions (e.g., BK, metapneumovirus, rubella)	Adenoviruses, BK virus, group A coxsackieviruses (not all types), group B coxsackieviruses, cytomegalovirus, echoviruses, enterovirus types 68–71, herpes simplex viruses, influenza viruses, measles virus, metapneumovirus, mumps virus, parainfluenza viruses, polioviruses, reoviruses, respiratory syncytial virus, rhinoviruses, rubella virus, varicella-zoster virus
Leukocyte cultures	Limited availability (e.g., public health laboratories and specialized research or reference laboratories)	Human herpesvirus 6,[a] human immunodeficiency viruses, human T-cell lymphotropic viruses, Epstein-Barr virus
Specialized cell culture techniques (e.g., organ cultures) and/or BSL 3 or BSL 4 facility design and practices	Limited availability (e.g., public health laboratories and specialized research or reference laboratories)	Arboviruses,[b,c] arenaviruses,[b,c] coronaviruses (e.g., SARS-CoV[b]), filoviruses,[b] hepatitis A virus, hepatitis E virus, parvovirus B19, poxviruses[b] (e.g., monkeypox, vaccinia, variola viruses), rabies virus,[b,c] rotaviruses
Animal hosts	Limited availability (public health laboratories and specialized research or reference laboratories)	Group A coxsackieviruses (several types require suckling mouse inoculation)
Noncultivable		Caliciviruses (*Norovirus, Sapovirus*), hepatitis viruses (B, C, D), JC virus, human papillomaviruses, molluscum contagiosum

[a] Has been cultivated in human lung fibroblast shell vial cultures (16a).

[b] Need to consider vaccination and BSL 3/BSL 4 recommendations.

[c] Animal inoculation may supplement cell culture for isolation of some agents and may be more sensitive than cell culture inoculation in some cases.

II. MATERIALS *(continued)*

2. Class II biological safety cabinet
It is highly recommended that separate cabinets be used for uninoculated and inoculated monolayers. If a single cabinet is used, uninoculated cell culture work should precede work with specimens or stock viruses, with decontamination in between. Before and after each use, wipe down all surfaces with 70% ethanol or a high-level disinfectant such as 2% alkaline glutaraldehyde. While 0.5% sodium hypochlorite is good for routine decontamination, it may corrode the stainless steel surfaces. Do not impede the airflow within the unit by blocking airflow vents. Avoid disrupting the air curtain at the front of the hood; minimize movements into and out of the hood, and avoid outside air turbulence such as that created by air conditioning units or open doors or windows. Do not use the unit for storage; remove all supplies at the completion of work.

3. Refrigerator at 2 to 8°C

4. Incubator (35 to 37°C)
◩ **NOTE:** CO_2 atmosphere is not necessary for closed containers.

III. ASSESSMENT, MAINTENANCE, AND QUALITY CONTROL OF UNINOCULATED CELL CULTURES

A. Assessment

Cell cultures may exhibit extensive lot-to-lot variation in appearance and quality. Assess each lot upon receipt by visually and microscopically observing the monolayers as described in Fig. 10.2–1 and Table 10.2–3.

B. Maintenance (refeeding)

Since cell cultures are used within 5 to 7 days of receipt, refeeding with fresh medium is generally not necessary until the time of inoculation. One indication for interim refeeding is a low pH (*see* Table 10.2–3).

1. Discard the medium (decant, aspirate, or pipette).
◩ **NOTE:** Be careful not to scrape the monolayer with the pipette.

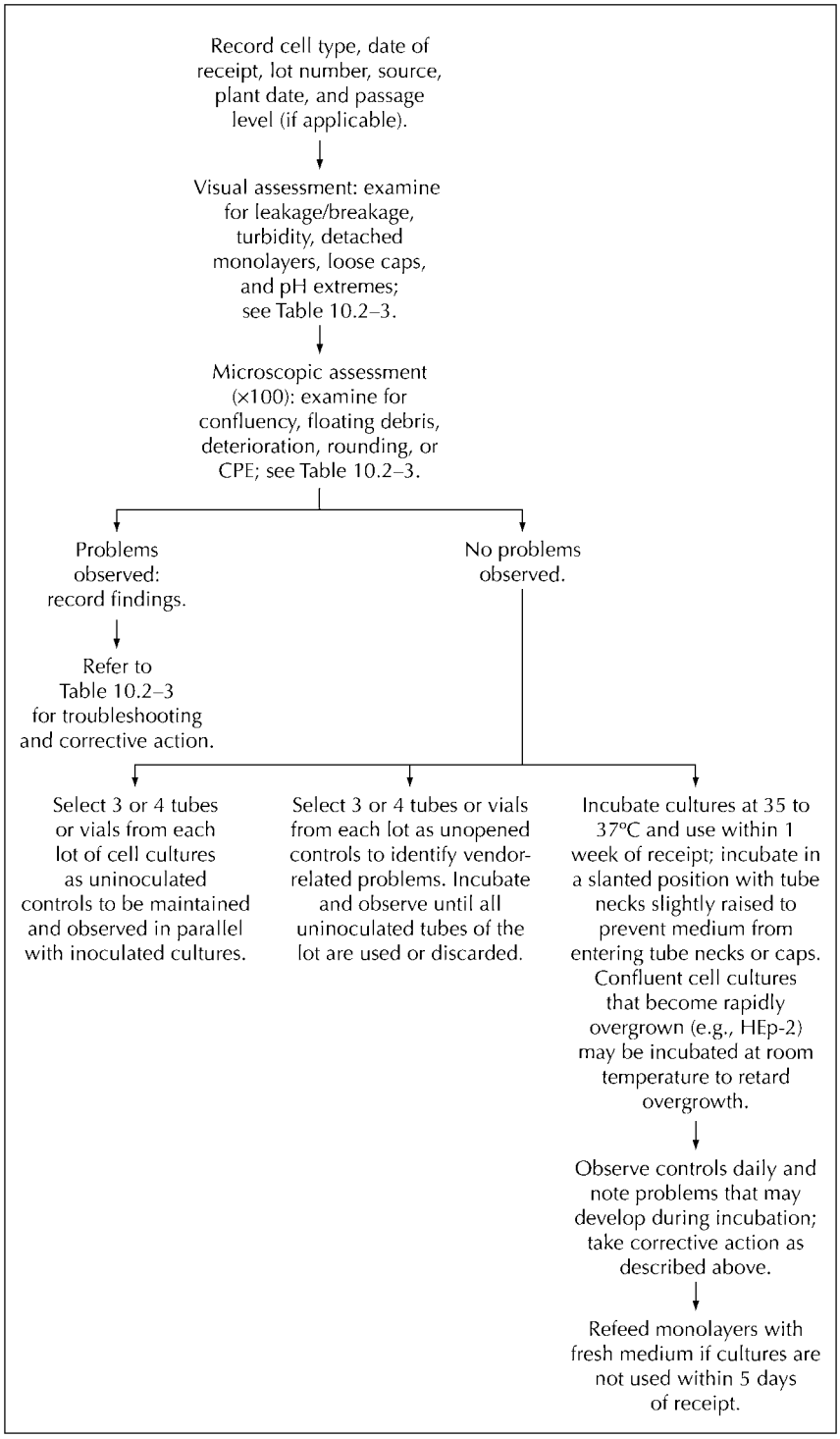
Record cell type, date of receipt, lot number, source, plant date, and passage level (if applicable).
Visual assessment: examine for leakage/breakage, turbidity, detached monolayers, loose caps, and pH extremes; see Table 10.2–3.
Microscopic assessment (×100): examine for confluency, floating debris, deterioration, rounding, or CPE; see Table 10.2–3.
Problems observed: record findings.
Refer to Table 10.2–3 for troubleshooting and corrective action.
No problems observed.
Select 3 or 4 tubes or vials from each lot of cell cultures as uninoculated controls to be maintained and observed in parallel with inoculated cultures.
Select 3 or 4 tubes or vials from each lot as unopened controls to identify vendor-related problems. Incubate and observe until all uninoculated tubes of the lot are used or discarded.
Incubate cultures at 35 to 37°C and use within 1 week of receipt; incubate in a slanted position with tube necks slightly raised to prevent medium from entering tube necks or caps. Confluent cell cultures that become rapidly overgrown (e.g., HEp-2) may be incubated at room temperature to retard overgrowth.
Observe controls daily and note problems that may develop during incubation; take corrective action as described above.
Refeed monolayers with fresh medium if cultures are not used within 5 days of receipt.

Figure 10.2–1 Assessment, incubation, and maintenance of uninoculated cell cultures.

Table 10.2–3 Assessment of monolayered cell cultures and troubleshooting

Component	Appearance	Possible cause(s)	Corrective action
Medium[a]	Clear yellow (low pH)	CO_2 accumulation	Examine microscopically for evidence of microbial contamination. If none, refeed with fresh maintenance medium and incubate. Reevaluate after 18–24 h. Retain approximately four to six tubes without medium change for observation.
	Clear purple (high pH)	Loose caps	Tighten caps; incubate. Notify vendor.
		Shipping trauma, chilling	Incubate for several hours or overnight and reevaluate.
		Poor cell quality, metabolically inactive cells; sparse monolayer	Notify vendor. Incubate; reject if not satisfactory within 24 h.
	Turbid or organisms visible	Bacterial/fungal contamination[b]	Notify vendor; reject. Do not open.
Monolayer[c]	Sparse (less than 60–70%)	Seeded too sparsely, cells failed to divide, medium or glassware problem	Notify vendor. Incubate and reject if not satisfactory within 24–48 h.
	Cells piled/detached	Seeded too heavily, old monolayer or poor cell quality, mycoplasmal contamination[d], medium or glassware problem, temperature extremes, inverted during shipment	Notify vendor. Reject if extensive.
	Cellular granularity and/or rounding	Shipping trauma, chilling, poor cell quality, bacterial/fungal contamination[b], mycoplasmal contamination[d], medium or glassware problem	Incubate for several hours or overnight, and reevaluate. Notify vendor; reject if improvement not observed.
	CPE and/or HAd	Endogenous or contaminating viruses, mycoplasmas	Primary simian cells are frequently contaminated with endogenous viruses. Perform appropriate QC to monitor extent of contamination and effect on sensitivity of cultures. May mimic human viral CPE, cause HAd, or interfere with the recovery of viruses from clinical samples. If a herpeslike CPE is observed, notify the vendor immediately and do not open the tubes because cell cultures may contain simian B virus, which is associated with a high mortality rate in humans. *Note:* Primary simian cell cultures may be obtained with medium containing antibodies to simian viruses (SV5 and SV40) which may temporarily retard the replication of these viruses.

[a] Should be clear and orange-red to cherry pink (pH 6.8 to 7.2).

[b] Perform Gram stain of sediment obtained from centrifuged culture supernatant; inoculate broth medium (e.g., THIO, TSB) or agar (e.g., blood, Sabouraud dextrose) with sediment and culture medium, and observe for bacterial or fungal growth for 10 to 14 days.

[c] Monolayered cell cultures should consist of a single layer of adherent cells exhibiting normal morphology at or near confluency (100% monolayer). Monolayers that are slightly subconfluent are preferred for viral culture.

[d] Mycoplasmal contamination can have a profoundly detrimental effect on viral isolation. Contamination with these organisms frequently does not result in obvious changes of the cell monolayer but may present as a generalized deterioration of the monolayer; occasionally a CPE mimicking viral growth may be observed. Obtain written certification from the vendor that cultures are mycoplasma free.

III. ASSESSMENT, MAINTENANCE, AND QUALITY CONTROL OF UNINOCULATED CELL CULTURES *(continued)*

2. Add 1 to 2 ml of cell culture maintenance medium to each tube or vial.

◩ **NOTE:** Use separate pipettes for each lot of each cell line; change pipettes frequently.

C. QC

1. Select monolayers from each lot to serve as controls as described in Fig. 10.2–1. Incubate and observe these in parallel with viral cultures.
2. Titration of viral reference material is useful for comparing different lots or sources of cell cultures or when there are concerns regarding sensitivity. For this purpose, use archived aliquots of characterized isolates or obtain refer-

III. ASSESSMENT, MAINTENANCE, AND QUALITY CONTROL OF UNINOCULATED CELL CULTURES *(continued)*

ence material from another laboratory or from ATCC, Manassas, Va. To assess relative sensitivities, perform parallel titrations using serial 10-fold dilutions of the virus stock (Appendix 10.5–1). Incubate and observe the titrations by following the procedure routinely employed for isolation of the particular virus.

IV. CELL CULTURE CONTAMINANTS

A. Bacterial and mycotic

Bacterial and fungal contamination may originate from many sources, including contaminated reagents or supplies, room air, work surfaces, or the technologist. A good QC program, rigorous training, and adherence to aseptic techniques are essential.

1. Ensure proper use of a certified and adequately maintained class II biological safety cabinet.
2. Use aseptic technique (Appendix 10.2–3).

B. Mycoplasmal

Mycoplasmal contamination of cell cultures may not be obviously apparent by routine macroscopic and microscopic evaluation, but it nonetheless may interfere with the quality and viability of serially propagated cell cultures.

1. Obtain confirmation from commercial suppliers that all cell lines are monitored for mycoplasmal contamination.
2. Contamination of continuous cell lines maintained in the laboratory can occur from several sources, including serum and trypsin. Monitor continuous cell lines at least quarterly for mycoplasmal contamination (*see* procedure 10.3).

C. Viral

Cell cultures may be contaminated with endogenous viruses, which can interfere with viral isolation, be mistaken for a true isolate, or even present a biohazard. While endogenous viruses are not limited to simian cells, culture contamination in the diagnostic laboratory is associated primarily with these cells. Primary monkey kidney cells are frequently contaminated with endogenous viruses which may produce cytopathic effect (CPE), as with simian virus 40 (SV40), or positive hemadsorption (HAd), as with SV5. Infections in humans caused by simian herpesvirus B are associated with a high mortality rate. While most human infections are associated with direct animal contact, the possibility exists that cell cultures may contain this virus.

Exogenous viral contamination, including cross-contamination of positive cultures, may occur as a consequence of using contaminated reagents or poor technique. The use of culture dishes and multiwell plates increases the risk for all types of contamination, including cross-contamination. Observation of uninoculated controls (Fig. 10.2–1) will facilitate detection of the presence of endogenous viruses.

D. Cellular

Cellular cross-contamination can occur by transferring floating or loosely adherent cells during pipetting processes. The contaminating cells may simply overlay the existing monolayer or actually develop foci of replicating cells (Fig. 10.2–2).

V. TOXICITY

Sources of toxicity include bacterial endotoxins in water and sera; high concentrations of antimicrobial agents; certain rubber, plastic, and cementing products used in cap liners and tubing; photoproducts of riboflavin and tryptophan; and residual detergent or chemicals on labware from washing and/or gas or steam sterilization. Signs of toxicity include a highly alkaline medium, granularity, cell rounding and detachment, and monolayer detachment.

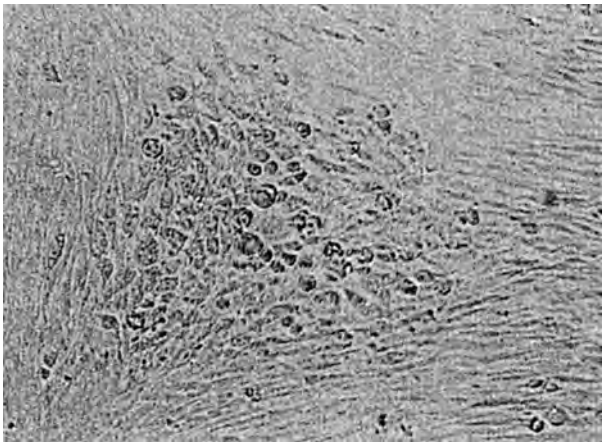

Figure 10.2–2 Focal area of replicating A-549 cells that have inadvertently cross-contaminated an MRC-5 monolayer.

REFERENCES

1. **Albert, M. J.** 1986. Enteric adenoviruses. *Arch. Virol.* **88:**1–17.
2. **Arens, M. Q., E. M. Swierkosz, R. R. Schmidt, T. Armstrong, and K. A. Rivetna.** 1986. Enhanced isolation of respiratory syncytial virus in cell culture. *J. Clin. Microbiol.* **23:**800–802.

2a. **Boivin, G., Y. Abed, G. Pelletier, L. Ruel, D. Moisan, S. Cote, T. C. T. Peret, D. D. Erdman, and L. J. Anderson.** 2002. Virological features and clinical manifestations associated with human metapneumovirus: a new paramyxovirus responsible for acute respiratory-tract infections in all age groups. *J. Infect. Dis.* **186:**1330–1334.

2b. **Chan, P. K. S., J. S. Tam, C. W. Lam, E. Chan, A. Wu, et al.** 2003. Human metapneumovirus detection in patients with severe acute respiratory syndrome. *Emerg. Infect. Dis.* **9:**1058–1063.

3. **Espy, M. J., C. Hierholzer, and T. F. Smith.** 1987. The effect of centrifugation on the rapid detection of adenovirus in shell vials. *J. Clin. Pathol.* **88:**358–360.
4. **Espy, M. J., T. F. Smith, M. W. Haromon, and A. P. Kendal.** 1986. Rapid detection of influenza virus by shell vial assay with monoclonal antibodies. *J. Clin. Microbiol.* **24:**677–679.

4a. **Fouchier, R. A., N. G. Hartwig, T. M. Bestebroer, B. Niemeyer, J. C. de Jong, J. H. Simon, and A. D. Osterhaus.** 2004. A previously undescribed coronavirus associated with respiratory disease in humans. *Proc. Natl. Acad. Sci. USA* **101:**6212–6216.

5. **Frank, A. L., R. B. Couch, C. A. Griffis, and B. D. Baxter.** 1979. Comparison of different tissue cultures for isolation and quantitation of influenza and parainfluenza viruses. *J. Clin. Microbiol.* **10:**32–36.

5a. **Gillim-Ross, L., J. Taylor, D. R. Scholl, J. Ridenour, P. S. Masters, and D. E. Wentworth.** 2004. Discovery of novel human and animal cells infected by the severe acute respiratory syndrome coronavirus by replication-specific multiplex reverse transcription-PCR. *J. Clin. Microbiol.* **42:**3196–3206.

6. **Gleaves, C. A., C. F. Lee, C. I. Bustamante, and J. D. Meyers.** 1988. Use of murine monoclonal antibodies for laboratory diagnosis of varicella-zoster infection. *J. Clin. Microbiol.* **26:**1623–1625.
7. **Gleaves, C. A., T. F. Smith, E. A. Shuster, and G. R. Pearson.** 1984. Rapid detection of cytomegalovirus in MRC-5 cells inoculated with urine specimens by using low-speed centrifugation and monoclonal antibody to an early antigen. *J. Clin. Microbiol.* **19:**917–919.
8. **Gleaves, C. A., D. J. Wilson, A. D. Wold, and T. F. Smith.** 1985. Detection and serotyping of herpes simplex virus in MRC-5 cells by use of centrifugation and monoclonal antibodies 16 h postinoculation. *J. Clin. Microbiol.* **21:**29–32.

8a. **Hamelin, M. E., and G. Boivin.** 2005. Human metapneumovirus: a ubiquitous and long-standing respiratory pathogen. *Pediatr. Infect. Dis. J.* **24:**S203–S207.

9. **Johnston, S. L. G., and C. S. Siegel.** 1990. Evaluation of direct immunofluorescence, enzyme immunoassay, centrifugation culture, and conventional culture for the detection of respiratory syncytial virus. *J. Clin. Microbiol.* **28:**2394–2397.

9a. **Ksiazek, T. G., D. Erdman, C. S. Goldsmith, et al.** 2003. A novel coronavirus associated with severe acute respiratory syndrome. *N. Engl. J. Med.* **348:**1953–1966.

10. **Landry, M. L., and D. Ferguson.** 1993. Comparison of quantitative cytomegalovirus antigenemia assay with culture methods and correlation with clinical disease. *J. Clin. Microbiol.* **31:**2851–2856.
11. **Lipson, S. M., R. Walderman, P. Costello, and K. Szabo.** 1988. Sensitivity of rhabdomyosarcoma and guinea pig embryo cell cultures to field isolates of difficult-to-cultivate group A coxsackieviruses. *J. Clin. Microbiol.* **26:**1298–1303.

REFERENCES *(continued)*

11a. **MacPhail, M., J. H. Schickli, R. S. Tang, J. Kaur, C. Robinson, et al.** 2004. Identification of small-animal and primate models for evaluation of vaccine candidates for human metapneumovirus (hMPV) and implications for hMPV vaccine design. *J. Gen. Virol.* **85:**1855–1863.

12. **Matthey, S., D. Nicholson, S. Ruhs, B. Alden, M. Knock, K. Schultz, and A. Schmuecker.** 1992. Rapid detection of respiratory viruses by shell vial culture and direct staining by using pooled and individual monoclonal antibodies. *J. Clin. Microbiol.* **30:**540–544.

13. **Minnich, L. L., F. Goodenough, and C. G. Ray.** 1991. Use of immunofluorescence to identify measles virus infections. *J. Clin. Microbiol.* **29:**1148–1150.

14. **Olsen, M. A., K. M. Shuck, A. R. Sambol, S. M. Flor, J. O'Brien, and B. J. Cabrera.** 1993. Isolation of seven respiratory viruses in shell vials: a practical and highly sensitive method. *J. Clin. Microbiol.* **31:**422–425.

15. **Paya, C. V., A. D. Wold, and T. F. Smith.** 1987. Detection of cytomegalovirus infections in specimens other than urine by the shell vial assay and conventional tube cell cultures. *J. Clin. Microbiol.* **25:**755–757.

15a. **Peret, T. C., G. Boivin, Y. Li, M. Couillard, C. Humphrey, et al.** 2002. Characterization of human metapneumoviruses isolated from patients in North America. *J. Infect. Dis.* **185:**1660–1663.

16. **Proffitt, M. R., and S. A. Schindler.** 1995. Rapid detection of HSV with an enzyme-linked virus inducible system (ELVIS™) employing a genetically modified cell line. *Clin. Diagn. Virol.* **4:**175–182.

16a. **Rogers, J., S. Rohal, D. Carrigan, S. Kusne, K. Knox, T. Gayowski, M. Wagener, J. J. Fung, and N. Singh.** 2000. Human herpesvirus 6 in liver transplant recipients: role in pathogenesis of fungal infections, neurologic complications, and outcome. *Transplantation* **69:**2566–2573.

17. **Schirm, J., J. M. Janneke, G. W. Pastoor, P. C. vanVoorst Vader, and F. P. Schroder.** 1989. Rapid detection of varicella-zoster virus in clinical specimens using monoclonal antibodies on shell vials and smears. *J. Med. Virol.* **28:**1–6.

18. **Schmidt, N. J., H. H. Ho, and E. H. Lennette.** 1975. Propagation and isolation of group A coxsackieviruses in RD cells. *J. Clin. Microbiol.* **2:**183–185.

19. **Smith, M. C., C. Creutz, and Y. T. Huang.** 1991. Detection of respiratory syncytial virus in nasopharyngeal secretions by shell vial technique. *J. Clin. Microbiol.* **29:**463–465.

20. **Stabell, E. C., S. R. O'Rourke, G. A. Storch, and P. D. Olivo.** 1993. Evaluation of a genetically engineered cell line and a histochemical β-galactosidase assay to detect herpes simplex virus in clinical specimens. *J. Clin. Microbiol.* **31:**2796–2798.

21. **Takiff, H. E., S. E. Straus, and C. F. Garon.** 1981. Propagation and in vitro studies of previously noncultivatable enteral adenoviruses in 293 cells. *Lancet* **ii:**832–834.

22. **Tobita, K., A. Sugiura, C. Enomoto, and M. Furuyama.** 1975. Plaque assay and primary isolation of influenza A viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. *Med. Microbiol. Immunol.* **162:**9–14.

22a. **Van den Hoogen, B. G., J. C. de Jong, J. Groen, T. Kuiken, R. de Groot, et al.** 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *Nat. Med.* **7:**719–724.

23. **Waris, M., T. Aiegler, M. Kivivirta, and O. Ruuskananen.** 1990. Rapid detection of respiratory syncytial virus and influenza A virus in cell cultures by immunoperoxidase staining with monoclonal antibodies. *J. Clin. Microbiol.* **28:**1159–1162.

24. **West, P. G., B. Aldrich, R. Hartwig, and G. J. Haller.** 1988. Increased detection rate for varicella-zoster virus with combination of two techniques. *J. Clin. Microbiol.* **26:**2680–2681.

APPENDIX 10.2–1

Cell Culture Sources

Diagnostic Hybrids, Inc.
350 W. State St.
Athens, OH 45701
(800) 344-5847
http://www.dhiusa.com

Neogenex
12811 Eighth Ave. West
Suite A102
Everett, WA 98204
(800) 334-4297
neogenex@earthlink.net

ViroMed Laboratories
6101 Blue Circle Dr.
Minnetonka, MN 55343
(800) 582-0077
http://www.viromed.com

APPENDIX 10.2–2

Cell Culture Maintenance Medium

Eagle's minimum essential medium (EMEM) in either HBSS or EBSS with 2 to 5% heat-inactivated FBS is the most widely used cell culture medium for the maintenance of monolayer cell cultures used for routine viral isolation. Serum-free medium is used by many laboratories for influenza and parainfluenza virus cultures. For this purpose, omit FBS in the formulation below; alternatively, specially formulated serum-free medium is commercially available.

Composition of balanced salt solutions and EMEM

Component	Amt (mg/liter) in:		
	EBSS	HBSS	Dulbecco's PBS[a]
Inorganic salts			
$CaCl_2$ (anhydrous)	200.00	140.00	
$CaCl_2 \cdot H_2O$			100.00
KCl	400.00	400.00	200.00
KH_2PO_4		60.00	200.00
$MgCl_2 \cdot 6H_2O$			100.00
$MgSO_4 \cdot 7H_2O$	200.00	200.00	
NaCl	6,800.00	8,000.00	8,000.00
$NaHCO_3$	2,200.00	350.00	
$Na_2HPO_4 \cdot 7H_2O$		90.00	2,160.00
$NaH_2PO_4 \cdot H_2O$	140.00		
Other components			
D-Glucose	1,000.00	1,000.00	
Phenol red	10.00	10.00	

[a] PBS, phosphate-buffered saline.

Cell culture medium

Component	Amt (mg/liter) in EMEM	
	EBSS	HBSS
Inorganic salts		
$CaCl_2$ (anhydrous)	200.00	140.00
$Ca(NO_3)_2 \cdot 4H_2O$		
KCl	400.00	400.00
KH_2PO_4		60.00
$MgSO_4 \cdot 7H_2O$	200.00	200.00
NaCl	6,800.00	8,000.00
$NaHCO_3$	2,200.00	350.00
$Na_2HPO_4 \cdot 7H_2O$		90.00
$NaH_2PO_4 \cdot H_2O$	140.00	
Other components		
D-Glucose	1,000.00	1,000.00
Glutathione (reduced)		
Phenol red	10.00	10.00
Amino acids		
L-Arginine HCl	126.40	126.40
L-Arginine (free base)		
L-Asparagine		
L-Aspartic acid		
L-Cystine	24.00	24.00
L-Glutamic acid		
L-Glycine		
L-Histidine HCl $\cdot H_2O$	41.90	41.90
L-Histidine (free base)		
Hydroxyl L-proline		
L-Isoleucine	52.50	52.50

(continued)

APPENDIX 10.2–2 *(continued)*

Cell culture medium *(continued)*

Component	Amt (mg/liter) in EMEM	
	EBSS	HBSS
Amino acids (cont.)		
L-Leucine	52.50	52.50
L-Lysine HCl	73.10	73.10
L-Methionine	14.90	14.90
L-Phenylalanine	33.00	33.00
L-Proline		
L-Serine		
L-Threonine	47.60	47.60
L-Tryptophan	10.20	10.20
L-Tyrosine	36.20	36.20
L-Valine	46.80	46.80
Vitamins		
p-Aminobenzoic acid		
D-Biotin		
D-Calcium pantothenate	1.00	1.00
Choline chloride	1.00	1.00
Folic acid	1.00	1.00
i-Inositol	2.00	2.00
Nicotinamide	1.00	1.00
Pyridoxine HCl	1.00	1.00
Riboflavin	0.10	0.10
Thiamine HCl	1.00	1.00
Vitamin B_{12}		

A. Viral cell culture maintenance medium

◩ **NOTE:** Observe manufacturer's expiration dates and storage recommendations for medium components and use aseptic technique.

EMEM in HBSS with 2% FBS and HEPES buffer

1. To 800 ml of sterile deionized pyrogen-free water, aseptically add the following.

EMEM in HBSS, 10×
(without L-glutamine or $NaHCO_3$) ....100.0 ml
L-glutamine, 200 mM solution ..... 10.0 ml
HEPES, 1 M solution ..... 20.0 ml
$NaHCO_3$, 7.5% solution ..... 29.3 ml
FBS, heat inactivated (see below) ..... 20.0 ml
gentamicin, 50 mg/ml ..... 0.20 ml
vancomycin, 50 mg/ml .....2.0 ml
nystatin (Mycostatin), 10,000 U/ml .....2.5 ml
q.s. to .....1,000 ml

2. Adjust the pH to between 7.2 and 7.4 if necessary using sterile 1 N NaOH or 1 N HCl.
3. Aseptically dispense the medium into sterile containers and perform a sterility check (*see* item D below).
4. Store at 4°C; use within 3 months of adding FBS. L-Glutamine is labile; use medium within 3 weeks of adding the glutamine. Add additional glutamine beyond 3 weeks as necessary.

◩ **NOTE:** L-Glutamine develops a precipitate when frozen and thawed but is readily resolubilized by warming briefly to 37°C. Avoid repeated freeze-thaw cycles; aliquot stock material into single-use volumes.

B. Medium modifications

1. When using MDCK cells for influenza virus isolation, supplement culture medium with TPCK-trypsin (1-tosylamide-2-phenylethyl chloromethyl ketone) at a final concentration of 1 to 2 μg/ml (1, 2).
2. HBSS is used with tightly capped culture vessels; EBSS is used when caps are loosened and incubation is done in a 5% CO_2 atmosphere.

APPENDIX 10.2–2 *(continued)*

C. Heat inactivation of FBS
FBS contains heat-labile inhibitors of viral replication. These can be inactivated by heating at 56°C for 30 min.
1. Thaw frozen FBS at room temperature or in a 37°C water bath.
2. Place thawed FBS in a 56°C water bath; the water level should match that of the FBS. *Note:* A thermometer inserted into a bottle containing the same volume of water can be used to assess the time required for the FBS to reach proper temperature.
3. Inactivate the FBS for 30 min, swirling every 10 min to ensure even heating.
◩ **NOTE:** Avoid repeated freeze-thaw cycles of FBS.

D. QC
1. FBS
FBS may contain heat-stabile inhibitors of viral replication. Since this may vary extensively from lot to lot, it is advisable to assess each new lot prior to use. To facilitate this effort, arrange to obtain samples of reserved lots prior to purchase. These include antibodies or other inhibitors of viral infection, particularly influenza viruses. Before use, assess each lot of FBS with a panel of viruses routinely detected by the laboratory.
2. Medium
   a. Assign a lot number to each batch of medium, and record the lot numbers, sources, and expiration dates of all medium components.
   b. Assess each lot of medium for sterility.
      (1) Inoculate broth medium (e.g., THIO, BHI, TSB) with 1-ml aliquots of each lot of medium.
      (2) Incubate and observe for 7 days. Do not use medium until sterility is confirmed.
   c. Assess each lot of medium for cell monolayer toxicity.
      (1) Feed two or three monolayers of each appropriate cell type with 1 to 2 ml of the medium to be assessed.
      (2) Incubate the cultures and observe for 10 to 14 days, comparing daily to uninoculated cultures maintained in an acceptable lot of medium. *Note:* Reduce the QC testing burden by purchasing or preparing single lots of media and reagents of the maximum size that can be used within the shelf life.
   d. Label reagents with date of expiration and date opened.

References

1. **Frank, A. L., R. B. Couch, C. A. Griffis, and B. D. Baxter.** 1979. Comparison of different tissue cultures for isolation and quantitation of influenza and parainfluenza viruses. *J. Clin. Microbiol.* **10:**32–36.
2. **Tobita, K., A. Sugiura, C. Enomoto, and M. Furuyama.** 1975. Plaque assay and primary isolation of influenza A viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. *Med. Microbiol. Immunol.* **162:**9–14.

APPENDIX 10.2–3

Summary of Aseptic Technique

A. Bacterial and fungal contamination may originate from many sources, including contaminated reagents or supplies, room air, or the technologist's hands. When preparing reagents or handling cell cultures, laboratory personnel must be aware of the need to practice aseptic technique.
B. Perform all cell culture manipulations in a class II biological safety cabinet and follow good operating practices.
C. Test all cell culture reagents for sterility before use and handle in a way to maintain sterility throughout their use.
D. Use only sterile supplies (e.g., pipettes, containers, caps, and stoppers); individually wrapped sterile pipettes are recommended.
E. Dispense sterile reagents in a manner that maintains sterility; pouring, rather than dispensing with a pipette or other dispenser, increases the risk for contamination.
F. Avoid touching the exposed necks, caps, or stoppers of opened containers.
G. Keep the work area and surfaces clean and free of dust.
H. Arrange materials and equipment within the safety cabinet to provide easy access and to minimize reaching.
I. Wear clean long-sleeved laboratory gowns or smocks when working with cell cultures and cell culture reagents.
J. Wipe reagent and medium bottles with 70% ethanol upon removal from storage; flaming bottle, tube, or flask necks is generally not performed when using a biological safety cabinet. Avoid leaving containers uncapped.

10.3 Cell Culture Techniques: Serial Propagation and Maintenance of Monolayer Cell Cultures

I. PRINCIPLE

Monolayer cell cultures are most frequently used in diagnostic virology. They are prepared by treating the tissue or subculture with proteolytic enzymes and/or chelating agents to dissociate the cells and then seeding the cell suspension in a culture vessel. The cells adhere, divide, and form a layer. Adherence to the vessel surface is important for the subsequent survival and growth of the cells.

Monolayer cell cultures may be divided into several types based on certain characteristics of the cells (Table 10.3–1). Primary cell cultures consist of cells explanted from tissues or organs obtained directly from an animal or human source. These cells have the same diploid chromosomes as the tissue of origin and are mainly epithelial. Subcultivation of primary cells is difficult, and in a short time, the cells become senescent and die. Diploid (or semicontinuous) cell lines can sometimes be established from subpassage of primary cells. At least 75% of these cells have two chromosomes that are structurally identical to those of the tissue of origin. These cells maintain their characteristic diploid chromosomal configuration through a number of serial passages but tend to become senescent by the 50th passage. Continuous or heteroploid cell lines are usually derived from tumor cells and possess nuclei that contain numbers of chromosomes other than the diploid number. These cells grow rapidly in culture and can be subpassaged indefinitely. In general, use of one of each type of cell line constitutes a satisfactory combination for the recovery of the major pathogenic viruses.

II. OBTAINING CELLS FOR CULTURE

All cell lines commonly used in diagnostic virology are available commercially. Laboratories may find it more practical to purchase cells already grown as monolayers in tubes. However, in-house preparation of cell cultures allows for the continued use of young, metabolically active cells and control over the quality and sensitivity of the culture system. The ATCC, the national repository for reference cell cultures, is a source of cell lines. Cells from the ATCC have been authenticated as to the species of origin; have been determined to be free of contaminating microorganisms such as mycoplasmas, bacteria, fungi, protozoa, and cytopathic viruses; and are provided in a cryopreserved state for use (3). These cells are seeded into a flask upon receipt by the laboratory and then serially propagated as described in this procedure. Cells from one or two flasks from an early passage should be cryopreserved for future use in the event of contamination or other problems. For

Table 10.3–1 Characteristics of monolayer cell cultures

Culture type	Synonym(s)	Subpassages	Source	Morphology	Examples[a]
Primary		Few[b]	Donor tissue	Epitheliallike	HNK, RMK, RK
Diploid	Semicontinuous	30–50	Monolayer	Fibroblastlike	MRC-5, WI-38, HNF
Heteroploid	Established, transformed, continuous, immortalized	Infinite	Monolayer	Epitheliallike	HEp-2, A-549, HeLa, Vero, McCoy, ML

[a] HNK, human neonatal kidney; RMK, rhesus monkey kidney; RK, rabbit kidney; MRC-5 and WI-38, human embryonic lung; HNF, human newborn foreskin; HEp-2, human epidermoid laryngial carcinoma; A-549, human epidermoid lung carcinoma; HeLa, human cervical carcinoma; Vero, African green monkey kidney; McCoy, mouse karyotype; ML, mink lung.

[b] May be subpassaged to yield secondary cultures.

II. OBTAINING CELLS FOR CULTURE *(continued)*

some laboratories, it may be more economical to purchase flasks of cells as needed for preparation of culture tubes, vials, and other vessels rather than serially propagating these cells in the laboratory. Most laboratories continue to purchase primary monkey kidney cells from commercial sources unless alternative cell lines are being used or primate facilities are available.

III. MATERIALS

A. Medium and reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

See procedure 10.2 for details on preparation of medium.

1. Cell culture medium

Eagle's minimum essential medium in either Earle's or Hanks' (HBSS) balanced salt solution and containing heat-inactivated (56°C, 30 min) fetal bovine serum (FBS). Growth medium is used for the rapid proliferation of cells and usually contains FBS at a final concentration of 7.5 to 10.0%. Maintenance medium contains only 2% FBS and is used to maintain the cells in a steady state of slow metabolic activity.

2. Enzymes for dissociation of cell monolayers: available commercially as sterile solutions or in powdered form

a. Trypsin-EDTA

Trypsin-EDTA (Versene) is a combination of enzyme and chelating agent most commonly used to dissociate cell monolayers. The mixture is prepared in a balanced salt solution without calcium and magnesium at a final concentration of 0.25% trypsin and 0.02% EDTA. Store reconstituted material at −20°C or lower and lyophilized material at 4°C, observing the manufacturer's expiration date. Once thawed, the trypsin-EDTA can be stored at 4°C for as long as 3 weeks or dispensed in single-use quantities. Avoid repeated freeze-thawing of trypsin-EDTA.

b. Alternative dissociation reagents include trypsin without EDTA, elastase, thermolysin, collagenase, protease, pancreatin (which includes amylase, trypsin, lipase, RNase, and protease), and a nonenzymatic solution containing EDTA, sodium citrate, and glycerol in a balanced salt solution.

3. HBSS or phosphate-buffered saline (PBS) without calcium or magnesium, pH 7.2 to 7.4. HBSS or PBS should be free of divalent cations such as Ca^{2+} and Mg^{2+}, since these ions stabilize bonds between cells and thereby prohibit effective dissociation of cells bound to substrates.

4. Dimethyl sulfoxide (DMSO) for cryopreservation

B. Supplies

1. Sterile plasticware or glassware (e.g., flasks, tubes, pipettes)

a. Sterile disposable plastic pipettes, flasks, and tubes, sterilized and chemically treated to provide a uniform and reproducible substrate for the attachment and growth of cells in monolayers, are commercially available and convenient to use.

b. Glassware must be meticulously cleaned and sterilized to be suitable for cell culture techniques. Processing of glassware requires presoaking of the soiled materials, washing with an appropriate detergent, thorough rinsing with tap and deionized water, and sterilization by dry heat. The cost and convenience of plasticware must be balanced against the time and labor involved in cleaning and sterilizing glassware. Also, *variations in preparation and sterilization of glassware can have significant adverse effects on the growth of cells in culture.*

2. Hemacytometer for cell enumeration

3. Racks designed to hold culture tubes at a 5 to 7° angle

4. Safety pipetting devices, protective clothing, infectious-waste disposal containers, adequate autoclave facilities

5. 0.5% Hypochlorite solution for cleaning work surfaces

6. Cryovials (e.g., Sarstedt, Corning, or Nunc) for freezing cell stocks

III. MATERIALS *(continued)*

C. Equipment

1. Inverted and standard light microscopes, ×100 to ×200 magnification
2. Class II biological safety cabinet
3. Refrigerator at 2 to 8°C
4. Centrifuge
5. Vacuum source and collection trap containing 0.5% sodium hypochlorite
6. Incubator at 37°C with or without CO_2
   The need for CO_2 depends on the use of Earle's balanced salt solution or HBSS and HEPES buffer in the cell culture medium.
7. −70°C freezer
8. Liquid nitrogen storage freezer

IV. QUALITY CONTROL

Cell cultures are extremely fragile and require meticulous care for optimum performance. Appropriate QC procedures and maintenance records should be established for all laboratories performing cell culture work. Cells should be observed routinely for indications of instability or deterioration. Examine the cells both macro- and microscopically for the presence of contaminating microorganisms; look for changes in characteristic cell morphology such as rounding, vacuolization, sloughing, or retraction; examine the medium for abnormal changes in the pH; and check all cell lines for continued susceptibility to viruses known to grow in each cell type.

A. Bacterial and fungal contamination
See Appendix 10.3–1.

B. Mycoplasmal contamination
See Appendix 10.3–2.

C. Contamination with endogenous viruses
Primary monkey kidney cells are often infected with simian virus 5 (SV5) and SV40 and the foamy retroviruses that may produce cytopathic effect or hemadsorption.

V. PROCEDURE

A. Subpassage of stock cell culture flasks

Cell lines may be kept active and available for seeding of tubes, vials, dishes, and plates by weekly subpassage. Subpassage ("splitting") of cell cultures is normally performed when the cells are at or near confluency.

1. Select flasks with confluent or nearly confluent monolayers for subpassage, and examine each flask prior to use.
2. Remove the medium from the flask by aspiration, and wash the monolayer once with a Ca^{2+}- and Mg^{2+}-free salt solution (refer to Table 10.3–2 for volume). Gently rock the flask back and forth to wash the residual medium from the cells. *Removal of residual medium is essential since medium components (i.e., serum, Ca^{2+}, Mg^{2+}) can inactivate the trypsin-EDTA.*
3. Aspirate the wash solution from the flask.
4. Add enough trypsin-EDTA solution to the flask to completely cover the monolayer of cells (refer to Table 10.3–2 for volume). Gently rock the flask back and forth for 10 to 20 s, and remove the solution by aspiration.
5. Add a fresh volume of trypsin-EDTA, and incubate the cell monolayer for 5 min at room temperature or for 2 to 3 min at 37°C.
6. Observe the cells during the incubation. When the cells begin detaching from the surface of the flask, hold the flask with one hand and firmly slap the side of the flask into the palm of your other hand to completely dislodge the cells. *Do not trypsinize cells for longer than needed.*

V. PROCEDURE *(continued)*

7. Add medium containing serum to the cell suspension as soon as possible to inhibit the enzymatic activity of the trypsin and to stop the action of the chelating agent (refer to Table 10.3–2 for medium volume).
8. Disperse the cells by gently pipetting them up and down enough times to break apart clumps and produce an even suspension.
9. If desired, perform a cell count (*see* Appendix 10.3–3).
10. For preparing new culture vessels, the split ratio method is commonly used in place of cell counting. This ratio is defined as the dilution at which cells will adequately replicate to form monolayers; it determines the number of new cell cultures that can be prepared from each cell suspension. For example, from a cell line having a split ratio of 1:3, the resuspended cells can be divided evenly among three new culture vessels of the same size, nine vessels that are three times smaller, one vessel that is triple in size, and so on. See Table 10.3–3 for the split ratios of commonly used cell lines in diagnostic virology.
11. Determine the number and types of new culture vessels needed, and label the vessels appropriately. Also, be certain to record the cell line used, date subcultured, new subpassage number, and number and types of vessels prepared.
12. Seed the resuspended cells into new culture vessels containing predispensed growth medium to make the final volumes (medium plus cell suspension) indicated in Table 10.3–4. It is recommended that 0.2 to 0.5 ml of medium be added per square centimeter of surface area (2). Make certain that the cell suspension remains completely resuspended during seeding to ensure delivery of the appropriate number of cells to each vessel.

Table 10.3–2 Reagent volumes for trypsinization of monolayer cell cultures

Size of source vessel (cm^2)	Amt (ml) of:		
	Wash reagent[a]	Enzyme[b]	Growth medium[c]
25	5	1	2
75	10	3	6
150	15	5	10

[a] PBS or balanced salt solution.

[b] Trypsin-EDTA (Versene).

[c] Minimum essential medium with 10% FBS. If a cell count is not going to be performed, resuspend the cells in a volume equal to the split ratio of the given cell line.

Table 10.3–3 Recommended split ratios for commonly used cell lines[a]

Cell line[b]	Split ratio
Diploid: MRC-5 and WI-38	1:3–1:4
Heteroploid	
HEp-2	1:6–1:8
A-549	1:6–1:8
ML	1:6–1:8
HeLa	1:5
Vero	1:4
LLC-MK_2	1:4
BHK-21	1:10
RK-13	1:3

[a] Adapted from Bird and Forrester (1).

[b] LLC-MK_2, rhesus monkey kidney; BHK-21, hamster kidney; RK-13, rabbit kidney. For other cell lines, see Table 10.3–1, footnote *a*.

Table 10.3–4 Surface area, cell yield, and final volume of growth medium for commonly used culture vessels[a]

Type of seed vessel	Surface area	Cell yield	Final vol (ml) of growth medium
Plates			
Microtiter (96 well)	32.0 mm^2	$\sim 10^5$	0.1
12 well	4.5 cm^2	$\sim 10^6$	2.0
24 well	2.0 cm^2	5.0×10^5	1.0
Petri dishes			
30 mm	6.85 cm^2	1.7×10^6	2.0
60 mm	21.0 cm^2	5.0×10^6	5.0
100 mm	55.0 cm^2	1.4×10^7	10.0
Flasks			
25 cm^2	25.0 cm^2	5.0×10^6	5.0–8.0
75 cm^2	75.0 cm^2	2.0×10^7	15.0–30.0
150 cm^2	150.0 cm^2	6.0×10^7	50.0–75.0
Shell vial	1.1 cm^2	3.0×10^5	2.0
Culture tube	$\sim$3.5 cm^2	$\sim 10^6$	2.0

[a] Adapted from Freshney (2).

V. PROCEDURE *(continued)*

13. Disperse the cells by gently swirling the vessels.
14. Incubate the vessels at 37°C with or without 5% CO_2 as needed. The caps of the vessels should remain slightly loose or completely tightened during the incubation depending on the buffering system used. Flasks, vials, and plates should be incubated in a flat position and tubes in a slanted position.
15. Observe the cells at 24 h and every other day thereafter for growth, contamination, and changes in the pH of the medium. If necessary, refeed cells with fresh growth medium.
16. When the monolayers are confluent, replace the growth medium with maintenance medium and maintain the cells as described in CMPH 8.5. Subpassage stock flasks of confluent monolayers weekly. Cell cultures of flasks, tubes, vials, or plates for virus growth should be used within 10 days of seeding.

B. Cryopreservation of cell lines

All diploid and continuous cell lines commonly used in the virology laboratory are routinely frozen and stored indefinitely in liquid nitrogen.

1. Use healthy, actively growing cells known to be free of contamination.
2. Follow steps V.A.1 through 8 above.
3. Perform a viable cell count on the cells (*see* Appendix 10.3–3). Freeze cells at high concentrations, usually 10^6 to 10^7 viable cells per ml.
4. Prepare freezing medium, which is composed of the growth medium without antimicrobial agents, 10 to 20% FBS, and 10% DMSO. The DMSO need not be sterilized but should be of cell culture quality and handled aseptically. Freezing medium should be freshly prepared.
5. Suspend cells in cold freezing medium to the concentration mentioned above.
6. Aliquot 1 ml of cell suspension into labeled cryovials. Seal the caps tightly.
7. Freeze the cells *slowly* by wrapping them in gauze or cotton and placing them in a styrofoam storage container that is sealed and stored at −70°C overnight.

V. PROCEDURE *(continued)*

8. Remove the vials from the styrofoam container, quickly place them in liquid nitrogen storage boxes or canes, and immediately place them in the vapor phase of liquid nitrogen (−196°C) for long-term storage.
9. Maintain inventory control records describing the cell line frozen, passage level, seed and freeze dates, number of vials frozen, size of source flask(s), and location of vials within the storage vessel.
10. Freeze all cell lines soon after they are received in the laboratory and when they are at low subpassage number.

C. Recovery of cryopreserved cells

1. Obtain a vial of frozen cells from liquid nitrogen storage and *quickly* thaw it by swirling it in a 37°C water bath.
2. Wipe the outside of the vial with 70% alcohol.
3. Transfer the contents of one vial to a flask of the same size as used for the source culture. If viability is expected to be low or if cells have been frozen for more than 1 year, a smaller flask may be used.
4. Slowly (over a period of 2 min) add the cell's growth medium to the flask to gradually dilute the cells and DMSO preservative. *Sudden dilution can cause severe osmotic damage and reduce cell survival.*
5. Incubate the cells at 37°C, and observe them at 24 h for adherence and growth.
6. Refeed cells with growth medium to remove all traces of DMSO and reincubate the cells.
7. Subpassage the flask when monolayer is confluent.

VI. PROCEDURE NOTES

A. Primary cell lines are normally purchased from commercial sources and are not subpassaged. These cells are refed with maintenance medium when they are received. The cells are incubated at 37°C until used or for a maximum of 14 days. Maintain inventory records on all cell lines received from commercial sources.

B. When preparing and maintaining stock flasks of cells, do not use antimicrobial agents in the growth or maintenance medium. Use medium with antimicrobial agents when preparing and maintaining tubes, vials, dishes, or plates. See CMPH 8.19 for details on the preparation of cell culture medium.

C. The ability to dissociate cells and remove them from the culture vessel surface is dependent on the cell type, confluency of the cells, completeness of serum and medium removal before the addition of trypsin-EDTA, and time elapsed since the cells were last subcultured. The time for trypsin-EDTA treatment may vary accordingly, although it should be kept to a minimum to avoid cell damage.

D. Once dissociated, cells are resuspended in growth medium for preparation of new culture vessels; delays may lead to cell loss due to adherence of cells to the source flask surface. This may lead to new vessels of lower numbers of cells than anticipated and subsequent delays or total inability to achieve confluent monolayers.

E. The number of cells used to seed culture vessels can be varied to yield confluent monolayers rapidly, e.g., within 24 h, as for chlamydial cultures, or within 3 to 5 days.

F. When seeding vessels containing coverslips, remove trapped air bubbles from under the coverslips by gently tapping the bottoms of the vessels.

G. It is recommended that cultures be refed with fresh maintenance medium the day before subpassage.

VI. PROCEDURE NOTES *(continued)*

H. When preparing tubes of HEp-2 or A-549 cells for the detection of respiratory syncytial virus, seed the cells at a lower density than normal to achieve slightly subconfluent monolayers at the time these cells will be used for detection of this virus. *Syncytium formation by respiratory syncytial virus is best observed in subconfluent monolayers and may not be seen at all if confluent cells are used for specimen inoculation.*

I. When freezing cells in liquid nitrogen, make certain that the vials have been properly sealed to avoid leaks, and *do not store the vials in the liquid phase because they may violently explode upon removal. The vials can be wrapped with sterile gauze immediately after removal and while opening to protect hands and eyes from flying glass or plastic. Always wear gloves and a protective face mask, and shield the water bath when thawing vials.*

J. If it is necessary to remove the DMSO when recovering cells from cryopreservation, resuspend the 1.0 ml of thawed cells in 10 ml of growth medium in a 15-ml conical centrifuge tube. Centrifuge the cells at 400 $\times$ *g* for 10 min at room temperature. Then, resuspend the cells in an appropriate amount of growth medium, and transfer them to a new culture vessel.

K. The use of nonadherent cell cultures and lines is limited to specialized applications. Because these cells do not form monolayers, the use of proteolytic enzymes for subpassage is obviated.

REFERENCES

1. **Bird, B. R., and F. T. Forrester.** 1981. *Basic Laboratory Techniques in Cell Culture.* U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta, Ga.
2. **Freshney, R. I.** 1983. *Culture of Animal Cells: a Manual of Basic Technique.* Alan R. Liss, Inc., New York, N.Y.
3. **Hay, R. J.** 1985. *ATCC Quality Control Methods for Cell Lines.* American Type Culture Collection, Rockville, Md.

APPENDIX 10.3–1

Detection of Bacterial and Fungal Contamination in Cultured Cells

A. Macro- and microscopic observation of cell cultures is very helpful in detecting bacterial and fungal contamination. Changes in cell morphology, abnormal shifts in pH, cloudiness of the medium, a decrease in cell growth over time, unusual odors, and direct visualization of microorganisms in the culture are all signs of probable contamination.

B. Simple procedures such as a Gram stain of sediments from centrifuged supernatant fluid of the culture cells or inoculation of the cell suspension to enriched broth medium (e.g., THIO, TSB, BHI) or agar (e.g., blood and Sabouraud dextrose agar plates) can be beneficial when contamination is suspected. It is recommended that broth medium be inoculated at a ratio of 1:10 and agar medium be inoculated with samples of 0.1 to 1.0 ml. Incubate the inoculated media at appropriate temperatures, and observe for bacterial and fungal growth for 7 to 14 days. Check sterility of all media and reagents prepared in-house for cell culture by placing a volume in broth medium and observing for growth of unwanted microorganisms. Quarantine the materials, and do not use them until sterility is confirmed. All solutions of purchased reagents undergo QC by the suppliers and need not be retested on a routine basis. However, it is prudent to periodically test these reagents for sterility and to check with each manufacturer to determine whether appropriate testing procedures are being performed.

APPENDIX 10.3–1 *(continued)*

C. The primary sources of bacterial and fungal contamination are many and can include any of the number of reagents, supplies, and equipment used to perform cell culture. However, it is more likely that a lapse in aseptic technique while using these materials is the origin of the contamination. Cell culture techniques can be easily performed free of contamination but only if laboratory personnel are constantly aware of the consequences of poor technique and if appropriate preventive and QC measures have been established in the laboratory. These should include a system for monitoring and detecting bacterial and fungal contamination and adequate methods for removing the contaminants.

D. When cell cultures are proven to be contaminated, immediately remove them from the tissue culture area and dispose of them properly. Unless absolutely necessary, do not attempt to treat the cells to eliminate the contaminant(s).

APPENDIX 10.3–2

Detection of Mycoplasmas in Cultured Cells

A. Contamination with mycoplasmas is difficult to detect without proper testing, and the organism is one of the contaminants most feared by individuals working with cell cultures. Mycoplasmas produce few recognizable gross changes in the cultured cells but can have a profound effect on the growth, metabolic activity, and susceptibility to viral infection of cell lines. They can also produce a cytopathic effect that may be confused with viral cytopathic effect. Generalized deterioration of cells and a rapid drop in the pH of the medium may indicate mycoplasmal contamination. Because mycoplasmas have no cell wall, the medium will not become turbid, as with other bacterial contaminants.

B. The source of mycoplasmal contamination is thought to be human, bovine, or porcine. Mycoplasmas are routinely found as commensal organisms in the respiratory and genital tracts of almost all humans (e.g., *Mycoplasma orale, M. hominis, M. buccale, M. fermentans,* and *M. salivarium*). Commercial sera produced from animals are the most likely source of the bovine (*M. arginini* and *Acholeplasma laidlawii*) and porcine (*M. hyorhinis*) mycoplasmas. Although commercial companies routinely check sera, trypsin, and cells for mycoplasmas, it may be beneficial to periodically retest these potential sources of contamination when they are received in the laboratory.

C. *It is strongly recommended that cultured cells be initially obtained from commercial sources that have tested and verified the cells to be free of mycoplasmas.* Avoid obtaining cell lines from colleagues, or strictly quarantine such cells until they have been appropriately tested for mycoplasmas.

D. Make certain that cells to be tested for mycoplasmas have been grown in antimicrobial agent-free medium for at least 3 days before testing is initiated and that at least 10^2 to 10^3 cells are used.

E. Testing should include a culture method and one of the many indirect methods available (1–3). Mycoplasmal detection services are also available through the ATCC and other commercial sources.

F. A defined schedule of testing for mycoplasmas should be developed in the laboratory. Test cells monthly that are continuously subpassaged, test frozen cells both before and after freezing, test cells obtained from outside sources before use, and check reagents as necessary. Keep a written inventory of the mycoplasmal testing performed.

References

1. **Bird, B. R., and F. T. Forrester.** 1981. *Basic Laboratory Techniques in Cell Culture.* U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta, Ga.
2. **McGarrity, G. J.** 1982. Detection of mycoplasma infection of cell cultures, p. 99–131. *In* K. Maramorosch (ed.), *Advances in Cell Culture.* Academic Press, Inc., New York, N.Y.
3. **Schmidt, N. J.** 1989. Cell culture procedures for diagnostic virology, p. 51–100. *In* N. J. Schmidt and R. W. Emmons (ed.), *Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections,* 6th ed. American Public Health Association, Washington, D.C.

APPENDIX 10.3–3

Cell Counting with a Hemacytometer

Cell counting with a hemacytometer can be used to count viable cells in the total cell suspension.

A. Obtain a Neubauer hemacytometer with coverslip. The hemacytometer has two chambers containing nine identical ruled squares, each measuring 1 by 1 mm. When the coverslip is placed over the chambers, the space between the coverslip and the ruled squares is 0.1 mm. Therefore, the volume of one ruled square is 0.1 mm^3, or 10^{-4} cm^3.
B. Prepare a dilution of the cell suspension in a balanced salt solution for total cell counts or in a 0.4% solution of trypan blue for viable cell counts. The dilution chosen should provide a cell count of approximately 2×10^5 to 5×10^5 cells per ml. Additional dilution or concentration of the suspension may be necessary to achieve this goal. Thoroughly mix the diluted cells to receive an even suspension without excessive clumping.
C. With the coverslip in place, transfer 10 μl of diluted cells to each chamber of the hemacytometer by using the V notches provided. The cell mixture will fill the chambers by capillary action.
D. Place the counting chamber on the stage of a light microscope. Using the low-power objective, focus on the ruled squares, and count all of the cells in the 1-mm^2 center square and the four 1-mm^2 corner squares. Do the same for the second chamber. Do not count cells lying outside the borders of the squares. If needed, keep a separate count of viable and nonviable cells.
E. Divide the total number of viable cells in the 10 squares by 10 to determine the mean count per square. This represents the number of cells per 0.1 mm^3. This number is multiplied by 10,000 to determine the number of cells per cubic centimeter. Since 1 cm^3 is equivalent to 1 ml, the cell number can now be expressed per milliliter. Adjust the final number by the appropriate dilution factor.
F. The percentage of viable cells can be determined by dividing the total number of viable cells by the total number of stained and unstained cells and multiplying by 100.
G. By knowing the total number of viable cells per milliliter and the split ratio of different cell lines, appropriate dilutions of cell suspensions can be made to prepare various types and numbers of culture vessels. It is recommended that concentrations of 100,000 to 300,000 cells per ml be used to seed new culture vessels and that diploid cells be seeded at a higher concentration than heteroploid cells (1).

Reference

1. **Bird, B. R., and F. T. Forrester.** 1981. *Basic Laboratory Techniques in Cell Culture.* U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta, Ga.

10.4 Specimen Collection and Processing

I. PRINCIPLE

Successful viral culture requires careful attention to the selection, collection, transport, and assessment of specimens. The information contained in this procedure is essential not only to those laboratories performing viral cultures on site but also to those outsourcing specimens. The laboratory must provide written guidelines to collection sites (nursing station, clinic, physician's office, and emergency room) detailing cultures that are available and instructions for submitting specimens. Instructions should include laboratory hours of operation and contact person(s); instructions for specimen collection, labeling, storage, and transport; source and storage conditions for transport media and containers; information required for adequate testing; turnaround time; reporting procedures and values; and testing limitations.

II. SPECIMEN COLLECTION

For best correlation between viral recovery and disease etiology, the specimen should reflect the target organ whenever possible. For example, isolation of an enterovirus from a throat or rectal swab obtained from an individual with aseptic meningitis is less meaningful than isolation from CSF. Specimens most frequently submitted for viral testing are presented in Table 10.4–1.

A. Timing

Collect specimens as soon after onset of symptoms as possible, because the likelihood of obtaining positive results is generally greatest within the first 3 days after onset of symptoms and diminishes rapidly as the course of infection proceeds in otherwise healthy and immunocompetent individuals. Viruses may be recovered from clinical samples for prolonged periods with disseminated or persistent infections.

Collect autopsy specimens as soon after death as possible.

B. Collection

Specimen collection practices are summarized in Fig. 10.4–1. Refer to Table 10.4–1 for specimen collection guidelines. Collect specimens as aseptically as possible since microbial contamination will interfere with testing. Place each specimen into a separate sterile leakproof container labeled with the patient's name and identification number, the collection site, and the date and time of collection. Collect fluid (e.g., CSF, urine) and bulk (e.g., stool, autopsy tissues) specimens in a sterile, dry, leakproof container.

Collect swab specimens by using sterile Dacron- or rayon-tipped swabs with plastic or aluminum shafts; do not use cotton, calcium alginate (2), or wooden-shafted swabs (9). Use flexible aluminum-shafted, small-tipped swabs for sampling sites (urethra, nasopharynx) for which larger swabs with rigid shafts would be inappropriate. Place swabs, scrapings, and small pieces of tissue into tubes or vials containing viral transport medium (VTM).

Table 10.4–1 Specimen collection for viral cultures[a]

Specimen	Collection[b]	Representative virus(es)	Comment(s)
Amniotic fluid	Collect 5–8 ml in a sterile container.		
Blood	Collect 8–10 ml in anticoagulant (sodium citrate, EDTA, or heparin). For pediatric specimens smaller volumes are acceptable, but less than 1–2 ml may be inadequate for testing.	Arboviruses, arenaviruses, CMV, HSV, HIV-1, and other retroviruses	Useful for persistent and disseminated infections in immunocompromised patients and neonates; do not refrigerate if cell separation techniques are to be performed. Do not freeze blood.
Bone marrow	Collect at least 2 ml in anticoagulant (sodium citrate, EDTA, heparin).	CMV	
CSF	Collect 2–5 ml in a sterile container.	Arboviruses, CMV, enteroviruses[c] other than poliovirus, HHV-6, HSV, LCMV, mumps virus	CMV: culture useful with polyradiculopathy, but not encephalitis. HSV: culture useful with meningitis or meningoencephalitis, but not encephalitis; useful for some arboviruses.
Gastrointestinal			
Rectal swab	Insert swab 4–6 cm into rectum, and roll swab against mucosa. Place swab into VTM.	Adenovirus, enteroviruses,[c] CMV, HSV	Not equivalent to stool sample. Useful for proctitis.
Stool	Place 2–4 g (ca. 3 tsp) into a sterile container. Add 8–10 ml of VTM if transport to laboratory is not immediate.	Adenoviruses, enteroviruses,[c] reoviruses	
Genitourinary			
Cervical swab	Remove exocervical mucus with swab and discard swab. Insert fresh swab at least 1 cm into cervical canal, and rotate swab against surface for 10 s. Place into VTM.	HSV, CMV	Asymptomatic HSV shedding in women: collection of a vulvar swab in addition to a cervical swab may increase viral recovery (12).
Urine	Collect 10–20 ml of midstream clean-voided urine in a sterile container.	Adenoviruses, BK virus, CMV, HSV, mumps virus, rubella virus	Low clinical specificity for CMV disease in immunocompromised individuals.
Lesion swab	Wipe vesicle with saline. Disrupt vesicle, and collect fluid with swab. With same swab, collect cells from base of the lesion. For nonvesicular lesions, collect cells from base of lesion by using a swab premoistened with saline. Place swab into VTM.	Group A coxsackieviruses, echoviruses, HSV, poxviruses, VZV	

Table 10.4–1 *(continued)*

Specimen	Collection[b]	Representative virus(es)	Comment(s)
Mucosal swab	Swab back and forth across involved surface and place swab into VTM.	Oral: group A coxsackieviruses, HSV. Anogenital: HSV	
Ocular			
Conjunctival swab	Swab lower conjunctiva with flexible fine-shafted swab premoistened with sterile saline. Place swab into VTM.	Adenoviruses, group A coxsackieviruses, CMV, enterovirus type 70, HSV	
Corneal or conjunctival scraping	Place scraping into VTM. Specimen should be obtained only by an ophthalmologist or other trained individual.	Same as for conjunctival swab	
Pericardial fluid	Place at least 2 ml in a sterile container.	Group B coxsackieviruses	
Pleural fluid	Place at least 2 ml in a sterile container.	Group B coxsackieviruses	
Respiratory swabs			
Nasal	Insert flexible fine-shafted swab into nostril, rotate swab, and let it rest for several seconds to absorb secretions. Place into VTM. Use separate swabs for each nostril; both swabs may be placed into the same transport vial.	Rhinoviruses	Not recommended for other respiratory viruses; prompt inoculation into cell culture is recommended.
Nasopharyngeal	Insert flexible fine-shafted swab through nostril into nasopharynx and rotate the swab gently a few times. Place swab into VTM.	Adenoviruses, CMV, enteroviruses,[c] HSV, human metapneumovirus, influenza viruses, measles virus, mumps virus, parainfluenza viruses, RSV, reoviruses, rhinoviruses, rubella virus, SARS-CoV, VZV	Preferred to pharyngeal swabs for respiratory viruses; prompt inoculation into cell culture is recommended for RSV.
Pharyngeal (throat)	Vigorously swab tonsillar areas and posterior nasopharynx. Use tongue blade to depress tongue to prevent contamination of swab with saliva. Place swab into VTM.	Same as for nasopharyngeal swab	
Respiratory fluids			
Lower respiratory tract: bronchoalveolar lavage, bronchial wash	Place 8–10 ml in sterile container.	Same as for nasopharyngeal swab	
Nasopharyngeal aspirate	Using mucus collection device, insert appropriate-sized catheter nasally into posterior nasopharynx. Apply suction, using intermittent suction as catheter is withdrawn. Wash aspirate through tubing with 5–8 ml of VTM or sterile saline, and transport material from trap to sterile container.	Same as for nasopharyngeal swab	Preferred upper respiratory tract specimen

(continued)

Table 10.4–1 Specimen collection[a] *(continued)*

Specimen	Collection[b]	Representative virus(es)	Comment(s)
Saliva	Collect saliva with one or two swabs from anterior floor of mouth and near Stenson's ducts. Place swabs into VTM.	CMV, mumps virus, rabies virus	
Tissues	Place small samples (e.g., lung biopsy specimen) into VTM to prevent drying. Place larger specimens (1–2 g; e.g., autopsy tissues) into sterile container. Add 8–10 ml of VTM.	Many viruses can be recovered from tissues during disseminated or visceral disease (e.g., CMV, HSV, adenoviruses); arboviruses, rabies virus, HSV, and other viruses associated with encephalitis can be recovered from brain tissue.	An understanding of the pathogenesis of viral infections is essential for selecting appropriate specimens. Autopsy tissues: several organs are usually sampled (liver, lung, spleen) and any organs or tissues for which involvement is suggested by clinical history or pathologic findings (e.g., brain, adrenal glands)
Vesicle aspirate	Wipe area with sterile saline. Aspirate fluid from vesicle with a 26- or 27-gauge needle attached to a tuberculin syringe. Immediately rinse the syringe in 1–2 ml of VTM.		Preferred specimen for VZV

[a] Certain agents that may be present in clinical specimens (e.g., avian influenza virus, SARS-CoV, variola virus) require special handling and/or a biosafety level that exceeds that of many clinical laboratories. The laboratory must provide information to individuals submitting specimens regarding these issues and emphasize the importance of providing clinical history information, including pertinent travel, animal contact, and/or exposure history. Information is available at http://www.cdc.gov and http://www.asm.org, including at the following links: http://www.asm.org/Policy/Index.asp?bid=6342, http://www.cdc.gov/ncidod/SARS/lab.htm, http://www.cdc.gov/flu/avian/professional/han020302.htm, and http://www.bt.cdc.gov/agent/smallpox/diagnosis/rashtestingprotocol.asp. Abbreviations: tsp, teaspoons; HHV-6, human herpesvirus type 6; HIV-1, HIV type 1; HSV, herpes simplex virus; LCMV, lymphocytic choriomeningitis virus; VZV, varicella-zoster virus.

[b] Use sterile screw-cap containers for fluid (other than blood) and bulk samples. For collection of swab samples, Dacron-tipped plastic- or wire-shafted swabs are recommended; do not use cotton or wooden-shafted swabs.

[c] Includes group A and group B coxsackieviruses, echoviruses, enterovirus types 68 to 71, and polioviruses.

II. SPECIMEN COLLECTION *(continued)*

VTM prevents specimen drying, maintains viability, and retards the growth of microbial contaminants. Most laboratories use a liquid transport medium, but other types are also used (reviewed in reference 9). Formulations are presented in Appendix 10.4–1.

1. Liquid transport media

VTM typically consists of a protein source such as bovine serum albumin, gelatin, or serum and antimicrobial agents in a buffered salt or sucrose solution. A sucrose-containing medium such as 2-SP is frequently used as a chlamydial transport medium and appears to protect labile viruses and may be particularly useful in situations requiring extended refrigeration or frozen shipment (6, 9). Transport tubes usually contain 2 to 3 ml of medium and small glass beads to facilitate specimen preparation; some sources package collection tubes and swabs together as a convenient collection kit. Use larger volumes (5 to 7 ml) of VTM to transport bulk (e.g., tissue) samples; these volumes should not be used for swabs because of the greater dilution effect.

2. Agar-containing transport systems (Stuart, Amies)

Stuart and Amies media are sometimes used to reduce the number of transport systems in an institution. Upon receipt in the laboratory, swabs are transferred to one of the liquid transport media described above. Stuart transport medium may be less satisfactory than liquid medium (9).

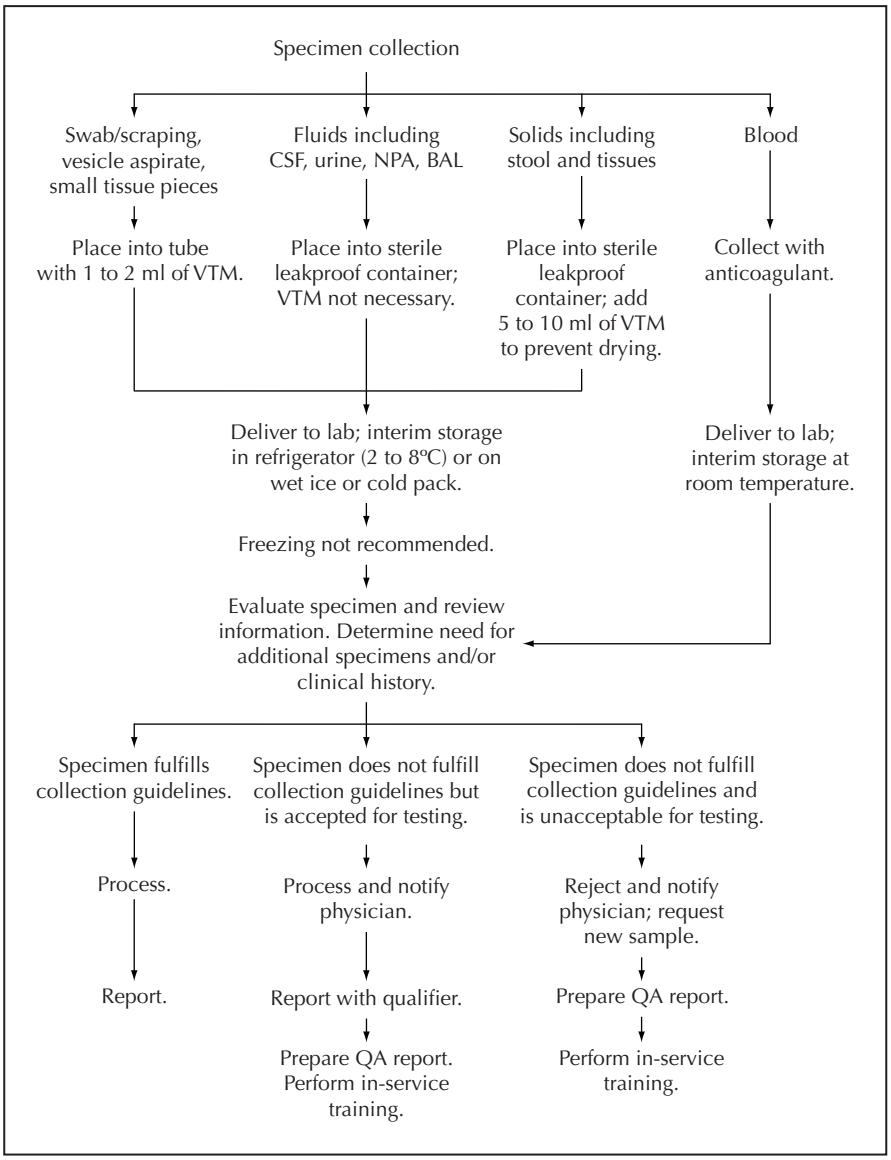
Specimen collection
Swab/scraping, vesicle aspirate, small tissue pieces
Fluids including CSF, urine, NPA, BAL
Solids including stool and tissues
Blood
Place into tube with 1 to 2 ml of VTM.
Place into sterile leakproof container; VTM not necessary.
Place into sterile leakproof container; add 5 to 10 ml of VTM to prevent drying.
Collect with anticoagulant.
Deliver to lab; interim storage in refrigerator (2 to 8ºC) or on wet ice or cold pack.
Deliver to lab; interim storage at room temperature.
Freezing not recommended.
Evaluate specimen and review information. Determine need for additional specimens and/or clinical history.
Specimen fulfills collection guidelines.
Specimen does not fulfill collection guidelines but is accepted for testing.
Specimen does not fulfill collection guidelines and is unacceptable for testing.
Process.
Process and notify physician.
Reject and notify physician; request new sample.
Report.
Report with qualifier.
Prepare QA report.
Prepare QA report. Perform in-service training.
Perform in-service training.

Figure 10.4–1 Summary of collection, transport, and evaluation of specimens for viral culture. NPA, nasopharyngeal aspirate; BAL, bronchoalveolar lavage fluid.

III. TEST ORDERING AND PATIENT INFORMATION

Information must be provided that uniquely identifies the patient and the ordering physician and states the viral agent(s) for which the specimen is to be tested. Laboratory utilization and cost containment issues preclude the practice of ordering "viral studies" on all specimens for which a viral laboratory diagnosis is being sought, and most requests now state the specific virus(es) being considered. However, a general "viral studies" request is usually ordered if the candidate etiologic agent(s) is not clear or symptoms are not typical, or if multiple agents are associated with the same clinical presentation. In these situations it is important to obtain information (clinical history, date of onset, history of recent exposure or vaccination, pertinent travel history, and animal or arthropod bite or exposure) that will enable the laboratory scientist to select an appropriate testing algorithm and determine the need for additional assays or specimens.

IV. TRANSPORT AND STORAGE

Place the tightly capped specimen container and the laboratory requisition form into separate compartments of a plastic specimen transport bag. Deliver all specimens to the laboratory as soon after collection as possible; a loss of infectivity occurs over time, resulting in the diminished likelihood of a positive result. Loss of viability is slower at refrigeration temperatures than at ambient temperature; samples containing labile viruses (e.g., respiratory syncytial virus [RSV] or cytomegalovirus [CMV]) at low titers are those most likely to show loss of infectivity with delayed transport. If immediate delivery to the laboratory is not possible, store specimens in a refrigerator (2 to 8°C) or place them on wet ice or a cold pack. Store and transport anticoagulated blood samples at ambient temperature because chilling may interfere with cell separation techniques.

Store specimens for which culture will be delayed beyond 48 h after collection at −70°C or lower; avoid freezing at higher temperatures and freeze-thaw cycles. If possible, snap-freeze specimens in a slurry of dry ice and acetone prior to storage. With few exceptions (e.g., arboviral studies), frozen blood specimens are unsuitable for testing. Transport frozen specimens on sufficient dry ice to ensure that specimens remain frozen until receipt by the laboratory. Pack, label, and ship specimens in compliance with regulatory guidelines.

V. SPECIMEN ACCESSIONING AND EVALUATION

It is essential to utilize a manual or computerized accessioning system that uniquely identifies each specimen and stores information required for laboratory records, including patient and physician identification, specimen type, date and time of collection, specimen condition, and test(s) requested. A system must also be in place for bringing unusual requests to the immediate attention of the laboratory director or supervisor.

No laboratory procedure can compensate for improper or inappropriate specimen collection and handling. Conditions that warrant specimen rejection include drying or warm-temperature storage. In some cases, when it is not reasonable or possible to obtain a replacement sample, it is recommended that a qualifier be included in the report when reporting a negative result. Maintain a record of rejected specimens, and review the information at least monthly to identify areas where in-service training or other intervention is necessary. Periodically review collection and transport guidelines with laboratory staff, and develop a system for identifying and correcting problems with specimen collection and transport. Prepare QA reports consistent with institutional policy, and include the corrective action taken. Establish a system to follow up with corrective action and monitor compliance with recommendations.

VI. SAFETY CONSIDERATIONS

While viruses encountered in the diagnostic laboratory usually encompass BSL 2 agents, a laboratory may be called on to assist in collection and referral of specimens requiring BSL 3 or BSL 4 practices (11). Fortunately, infections involving the latter category (e.g., arboviruses, arenaviruses, filoviruses, poxviruses, and rabies) are usually associated with specific clinical presentations, travel history, and/or animal or insect vector exposures.

VII. SPECIMEN PROCESSING

A. Fluids and aspirates

◩ **NOTE:** Process vesicle aspirates collected in VTM as described for swab and scraping specimens.

Fluid specimens contain various amounts and types of cells, with virus both extra- and intracellular. Laboratories vary with respect to preinoculation specimen preparation procedures. For example, the addition of antimicrobial agents and thorough suspension of the sample is a simple but satisfactory preparatory

VII. SPECIMEN PROCESSING
(continued)

method for most fluid specimens. Alternatively, fluid specimens may be processed by suspending the cells in a small amount of VTM, diluent, or supernatant, after pelleting them by low-speed centrifugation. Yet another approach is to use clarified supernatant after cell disruption by sonication or vigorous vortexing with glass beads. Although one might suspect that the greatest culture positivity, particularly with a cell-associated virus such as CMV, would be achieved by using the cellular component, it has been demonstrated that clarified supernatant or a well-suspended, unprocessed bronchoalveolar lavage fluid sample can yield higher isolation rates than do pelleted cells suspended in VTM (1).

1. Materials
   a. Pipettes (assorted) and pipetting device
   b. Antimicrobial agent concentrate (*see* Appendix 10.4–1)
   c. Vortex mixer

2. For the procedure, see Fig. 10.4–2.

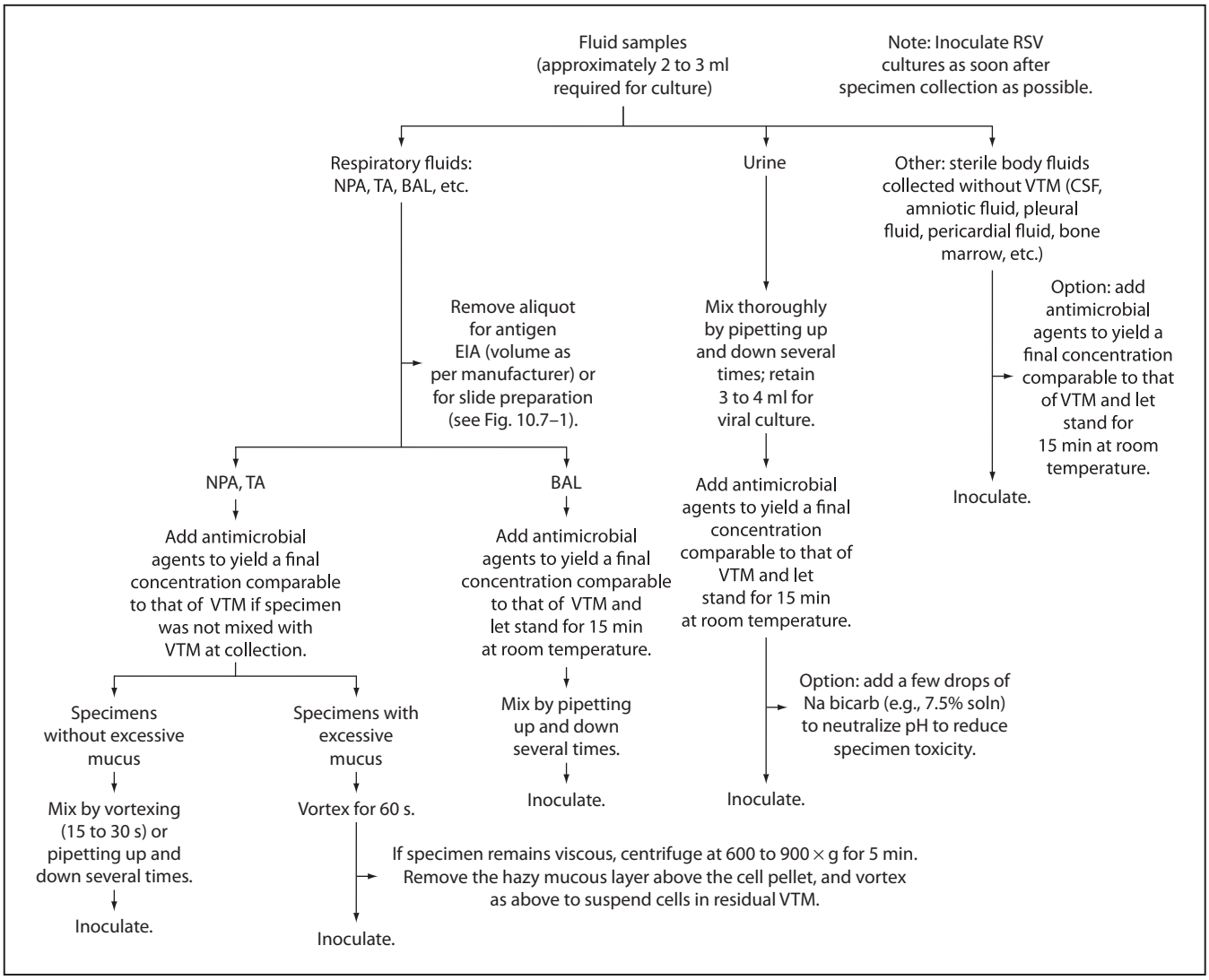
Fluid samples (approximately 2 to 3 ml required for culture)
Note: Inoculate RSV cultures as soon after specimen collection as possible.
Respiratory fluids: NPA, TA, BAL, etc.
Urine
Other: sterile body fluids collected without VTM (CSF, amniotic fluid, pleural fluid, pericardial fluid, bone marrow, etc.)
Remove aliquot for antigen EIA (volume as per manufacturer) or for slide preparation (see Fig. 10.7–1).
Mix thoroughly by pipetting up and down several times; retain 3 to 4 ml for viral culture.
Option: add antimicrobial agents to yield a final concentration comparable to that of VTM and let stand for 15 min at room temperature.
Inoculate.
NPA, TA
BAL
Add antimicrobial agents to yield a final concentration comparable to that of VTM if specimen was not mixed with VTM at collection.
Add antimicrobial agents to yield a final concentration comparable to that of VTM and let stand for 15 min at room temperature.
Add antimicrobial agents to yield a final concentration comparable to that of VTM and let stand for 15 min at room temperature.
Option: add a few drops of Na bicarb (e.g., 7.5% soln) to neutralize pH to reduce specimen toxicity.
Specimens without excessive mucus
Specimens with excessive mucus
Mix by pipetting up and down several times.
Inoculate.
Inoculate.
Mix by vortexing (15 to 30 s) or pipetting up and down several times.
Vortex for 60 s.
If specimen remains viscous, centrifuge at 600 to 900 × g for 5 min. Remove the hazy mucous layer above the cell pellet, and vortex as above to suspend cells in residual VTM.
Inoculate.
Inoculate.

Figure 10.4–2 Preparation of fluid specimens. soln, solution; NPA, nasopharyngeal aspirate; TA, tracheal aspirate; BAL, bronchoalveolar lavage fluid.

VII. SPECIMEN PROCESSING *(continued)*

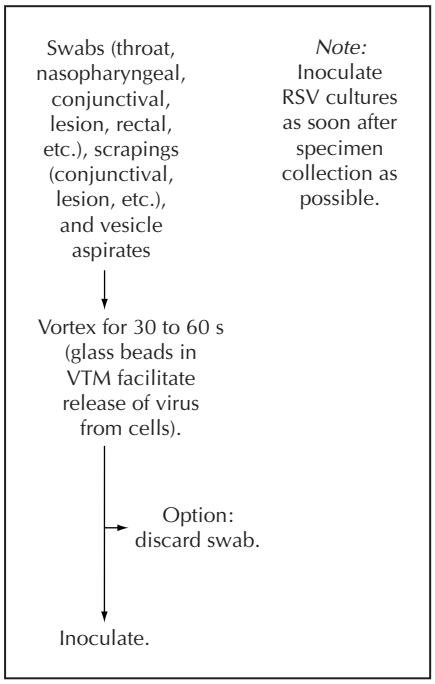
Swabs (throat, nasopharyngeal, conjunctival, lesion, rectal, etc.), scrapings (conjunctival, lesion, etc.), and vesicle aspirates
Note: Inoculate RSV cultures as soon after specimen collection as possible.
Vortex for 30 to 60 s (glass beads in VTM facilitate release of virus from cells).
Option: discard swab.
Inoculate.

Figure 10.4–3 Preparation of swabs, scrapings, and vesicle aspirates collected in VTM.

B. Swabs, scrapings, and aspirates collected in VTM

1. Materials
- **a.** Forceps
- **b.** Vortex mixer

2. For the procedure, see Fig. 10.4–3.

C. Solid specimens

1. Materials
- **a.** Pipettes (assorted) and pipetting device
- **b.** 15-ml sterile screw-cap centrifuge tubes
- **c.** Tongue blades
- **d.** Sterile petri plates
- **e.** Scalpels (for tissue samples)
- **f.** Sterile disposable tissue grinders
- **g.** Diluent: VTM or Hanks' balanced salt solution (HBSS) with antimicrobial agents
- **h.** Vortex mixer
- **i.** Centrifuge, refrigerated, with swinging bucket rotor

2. Procedure
- **a.** For details on tissue samples, see Fig. 10.4–4.
- **b.** For details on stool samples, see Fig. 10.4–5.

D. Anticoagulated blood

Viruses may be associated with various components of the blood, including PMNs (CMV), plasma (enteroviruses, arboviruses), or mononuclear cells (human immunodeficiency virus [HIV], enteroviruses, measles virus, Epstein-Barr virus). Mixed leukocyte populations (PMNs, lymphocytes, monocytes) can be recovered from anticoagulated blood by allowing RBCs to settle by gravity and harvesting the leukocyte-rich plasma. Dextran, an erythrocyte-aggregating agent, accelerates RBC sedimentation, and this method is preferred to sedimentation of untreated anticoagulated blood. Leukocytes separated by gradient centrifugation methods (3, 4) have been shown to yield higher rates of viral recovery than buffy coat preparations derived by gravity separation alone (5, 8). More recently, a modification of a rapid NH_4Cl treatment method (4) has been shown to recover WBC types in proportions similar to those found in whole blood and was useful for preparation of leukocytes for CMV culture (10).

Several cell separation products are available and should be used by following the manufacturers' instructions.

Ammonium chloride lysis method

1. Materials
- **a.** Sterile pipettes (assorted) and pipetting device
- **b.** Sterile 15-ml screw-cap centrifuge tubes
- **c.** NH_4Cl lysis: 0.155 M NH_4Cl, pH 7.2 (Appendix 10.4–2)
- **d.** Centrifuge, swinging bucket rotor

2. Procedure

NH_4Cl lysis: Fig. 10.4–6

E. Semen

Semen is not generally collected for diagnostic viral cultures, but it is sometimes used in the fertility practice and research settings (e.g., CMV, HIV type 1). Toxicity is extensive with this sample and has been shown to be associated with the enzyme-rich fluid fraction rather than the pelleted cellular elements when the sample is fractionated by centrifugation (7).

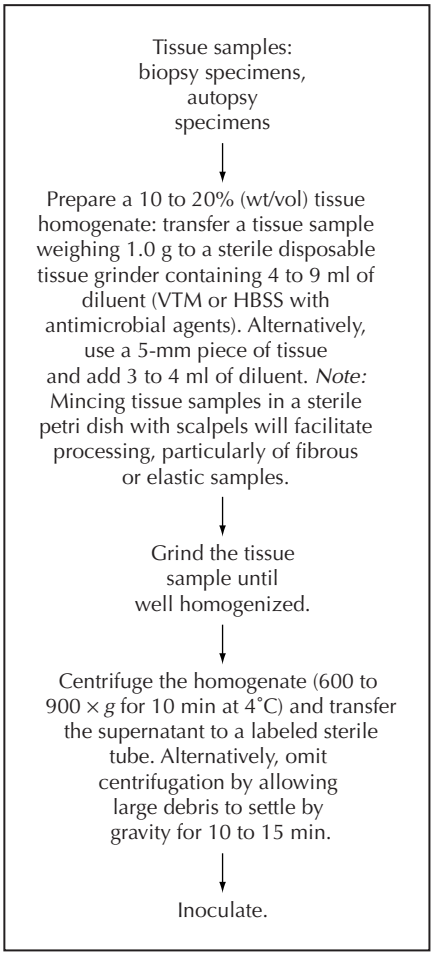
Tissue samples:
biopsy specimens,
autopsy
specimens
Prepare a 10 to 20% (wt/vol) tissue homogenate: transfer a tissue sample weighing 1.0 g to a sterile disposable tissue grinder containing 4 to 9 ml of diluent (VTM or HBSS with antimicrobial agents). Alternatively, use a 5-mm piece of tissue and add 3 to 4 ml of diluent. *Note:* Mincing tissue samples in a sterile petri dish with scalpels will facilitate processing, particularly of fibrous or elastic samples.
Grind the tissue sample until well homogenized.
Centrifuge the homogenate (600 to 900 × *g* for 10 min at 4°C) and transfer the supernatant to a labeled sterile tube. Alternatively, omit centrifugation by allowing large debris to settle by gravity for 10 to 15 min.
Inoculate.

Figure 10.4–4 Preparation of tissue samples.

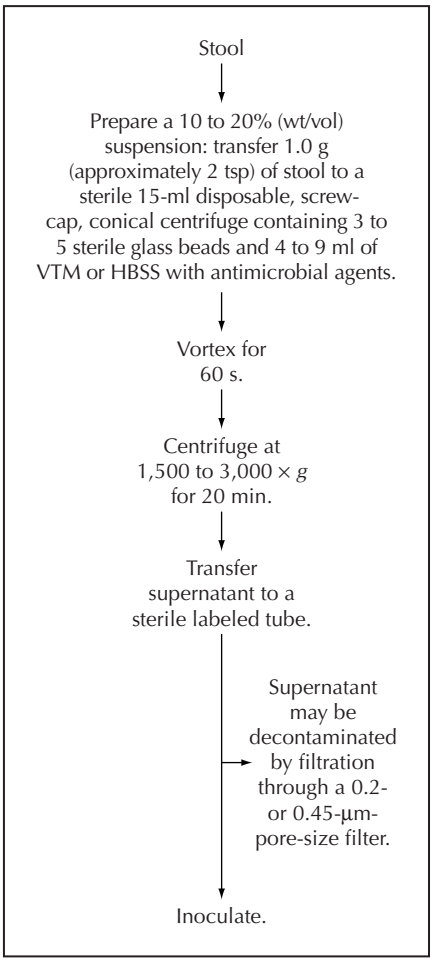
Stool
Prepare a 10 to 20% (wt/vol) suspension: transfer 1.0 g (approximately 2 tsp) of stool to a sterile 15-ml disposable, screw-cap, conical centrifuge containing 3 to 5 sterile glass beads and 4 to 9 ml of VTM or HBSS with antimicrobial agents.
Vortex for 60 s.
Centrifuge at 1,500 to 3,000 × *g* for 20 min.
Transfer supernatant to a sterile labeled tube.
Supernatant may be decontaminated by filtration through a 0.2- or 0.45-μm-pore-size filter.
Inoculate.

Figure 10.4–5 Preparation of stool samples. tsp, teaspoons.

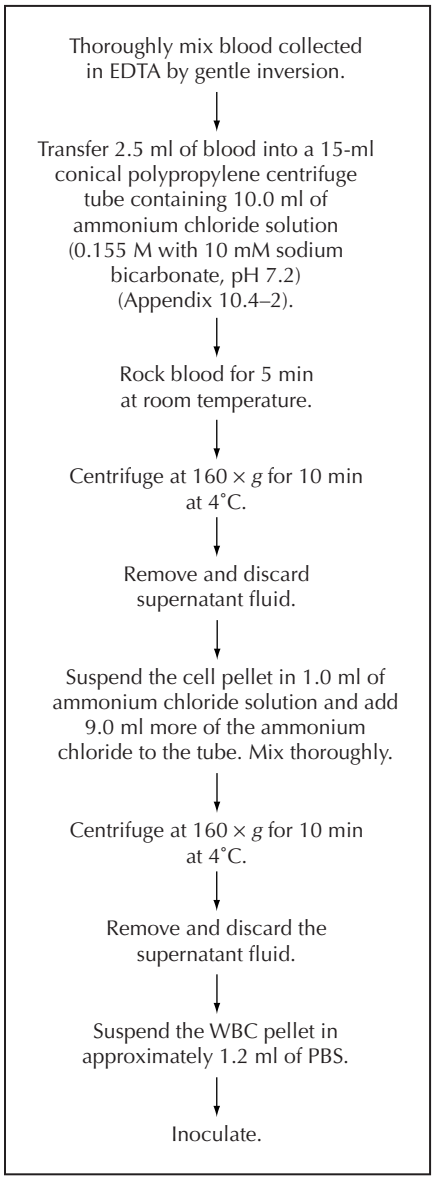
Thoroughly mix blood collected in EDTA by gentle inversion.
Transfer 2.5 ml of blood into a 15-ml conical polypropylene centrifuge tube containing 10.0 ml of ammonium chloride solution (0.155 M with 10 mM sodium bicarbonate, pH 7.2) (Appendix 10.4–2).
Rock blood for 5 min at room temperature.
Centrifuge at 160 × *g* for 10 min at 4°C.
Remove and discard supernatant fluid.
Suspend the cell pellet in 1.0 ml of ammonium chloride solution and add 9.0 ml more of the ammonium chloride to the tube. Mix thoroughly.
Centrifuge at 160 × *g* for 10 min at 4°C.
Remove and discard the supernatant fluid.
Suspend the WBC pellet in approximately 1.2 ml of PBS.
Inoculate.

Figure 10.4–6 Preparation of leukocytes from anticoagulated blood by the ammonium chloride method. For details, see reference 10. PBS, phosphate-buffered saline.

REFERENCES

1. **Clarke, L. M., B. J. Daidone, R. Inghida, M. Kirwin, and M. F. Sierra.** 1992. Differential recovery of cytomegalovirus from cellular and supernatant components of bronchoalveolar lavage specimens. *J. Clin. Pathol.* **97:**313–317.
2. **Crane, L. R., P. A. Gutterman, T. Chapel, and A. M. Lerner.** 1980. Inoculation of swab materials with herpes simplex virus. *J. Infect. Dis.* **131:**531.
3. **Eggleton, P., R. Gargan, and D. Fisher.** 1989. Rapid method for the isolation of neutrophils in high yield without the use of dextran or density gradient polymers. *J. Immunol. Methods* **121:**105–113.
4. **English, D., and B. R. Anderson.** 1974. Single-step separation of red blood cells, granulocytes and mononuclear leukocytes on discontinuous density gradients of Ficoll-Hypaque. *J. Immunol. Methods* **5:**249–259.
5. **Ferrante, A., and Y. H. Thong.** 1980. Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leukocytes from human peripheral blood by the Hypaque-Ficoll method. *J. Immunol. Methods* **36:**109–117.
6. **Howell, C. L., and M. J. Miller.** 1983. Effect of sucrose phosphate and sorbitol on infectivity of enveloped viruses during storage. *J. Clin. Microbiol.* **18:**658–662.
7. **Howell, C. L., M. J. Miller, and D. A. Bruckner.** 1986. Elimination of toxicity and enhanced cytomegalovirus detection in cell cultures inoculated with semen from patients with acquired immunodeficiency syndrome. *J. Clin. Microbiol.* **24:**657–660.
8. **Howell, C. L., M. J. Miller, and W. J. Martin.** 1979. Comparison of rates of virus isolation from leukocyte populations separated from blood by conventional and Ficoll-Paque/Macrodex methods. *J. Clin. Microbiol.* **10:**533–537.
9. **Johnson, F. B.** 1990. Transport of viral specimens. *Clin. Microbiol. Rev.* **3:**120–131.
10. **Menegus, M. A., C. M. Mayer, C. F. Mellen, and J. R. Zeller.** 1994. A simple and rapid method for the preparation of white blood cells (WBC) suitable for CMV culture. Abstr. Tenth Annual Clinical Virology Symposium, Clearwater, Fla.
11. **U.S. Public Health Service, Centers for Disease Control and Prevention, and National Institutes of Health.** 1999. *Biosafety in Microbiological and Biomedical Laboratories.* Publication no. (NIH) 93-8395, 4th ed. U.S. Government Printing Office, Washington, D.C.
12. **Warford, A. L., R. A. Levy, K. A. Rekrut, and E. Steinberg.** 1986. Herpes simplex virus testing of an obstetric population with an antigen enzyme-linked immunosorbent assay. *Am. J. Obstet. Gynecol.* **154:**21–28.

APPENDIX 10.4–1

VTM

A. HBSS with bovine serum albumin

1. Aseptically combine the following sterile reagents.

bovine serum albumin, 7.5% sterile solution	33.00 ml
HEPES, 1 M solution	10.00 ml
antimicrobial agents	variable, depending on concentrations used
HBSS, q.s. to	500.0 ml

2. The final pH should be 7.2 to 7.4 (reddish-orange color). Adjust the pH with 7.5% sodium bicarbonate solution before adding the HEPES.
3. Dispense 2.0- to 3.0-ml volumes into sterile 4-ml screw-cap tubes; dispense 5.0- to 7.0-ml volumes into 15-ml centrifuge tubes. Store frozen at −20°C for up to 6 months. Thaw just before use.

   ◩ **NOTE:** Nystatin is insoluble in water; keep material in suspension during preparation and dispensing.

   ◩ **NOTE:** Include three to five small (e.g., 5-mm) sterile glass beads in each vial and tube to facilitate specimen processing. Tubes should be able to withstand freezing at −70°C or lower for specimen storage (e.g., Nunc cryotubes; polypropylene centrifuge tubes).

APPENDIX 10.4–1 *(continued)*

B. 2-SP (0.2 M sucrose–0.02 M phosphate) medium
   1. Dissolve the following ingredients separately in approximately 300 ml of deionized water.

      KH_2PO_4 ..... 2.01 g
      Na_2HPO_4 ..... 1.13 g
      sucrose .....68.46 g

   2. Combine the solutions and add deionized water to 1,000 ml.
   3. Adjust the pH to 7.2 to 7.4.
   4. Add HEPES to a final concentration of 20 mM (20.0 ml of 1 M solution per 1,000 ml).
   5. Sterilize by using a 0.2-μm-pore-size filter.
   6. Aseptically add sterile antimicrobial agent concentrates.
   7. Dispense 2- to 3-ml volumes into sterile 4-ml screw-cap tubes; dispense 5- to 7-ml volumes into 15-ml centrifuge tubes.
   8. Store frozen at −20°C for up to 6 months. Thaw just before use. See notes above regarding nystatin and glass beads.

C. Concentration of antimicrobials in VTM

Three commonly used antimicrobial combinations are gentamicin-vancomycin-amphotericin B (or nystatin [Mycostatin]), penicillin-streptomycin-gentamicin-amphotericin B, and penicillin-streptomycin-amphotericin B (or nystatin). The following ranges (i.e., final concentrations per milliliter of transport medium or processed specimen) have been used: amphotericin, 2 to 40 μg/ml; vancomycin, 25 to 1,000 μg/ml; gentamicin, 25 to 150 μg/ml; penicillin, 50 to 55 U/ml; streptomycin, 50 to 500 μg/ml; and nystatin, 50 to 200 U/ml. The higher concentrations may be useful for specimens likely to contain high rates of microbial contamination (e.g., stool); however, high antimicrobial agent concentrations may be toxic to cell culture monolayers. Monitor toxicity by observing cell cultures that have been mock inoculated with VTM or diluent containing the antimicrobial agents.

APPENDIX 10.4–2

NH_4Cl Lysis

Ammonium chloride lysing solution (0.155 M), with 10 mM $NaHCO_3$, pH 7.2

A. Place 4.15 g of NH_4Cl and 0.42 g of $NaHCO_3$ in a 500-ml volumetric flask, add approximately 300 ml of double deionized water, swirl to dissolve, and then q.s. to 500 ml.
B. Sterilize by filtration using a 0.22-μm-pore-size filter.
C. Store at 2 to 8°C (up to 6 months).

10.5 Viral Culture: Isolation of Viruses in Cell Cultures

I. PRINCIPLE

Viral culture laboratories generally use a combination of tube and shell vial cultures. A number of variables can influence the sensitivity of viral cultures, including cell culture type, age and confluence of the monolayer, the number of tubes or vials inoculated, the inoculation and incubation conditions, and the method and reagent used for isolate detection or identification.

A. Tube cultures

Adherent cell culture monolayers are inoculated and observed for evidence of viral replication, most commonly observed as cytopathic effect (CPE) or hemadsorption (HAd). Once viral CPE or HAd is observed, isolate identification is confirmed, usually by immunofluorescence (IF).

B. Shell vial cultures

The shell vial culture method uses centrifugation-enhanced inoculation of cells monolayered on a coverslip contained in a flat-bottomed shell vial. After incubation for 1 to 5 days, the monolayers are fixed and examined, most frequently by IF, for evidence of viral infection. The overnight pre-CPE detection of cytomegalovirus (CMV) in shell vial cultures, by using a monoclonal antibody directed against a viral protein expressed in the cell nucleus within several hours after infection, introduced the concept of rapid viral cultures (10, 18). Unlike for most CMV shell vial cultures, CPE may be apparent for other viral cultures by the time staining is performed, depending on the length of incubation.

Despite the great utility of shell vial cultures, this methodology does not eliminate the utility of tube cultures. Furthermore, the use of shell vial cultures does not eliminate the need to develop cell culture expertise, including the ability to identify viral CPE and differentiate it from problems that may arise in inoculated cell cultures, even during brief incubation.

PREANALYTICAL CONSIDERATIONS

II. SPECIMENS

A. Prepare specimens as described in procedure 10.4.

B. Thaw frozen specimens rapidly and completely by swirling in a 37°C water bath until they are *almost* thawed; do not allow thawed specimens to remain at 37°C.

III. MATERIALS

A. Supplies

1. Racks designed to hold culture tubes in a slightly slanted horizontal position with the necks slightly raised
2. Sterile individually wrapped pipettes (1-, 2-, 5-, and 10-ml sizes) and safety pipetting devices
3. Cold blocks or ice
4. Personal protective gear
5. Waste containers
   Discard specimens, cell cultures, and other infectious waste based on local regulations.
6. Microscope slides (1 by 3 in.) for mounting shell vial coverslips
7. Teflon-coated microscope slides with 5- to 8-mm wells and no. 1 coverslips for IF
8. 0.22-μm-pore-size filters for culture decontamination
9. Sterile screw-cap vials (2 and 4 ml) suitable for freezing isolates at −70°C
10. Sterile 15-ml polypropylene conical centrifuge tubes
11. Labels for tubes and marking pens
12. Dry ice

B. Cell cultures

Select cell culture types based on the guidelines presented in procedure 10.2.

III. MATERIALS *(continued)*

Include QC information on reagent container and in QC records.

C. Reagents

1. Cell culture medium (*see* procedure 10.2)

2. Immunologic reagents
Reagents are available from several sources. Most antibody reagents used for viral identification are monoclonal antibodies (MAbs); because of their high specificity and lower binding efficiencies, a mixture of MAbs directed against different viral epitopes is generally more useful than individual MAb preparations. Mixtures of MAbs (blends, pools) directed against two or more viruses are also available for detecting multiple viruses with a single reagent (13, 15, 19). Recently, mixed antibody preparations labeled with different fluorescing reagents have been introduced for the simultaneous identification of common virus pairs (e.g., influenza virus types A and B [1, 3], herpes simplex virus [HSV], and CMV).

3. Sterile phosphate-buffered saline (PBS), pH 7.2 to 7.6

4. Hanks' balanced salt solution (HBSS)

5. Buffered glycerol mounting medium, pH 8.0, for IF

6. Acetone or other fixative recommended by the antibody manufacturer

7. Guinea pig blood in Alsever's solution for HAd

8. Disinfectants
Hypochlorite solution (0.5%), Wescodyne, 70% ethanol

D. Equipment

1. Microscope ($\times 100$)
Shell vials can be examined by using an inverted microscope with a glass microscope slide over the objective to support the vial. Tube cultures can be examined by using a standard or inverted microscope; tubes can be held in place with a holder consisting of two parallel tracks glued to the stage.
◩ **NOTE:** One end of the holder should be slightly higher so that the culture medium does not flow into the caps during observation.

2. Class II biological safety cabinet (certified semiannually)
Wipe down all interior cabinet surfaces with 70% ethanol or another appropriate reagent before and after each use.
◩ **NOTE:** 10% Clorox is used by many laboratories to wipe down bench surfaces; this material will cause pitting and deterioration of the stainless steel surfaces of the biological safety cabinet.

3. Centrifuge
Swinging bucket rotor with carriers to accommodate 15-ml centrifuge tubes and shell vials (15 by 45 mm); size 00 rubber stoppers may be used as cushions to adapt carriers to hold shell vials.

4. Incubator (34 to 37°C)
A CO_2 environment is not necessary for tube or shell vial cultures since these vessels are generally tightly capped during incubation. However, cell culture methods with plastic dishes, microtiter plates, or vented flasks require an atmosphere containing 5 to 8% CO_2.

5. Roller drum (optional) to hold tubes horizontally in incubator at 12 to 15 rph

6. Rotator (15 to 20 rpm) or rocker (for IF)

7. Fluorescence microscope equipped with filters suitable for fluorescein isothiocyanate (FITC) or fluorochrome of choice

8. Refrigerator at 2 to 8°C

9. Ultralow freezer, −70°C or lower

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Incubators

1. Temperature stability is important. If a universal incubation temperature of 35°C is selected, the acceptable range should be 34 to 37°C. Check incubators for hot spots and utilize a continuous temperature-monitoring system.

2. Temperatures above 36°C may adversely affect respiratory virus recovery, particularly that of rhinoviruses, which prefer 33°C.

IV. QUALITY CONTROL *(continued)*

B. Cell cultures

1. Record the lot number of cell cultures used for each specimen.

2. Uninoculated (negative) controls

a. Select several uninoculated controls from each lot. Inoculate, observe, and process in parallel with inoculated specimens.

b. Utilize uninoculated lot-matched cultures as negative controls for assays (e.g., HAd and IF).

3. Inoculated (positive) controls

a. Use viral QC stocks to assess cell culture sensitivity. Positive cell culture controls can be particularly useful when a sensitivity problem is suspected or when new culture types or sources are being evaluated. Cell culture susceptibility can be affected by several variables (e.g., monolayer density and age, mycoplasmal contamination, endogenous virus contamination), and quality can vary from lot to lot. A typical QC panel for a general viral culture laboratory consists of those viruses most likely to be encountered in the patient population served by the laboratory. Cell culture sensitivity is best determined by low virus doses or, ideally, by titrating characterized stocks (Appendix 10.5–1). However, the time and cost of performing titrations on each lot of cells are prohibitive, and laboratories generally limit use of titrations to special circumstances (e.g., reduction in isolation rate, evaluation of new cell lines or vendors, or development of a new isolation procedure). Communication among laboratories regarding suspected cell culture problems is invaluable for identifying problems with cell culture performance.

b. Use virus-inoculated cell cultures as controls for assays (e.g., HAd or IF).

◩ **NOTE:** Characterized viral QC material can be obtained from several sources, including proficiency testing samples, reference laboratories, or the ATCC (Manassas, Va., [800] 638-6597). Prepare stock material by inoculating several tubes and harvesting positive cultures as described for viral isolation; dispense in single-use aliquots and store at −70°C or lower.

C. Fetal bovine serum (FBS) and other cell culture reagents

1. Cell culture reagents must be sterile and nontoxic to cell culture monolayers.

2. Cell culture medium must maintain cells in a metabolically active condition and must not be inhibitory to viral replication.

V. PROCEDURES

A. Inoculation (Fig. 10.5–1)

1. The recommended method consists of adsorption of the prepared specimen onto a drained monolayer as described in Fig. 10.5–1. Alternatively, the specimen can be inoculated directly into the tube containing 1 to 2 ml of cell culture medium; refeed tubes with fresh medium before inoculation. The latter method may be particularly useful for specimens known to be very toxic to cell monolayers (stool suspensions, blood, bone marrow, and urine).

2. A variety of centrifugation forces and times have been used for shell vial cultures (reviewed in reference 11). Centrifugation forces of 700 to 900 × *g* for 30 to 45 min are most commonly used, although a shorter centrifugation at 3,500 × *g* has provided good results without adversely affecting monolayer integrity (5).

B. Incubation

1. Many laboratories use an incubation temperature of 35°C (34 to 37°C) for all viral cultures, while others incubate respiratory virus cultures at 33 to 34°C.

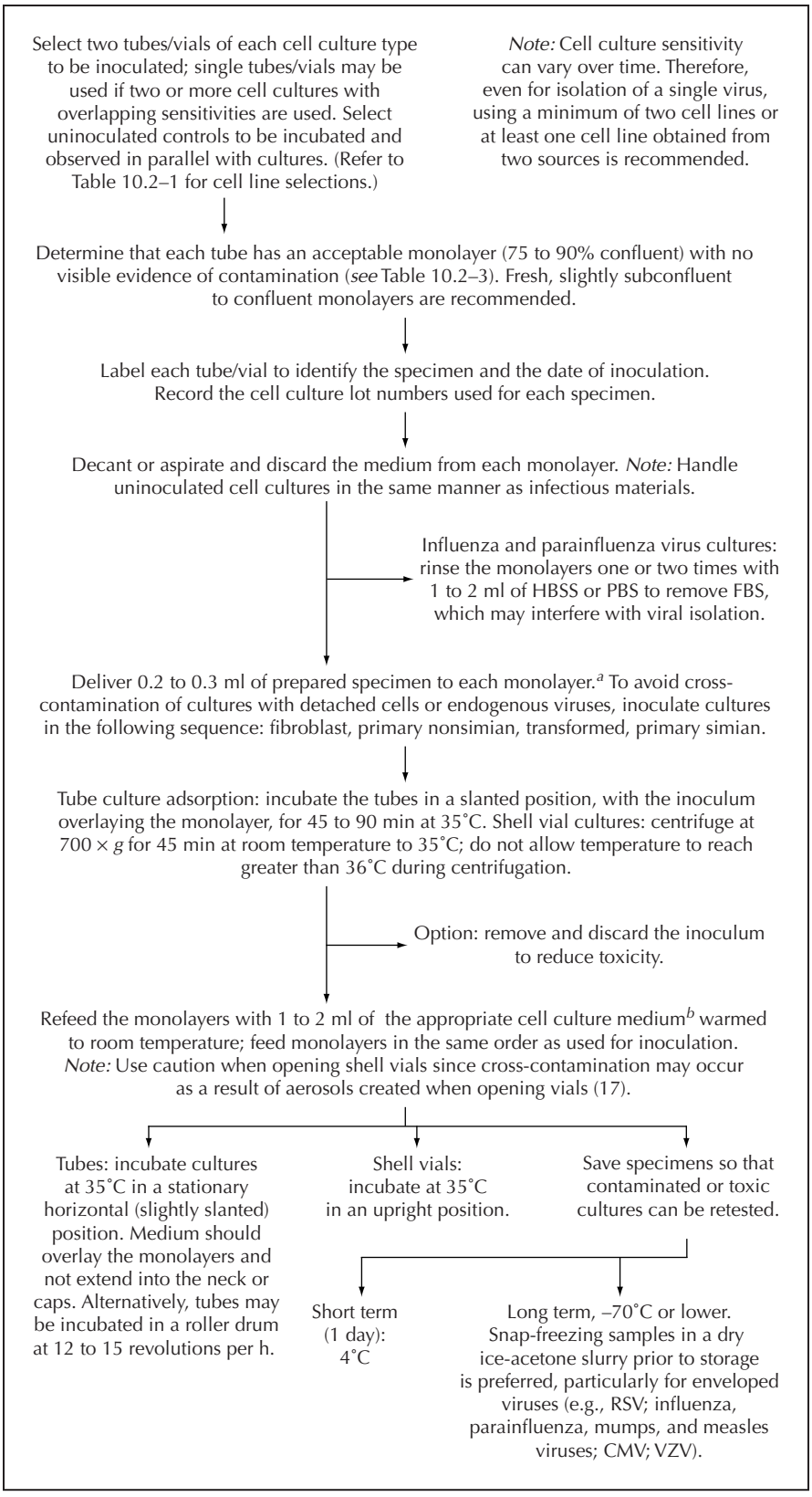
Select two tubes/vials of each cell culture type to be inoculated; single tubes/vials may be used if two or more cell cultures with overlapping sensitivities are used. Select uninoculated controls to be incubated and observed in parallel with cultures. (Refer to Table 10.2–1 for cell line selections.)
Note: Cell culture sensitivity can vary over time. Therefore, even for isolation of a single virus, using a minimum of two cell lines or at least one cell line obtained from two sources is recommended.
Determine that each tube has an acceptable monolayer (75 to 90% confluent) with no visible evidence of contamination (*see* Table 10.2–3). Fresh, slightly subconfluent to confluent monolayers are recommended.
Label each tube/vial to identify the specimen and the date of inoculation. Record the cell culture lot numbers used for each specimen.
Decant or aspirate and discard the medium from each monolayer. *Note:* Handle uninoculated cell cultures in the same manner as infectious materials.
Influenza and parainfluenza virus cultures: rinse the monolayers one or two times with 1 to 2 ml of HBSS or PBS to remove FBS, which may interfere with viral isolation.
Deliver 0.2 to 0.3 ml of prepared specimen to each monolayer.[a] To avoid cross-contamination of cultures with detached cells or endogenous viruses, inoculate cultures in the following sequence: fibroblast, primary nonsimian, transformed, primary simian.
Tube culture adsorption: incubate the tubes in a slanted position, with the inoculum overlaying the monolayer, for 45 to 90 min at 35˚C. Shell vial cultures: centrifuge at 700 × *g* for 45 min at room temperature to 35˚C; do not allow temperature to reach greater than 36˚C during centrifugation.
Option: remove and discard the inoculum to reduce toxicity.
Refeed the monolayers with 1 to 2 ml of the appropriate cell culture medium[b] warmed to room temperature; feed monolayers in the same order as used for inoculation. *Note:* Use caution when opening shell vials since cross-contamination may occur as a result of aerosols created when opening vials (17).
Tubes: incubate cultures at 35˚C in a stationary horizontal (slightly slanted) position. Medium should overlay the monolayers and not extend into the neck or caps. Alternatively, tubes may be incubated in a roller drum at 12 to 15 revolutions per h.
Shell vials: incubate at 35˚C in an upright position.
Save specimens so that contaminated or toxic cultures can be retested.
Short term (1 day): 4˚C
Long term, –70˚C or lower. Snap-freezing samples in a dry ice-acetone slurry prior to storage is preferred, particularly for enveloped viruses (e.g., RSV; influenza, parainfluenza, mumps, and measles viruses; CMV; VZV).

Figure 10.5–1 Inoculation of tube and shell vial cell cultures for the detection of cytopathic and hemadsorbing viruses (*see* Table 10.5–1). RSV, respiratory syncytial virus.

[a] CMV antigenemia or molecular assays (e.g., PCR) have largely supplanted cell culture for detecting CMV in blood samples. When using shell vial cultures for recovery of CMV from blood, inoculate three vials with approximately 1.5×10^6 leukocytes per vial. It has been reported that a total of at least 4×10^6 leukocytes is recommended for the sensitive detection of CMV viremia using human lung fibroblasts (2); concentrations of $>2 \times 10^6$ cells per vial are not generally recommended because of toxicity.

[b] Eagle minimal essential medium (EMEM) supplemented with 2% heat-inactivated FBS is a medium suitable for all or most viral cultures performed in the diagnostic setting with monolayered cell cultures; serum-free EMEM or other serum-free cell culture medium is used by many laboratories for the isolation of influenza and parainfluenza viruses. Medium containing trypsin is recommended for influenza virus cultures when using MDCK cells (*see* Table 10.2–1).

V. PROCEDURES *(continued)*

2. Incubate shell vials in an upright position. Tubes may be incubated in a stationary horizontal position or in a roller drum. Low-speed rolling (12 to 15 rph) has been shown to enhance recovery of several viruses (reviewed in reference 11).

   ◩ **NOTE:** Tissue degeneration may accelerate for some cell lines during roller incubation.

3. Observe inoculated tube cultures for 10 to 14 days after inoculation. Longer incubation times, up to 4 to 6 weeks, have traditionally been used for CMV tube cultures, particularly for isolation from blood and tissue specimens containing low concentrations of virus. However, extended culture times may have little clinical value. Time points for terminating shell vial cultures also vary. For example, some laboratories perform staining for CMV at 24 h, while others stain at 48 h, and yet others stain at both time points. Incubation times for shell vial cultures for other viruses range from 24 h to 4 to 5 days, depending on the virus and the laboratory's experience with sensitivity at different time points compared with that of tube cultures.

C. Observation of inoculated monolayers

1. Observe monolayers for the development of viral CPE and/or HAd (Fig. 10.5–2) and problems and conditions sometimes encountered with cell cultures (Fig. 10.5–3); record all observations and manipulations.
2. Most of the human viruses encountered in the diagnostic virology laboratory produce a characteristic CPE (Table 10.5–1). CPE represents a composite of morphological changes that may include swelling, rounding, clumping, and increased refractility of cells; cytoplasmic vacuolation, granulation, and nuclear condensation; and the development of multinucleated giant cells (syncytia). It is usually progressive and may lead to complete monolayer destruction. The appearance, the time required for the development of CPE, and the rate of progression are determined not only by the type and dose of virus in the inoculum but also by the monolayer type, age, and density. These characteristics also provide a basis for tentative identification of the viral isolate. Commonly encountered CPEs are illustrated in Fig. 10.5–4, parts 1 to 7.
3. Endogenous viruses can also produce CPE, including many simian viruses. Simian virus 40 (SV40) (vacuolating virus) produces cytoplasmic vacuoles, and simian foamy virus produces large vacuoles resembling soap foam. Simian adenoviruses, herpes B virus, enteroviruses, reovirus, and measles virus can produce CPE mimicking the CPE produced by their human counterparts and can cause human infections associated with high mortality (herpes B virus, Marburg virus).

D. HAd procedure

1. HAd with guinea pig RBCs (*see* Fig. 10.5–5) is a simple and inexpensive way to screen inoculated cell cultures to detect influenza, parainfluenza, mumps, and Newcastle disease viruses. Perform this procedure as shown in Fig. 10.5–6.
2. SV5, a simian parainfluenza virus, hemadsorbs guinea pig RBCs and is an important endogenous contaminant of primary monkey kidney cells.
3. HAd, the attachment of RBCs to infected cells, is mediated by hemagglutinins, viral envelope proteins that are inserted into the plasma membranes of infected cells during viral replication.
4. Not all hemadsorbing viruses produce CPE (Table 10.5–1).

E. Isolate detection and identification

1. Direct or indirect IF with MAbs is the method most frequently used by diagnostic laboratories to identify viral isolates in tube and shell vial cultures. FITC is the most commonly used fluorochrome, with specific staining appearing as bright to brilliant apple-green fluorescence.

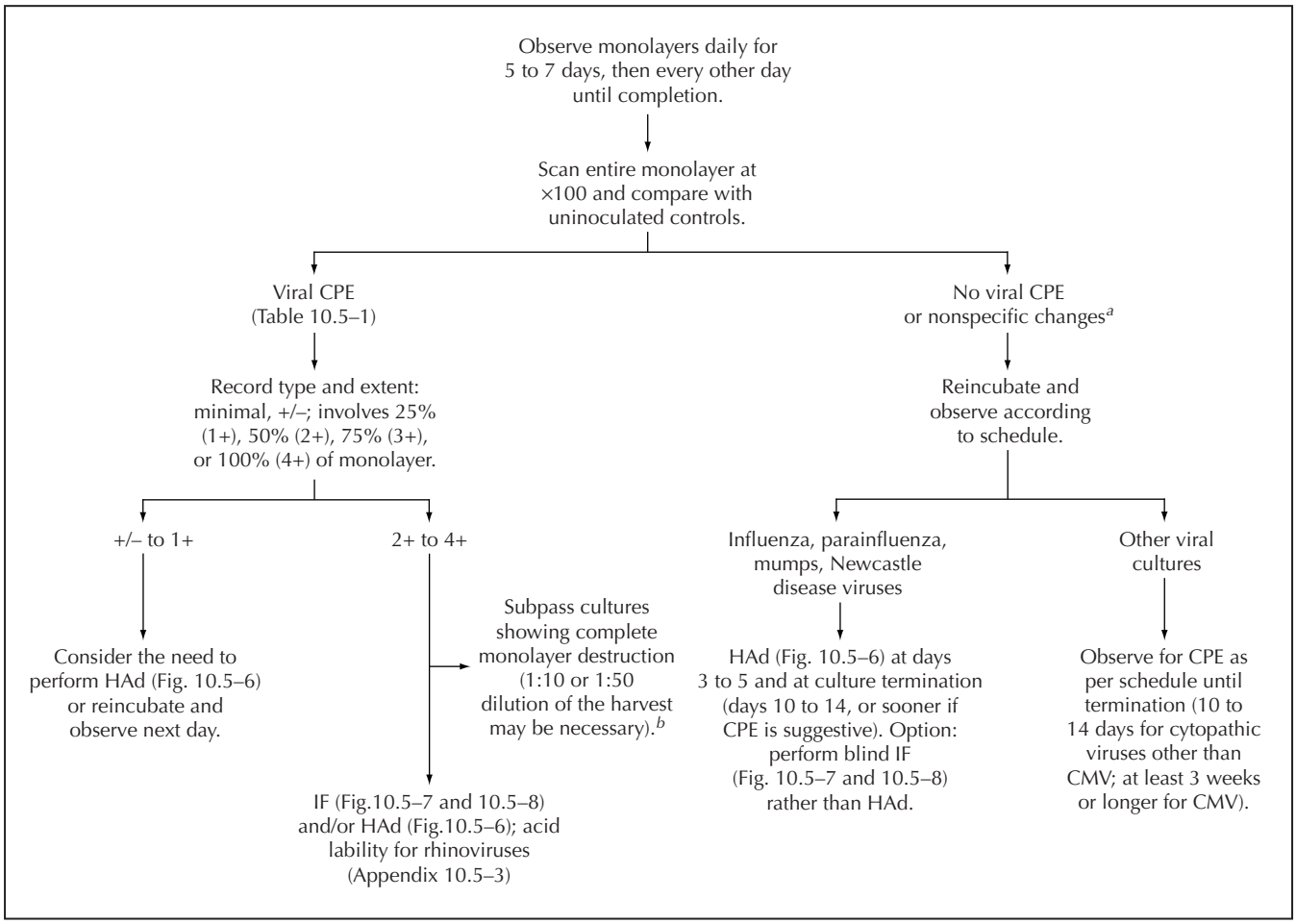
Observe monolayers daily for 5 to 7 days, then every other day until completion.
Scan entire monolayer at ×100 and compare with uninoculated controls.
Viral CPE (Table 10.5–1)
No viral CPE or nonspecific changes[a]
Record type and extent: minimal, +/–; involves 25% (1+), 50% (2+), 75% (3+), or 100% (4+) of monolayer.
Reincubate and observe according to schedule.
+/– to 1+
2+ to 4+
Influenza, parainfluenza, mumps, Newcastle disease viruses
Other viral cultures
Consider the need to perform HAd (Fig. 10.5–6) or reincubate and observe next day.
Subpass cultures showing complete monolayer destruction (1:10 or 1:50 dilution of the harvest may be necessary).[b]
HAd (Fig. 10.5–6) at days 3 to 5 and at culture termination (days 10 to 14, or sooner if CPE is suggestive). Option: perform blind IF (Fig. 10.5–7 and 10.5–8) rather than HAd.
Observe for CPE as per schedule until termination (10 to 14 days for cytopathic viruses other than CMV; at least 3 weeks or longer for CMV).
IF (Fig.10.5–7 and 10.5–8) and/or HAd (Fig.10.5–6); acid lability for rhinoviruses (Appendix 10.5–3)

Figure 10.5–2 Observation of inoculated monolayers: CPE and HAd. *Note:* Rarely, cells exhibiting minimal CPE revert to a normal appearance. Subpassage or reinoculation may be helpful in this instance. RSV, respiratory syncytial virus.
[a] See Fig. 10.5–3.
[b] Viral viability may decline rapidly with incubation in the absence of cells, particularly with labile viruses (e.g., RSV, CMV).

V. PROCEDURES *(continued)*

2. Prepare slides from tube cultures for isolate identification by IF or immunoperoxidase staining as described in Fig. 10.5–7. This procedure yields cell spots suitable for culture confirmation of isolates obtained in tube cultures.
3. Perform IF for tube culture confirmation as described in Fig. 10.5–8. IF staining patterns vary with the virus and the antibody preparation used (Fig. 10.5–9).
4. For shell vial cultures, establish the time points for fixing and staining monolayers and follow the procedure described in Fig. 10.5–10.
5. Perform IF on shell vial coverslips as described in Fig. 10.5–11. IF staining patterns vary with the virus and the antibody preparation used (Fig. 10.5–9).
6. Commercial reagents are supplied either at the appropriate working dilution or with instructions for preparing the optimal working dilution; products generally contain a counterstain such as trypan blue which stains cells to aid in visualization of cells and provide a contrasting background color for the IF staining.

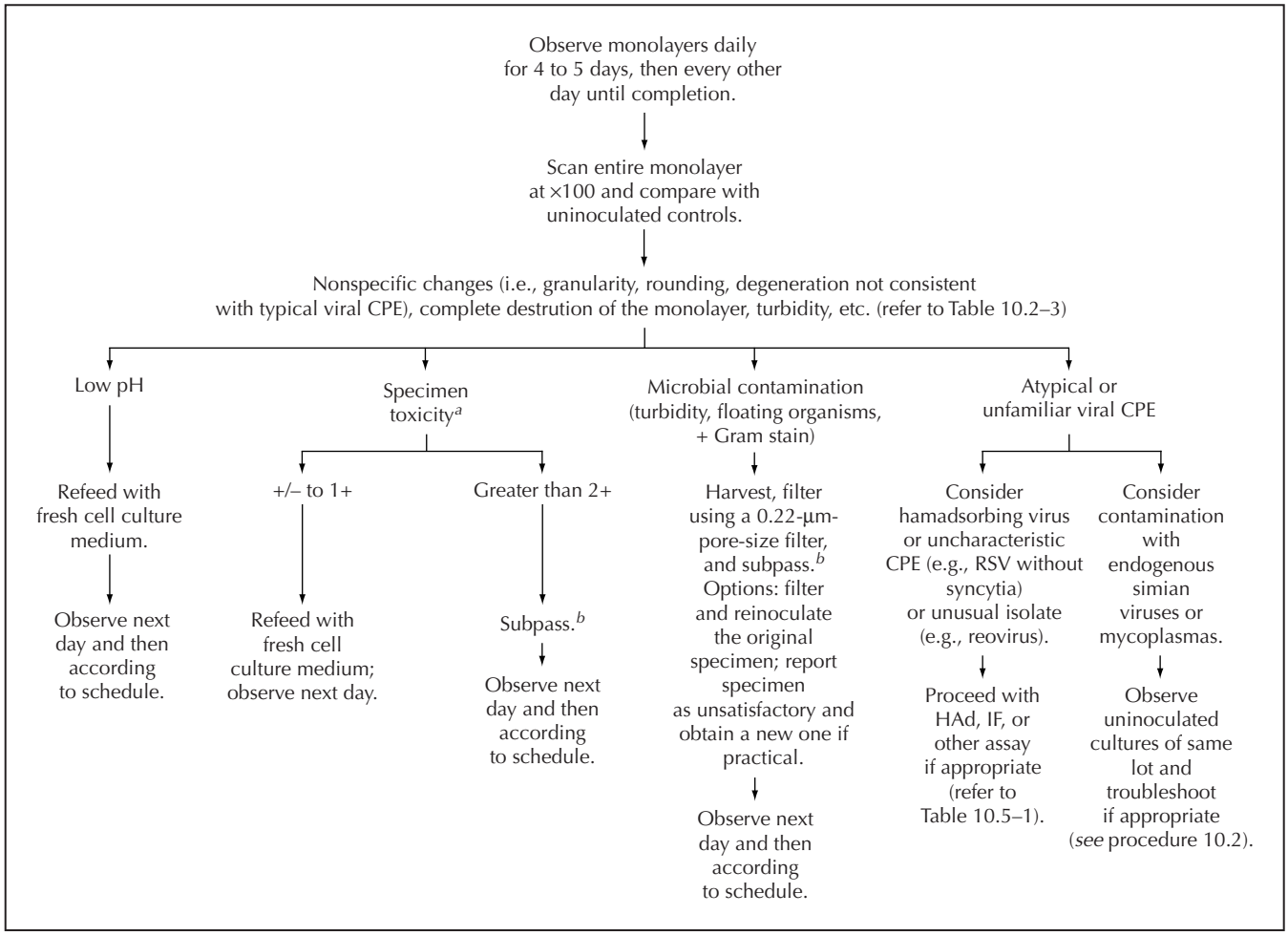
Observe monolayers daily for 4 to 5 days, then every other day until completion.
Scan entire monolayer at ×100 and compare with uninoculated controls.
Nonspecific changes (i.e., granularity, rounding, degeneration not consistent with typical viral CPE), complete destrution of the monolayer, turbidity, etc. (refer to Table 10.2–3)
Low pH
Refeed with fresh cell culture medium.
Observe next day and then according to schedule.
Specimen toxicity[a]
+/– to 1+
Refeed with fresh cell culture medium; observe next day.
Greater than 2+
Subpass.[b]
Observe next day and then according to schedule.
Microbial contamination (turbidity, floating organisms, + Gram stain)
Harvest, filter using a 0.22-µm-pore-size filter, and subpass.[b] Options: filter and reinoculate the original specimen; report specimen as unsatisfactory and obtain a new one if practical.
Observe next day and then according to schedule.
Atypical or unfamiliar viral CPE
Consider hamadsorbing virus or uncharacteristic CPE (e.g., RSV without syncytia) or unusual isolate (e.g., reovirus).
Proceed with HAd, IF, or other assay if appropriate (refer to Table 10.5–1).
Consider contamination with endogenous simian viruses or mycoplasmas.
Observe uninoculated cultures of same lot and troubleshoot if appropriate (*see* procedure 10.2).

Figure 10.5–3 Observation of inoculated cell cultures: toxicity, microbial contamination, and atypical changes. RSV, respiratory syncytial virus.
[a] In rare cases, specimen toxicity may be so great that two or three subpassages are required. Unlike viral CPE, toxicity may sometimes be observed at 3 to 4 h after inoculation.
[b] Viral viability may decline rapidly with incubation in the absence of cells, particularly with labile viruses (e.g., respiratory syncytial virus, CMV).

V. PROCEDURES *(continued)*

7. In addition to IF, immunoperoxidase staining, solid-phase EIA, or nucleic acid hybridization (8, 12) can also be used to identify isolates.
8. Other immunologic methods are occasionally used by the diagnostic laboratory (e.g., neutralization for typing enteroviruses, adenoviruses [Appendix 10.5–2]; HAd inhibition, hemagglutination inhibition [HI] for subtyping influenza virus isolates). Alternatively, laboratories may arrange to send isolates to reference or public health laboratories when typing or subtyping is important (e.g., influenza surveillance).
9. Immunologic reagents are not available for identification of rhinoviruses; these viruses are distinguished from enteroviruses with the acid lability assay (Appendix 10.5–3).

F. Rubella interference assay (*see* Appendix 10.5–4)

Table 10.5–1 Viral culture characteristics[a]

Virus	Characteristic CPE[e]				Isolate identification	Shell vial applications
	Appearance	Development[b] (days)	Progression	Comment(s)		
Adenoviruses	Enlarged, rounded cells in tightly associated grape-like clusters. Some isolates may produce a lattice-type arrangement of rounded cells.	4–7	Moderate	CPE is less characteristic in diploid fibroblasts.	IF (group-specific antibody), neutralization[c] useful for typing (Appendix 10.5–2)	Sensitivity may be low compared with that of tube cultures (15, 16).
Coronaviruses						
NL63	Foci of rounded cells and monolayer detachment and syncytium formation (6a)	7–10	Slow		PCR	
SARS-CoV	Foci of rounded and detached cells (4a, 11a)	2–5	Rapid	Replicates in RhMK, CMK, and ML cells without CPE after short-term incubation (7a)	PCR	
CMV	Plump, rounded cells in elongated foci parallel to the long axis of the cell	7–10	Slow	CPE may take 2–3 weeks or longer to develop with low viral concentrations; CPE may develop rapidly with specimens containing high viral concentrations. Some isolates may not progress beyond a few patches of CPE.	IF; identification of isolates with minimal CPE may be facilitated by subpassing harvested cells and fluid into centrifugation-enhanced shell vial cultures.	The shell vial technique has largely supplanted tube culture for this agent.
Enteroviruses	Rounded, highly refractile cells in loose clusters or dispersed throughout monolayer: varies with enterovirus group.	2–5	Moderate to rapid, depending on the virus		IF (panenterovirus MAb, polio blends, type-specific MAbs); isolates not confirmable by IF may be identified by neutralization[c] (Appendix 10.5–2). A panenterovirus MAb has been shown to be useful for culture confirmation of many isolates. Ruling out poliovirus is important and can be accomplished by using a blend of MAbs to the three poliovirus types.	

(continued)

Table 10.5–1 Viral culture characteristics[a] *(continued)*

Virus	Characteristic CPE[e]				Isolate identification	Shell vial applications
	Appearance	Development[b] (days)	Progression	Comment(s)		
HSV	Clusters of rounded, ballooned cells with or without syncytia. Early CPE is focal but then progresses throughout the monolayer.	1–3	Moderate to rapid	CPE may develop more slowly and be less characteristic in human fibroblasts. Simian B virus produces a similar CPE in simian cells.	IF (type-specific MAbs, type 1-type 2 blends)	Shell vials may yield a less dramatic improvement over tube cultures since most isolates can be detected from genital specimens collected from symptomatic individuals within 24–72 h after inoculation of tube cultures. However, shell vial cultures may improve the recovery of HSV in less sensitive cell lines and with specimens containing low viral concentrations (22).
Influenza virus	Variable. No CPE may be produced or may include granular and vacuolated appearance or nonspecific degeneration. Rounded refractile cells may be associated with type B.	3–5	Moderate	HAd with guinea pig RBCs; degree of HAd is independent of the presence or degree of CPE. Blind IF (e.g., at days 3–5 and/or at culture termination) rather than HAd may enhance isolation. Incorporate trypsin in medium when using MDCK cells (6, 20).	IF (type-specific MAbs, dual-labeled mixtures [1, 3], pools containing antibodies against several respiratory viruses). Subtyping by IF, HI	Shell vials have been shown to be useful for respiratory viruses (6, 12), including the use of MAb pools (13, 15, 16, 18). Staining performed at 1–4 days
Measles virus	Syncytium formation. Nuclei may encircle granular mass of giant cell. Extensive vacuolization may also be present.	5–10	Slow to moderate		IF	A-549 useful (urine specimens required longer [up to 5 days] than NP swabs [18–36 h] [14])
Metapneumovirus	Foci of rounded cells and monolayer detachment and/or syncytium formation (4a, 10a, 12a, 20a)	10–17	Slow	Replicates in HEp-2 cells without CPE (4a)	PCR, IF (15a)	

Mumps virus	Cell rounding and syncytium formation. May appear as nonspecific-looking granularity with progressive degeneration.	3–7	Moderate	HAd with guinea pig RBCs	IF	
Parainfluenza viruses	Variable, with increased rounding, granularity, and progressive degeneration; syncytium formation associated with types 2 and 3.	3–7; type 4 may require incubation beyond the usual 10–14 days	Moderate	HAd with guinea pig RBCs; type 4 hemadsorbs better at room temp or 37°C than at the usual incubation temp of 4°C (4) Blind IF (e.g., at days 3–5 and/or at culture termination) may enhance isolation. Incorporate trypsin in medium when using LLC-MK2 cells (7).	IF (type-specific MAbs, pools containing antibodies against several respiratory viruses, including parainfluenza virus types 1, 2, and 3)	Shell vials have been shown to be useful for respiratory viruses (12, 13, 15, 16, 19). Staining performed at 1–4 days.
Reoviruses	Nonspecific-looking granular appearance with progressive degeneration and detaching of the monolayer	7–10	Slow to moderate	CPE may be difficult to distinguish from nonspecific monolayer degeneration.	Neutralization[c] (Appendix 10.5–2)	
RSV[d]	Syncytia develop in some cell lines, particularly subconfluent HEp-2 cells. May also appear as granular progressive degeneration.	3–5	Moderate	Blind IF (e.g., at days 3–5 and/or at culture termination) may enhance isolation.	IF (type-specific MAbs, pools containing antibodies against several respiratory viruses)	Shell vials have been shown to be useful for respiratory viruses (12, 13, 15, 16, 19). Staining performed at 1–4 days; longer incubation shown to detect more isolates (15).
Rhinoviruses	Enterovirus-like	5–7	Moderate	Incubation at 33°C preferred.	Acid lability assay (Appendix 10.5–3)	
Rubella virus	CPE not produced on primary isolation				Interference assay (Appendix 10.5–4), IF	Shell vial technique appears useful (21).

(continued)

Table 10.5–1 Viral culture characteristics[a] *(continued)*

Virus	Characteristic CPE[e]				Isolate identification	Shell vial applications
	Appearance	Development[b] (days)	Progression	Comment(s)		
Vaccinia virus	Syncytia and clusters of rounded, enlarged, refractile cells with cytoplasmic strands frequently bridging foci of CPE	1–3	Moderate to rapid	HAd with chicken RBCs	IF, neutralization[c] (Appendix 10.5–2)	
VZV	Foci of enlarged, rounded, refractile cells with or without syncytia Cytoplasmic strands and granularity may be prominent as CPE progresses.	4–7	Slow to moderate		IF; identification of isolates with minimal CPE may be facilitated by subpassing harvested cells and fluid into centrifugation-enhanced shell vial cultures.	Shell vials (9) preferred; stain one vial at 24 to 48 h and second vial at day 5 or sooner if CPE develops.

[a] Certain viruses require specialized culture techniques or are noncultivatable. Refer to Table 10.5–2.

[b] Time generally required for majority of isolates to produce CPE or HAd.

[c] Neutralization assay involves mixing the isolate with antiserum containing neutralizing antibodies and, after a brief incubation, inoculating the cell cultures with the virus-antibody mixture(s). Neutralization by an antibody is determined by the failure to develop CPE, thereby establishing viral identity (Appendix 10.5–2).

[d] RSV, respiratory syncytial virus.

[e] See Fig. 10.5–4, parts 1 to 6.

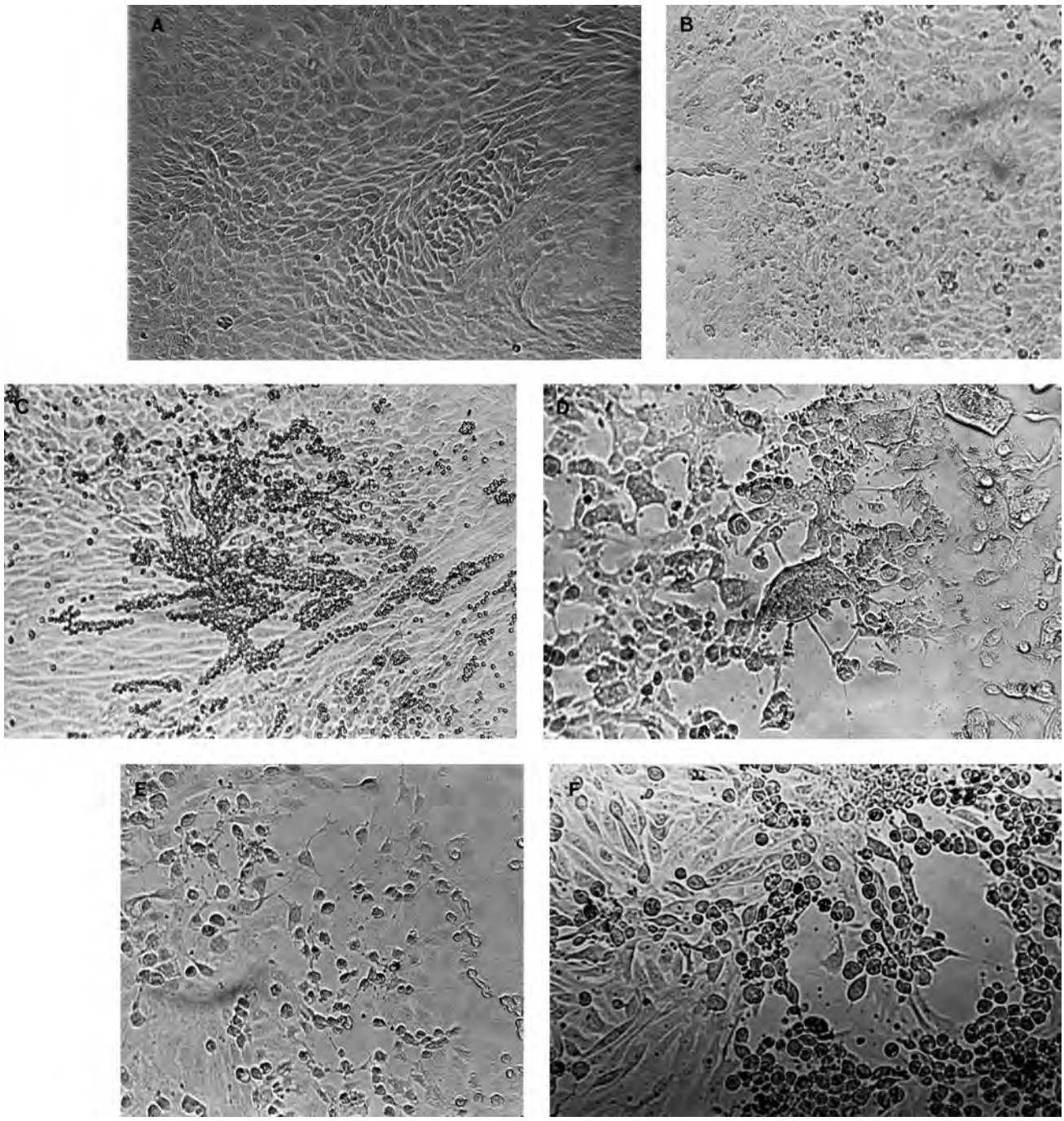

Figure 10.5–4 (part 1) Primary rhesus monkey kidney cell cultures. Original magnification, ×200. (A) Uninoculated confluent monolayer; (B) influenza virus type B-infected culture showing granular cytopathic changes produced by some influenza virus strains; (C) culture infected with influenza virus type A, showing HAd of guinea pig RBCs; (D) cellular rounding and syncytium formation produced by measles virus; (E) culture infected with coxsackievirus type B3 showing scattered rounded refractile cells frequently referred to as enterovirus-like CPE; (F) CPE produced by echovirus type 11 (courtesy of CDC).

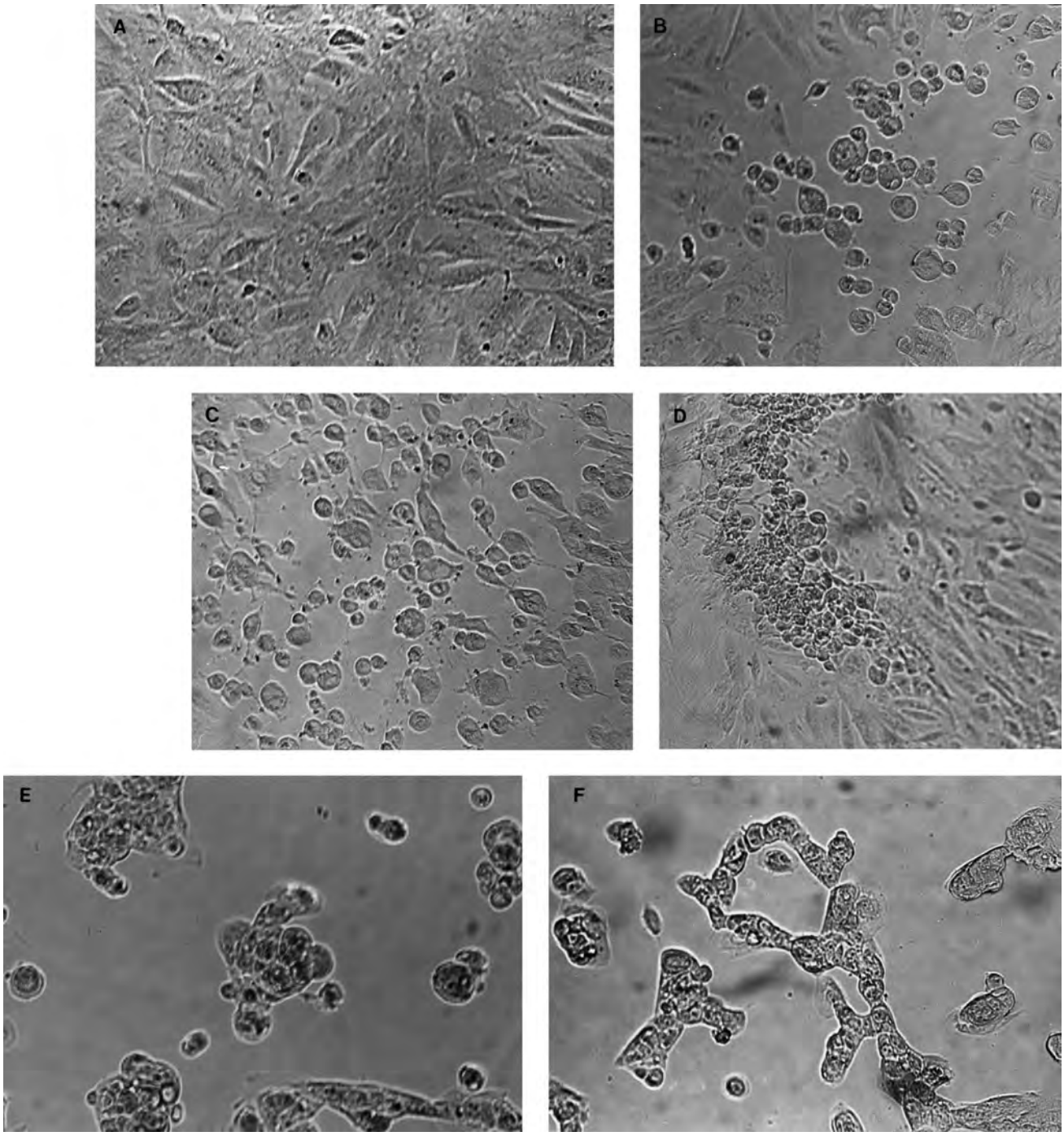

Figure 10.5–4 (part 2) Low-passage-number human neonatal kidney cell cultures. Original magnification, ×200. (A) Uninoculated confluent monolayer. (B) Early focus of enlarged rounded cells produced by HSV type 2. (C) Extensive involvement of the monolayer by the rapidly progressive CPE of HSV type 2. (D) Focal area of CPE produced by VZV. Progression of cytopathic changes produced by this virus is slower than with infection by HSV. (E) Culture infected with adenovirus type 3 showing the tightly overlapped rounded cells frequently referred to as grapelike clusters. (F) Latticed arrangement of cells that may result from infection by adenoviruses.

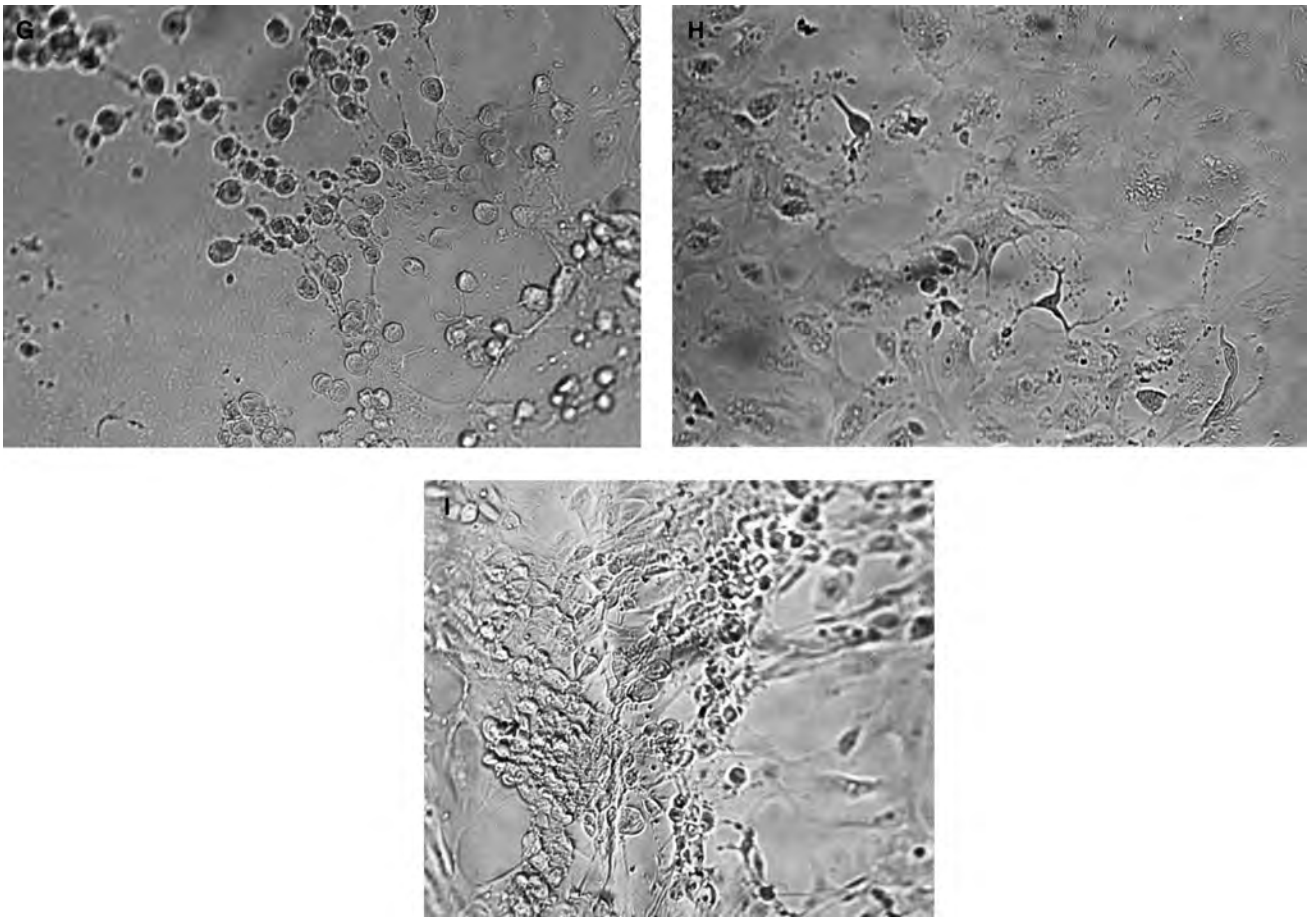

Figure 10.5–4 (part 2) *(continued)* (G) Generalized rounding and extensive monolayer destruction caused by echovirus type 6. (H) Early cytopathic changes produced by coxsackievirus type B3. (I) Nonspecific type of CPE observed with infection by reoviruses. Monolayer shows cellular rounding, degeneration, and lifting from the glass surface.

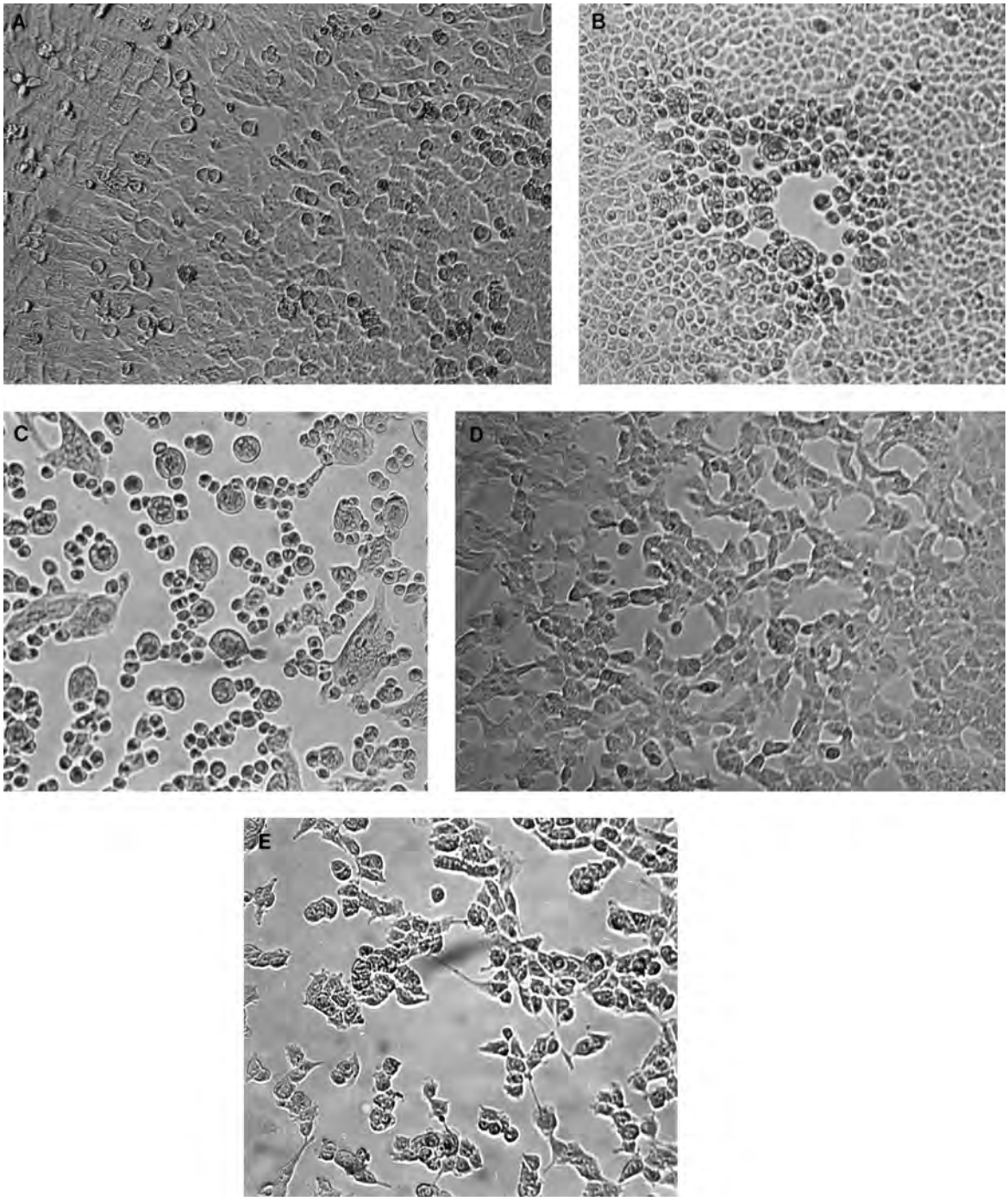

Figure 10.5–4 (part 3) A-549 cells. Original magnification, ×200. (A) Uninoculated culture showing overgrowth of the monolayer typical of rapidly growing continuous cell lines; (B) early focus of CPE produced by HSV type 2 against a background of uninfected confluent cells; (C) advanced CPE of HSV type 2; (D) early CPE of adenovirus type 3; (E) advanced CPE of adenovirus type 3.

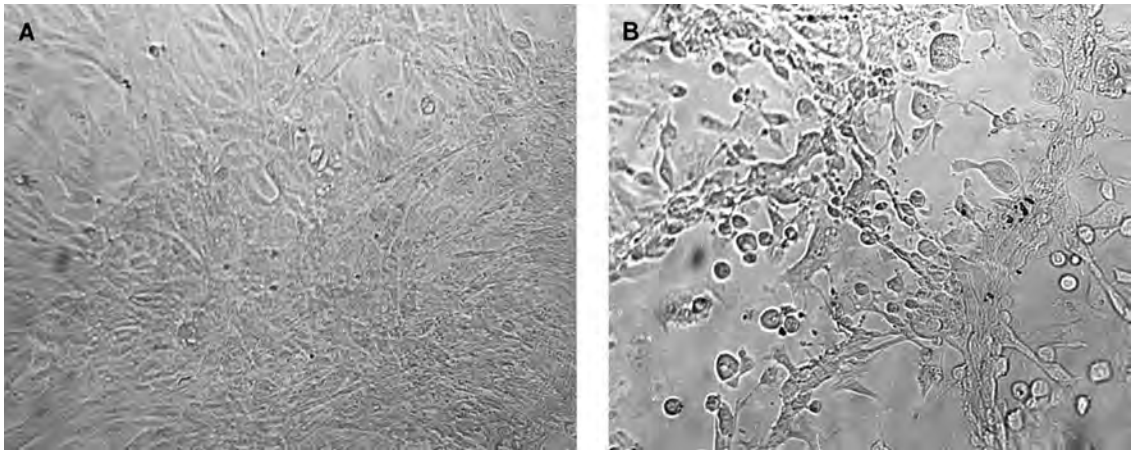

Figure 10.5–4 (part 4) Primary rabbit kidney cell cultures. Original magnification, ×200. (A) Uninoculated confluent monolayer; (B) rapidly progressive CPE produced by HSV type 2.

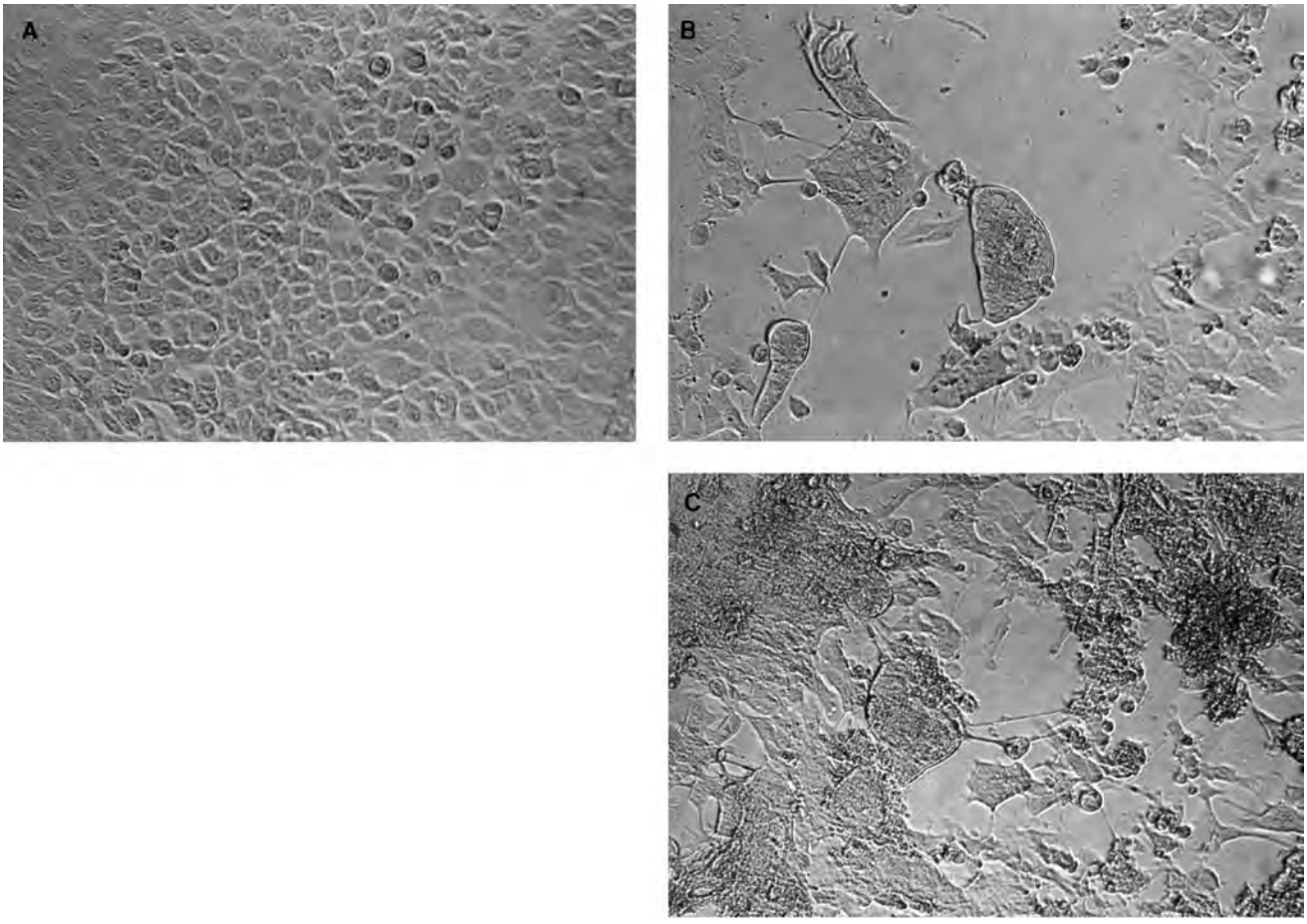

Figure 10.5–4 (part 5) HEp-2 cell cultures. Original magnification, ×200. (A) Uninoculated confluent monolayer; (B) CPE produced by respiratory syncytial virus showing numerous syncytia formed as a result of cell fusion; (C) syncytium formation resulting from infection with measles virus.

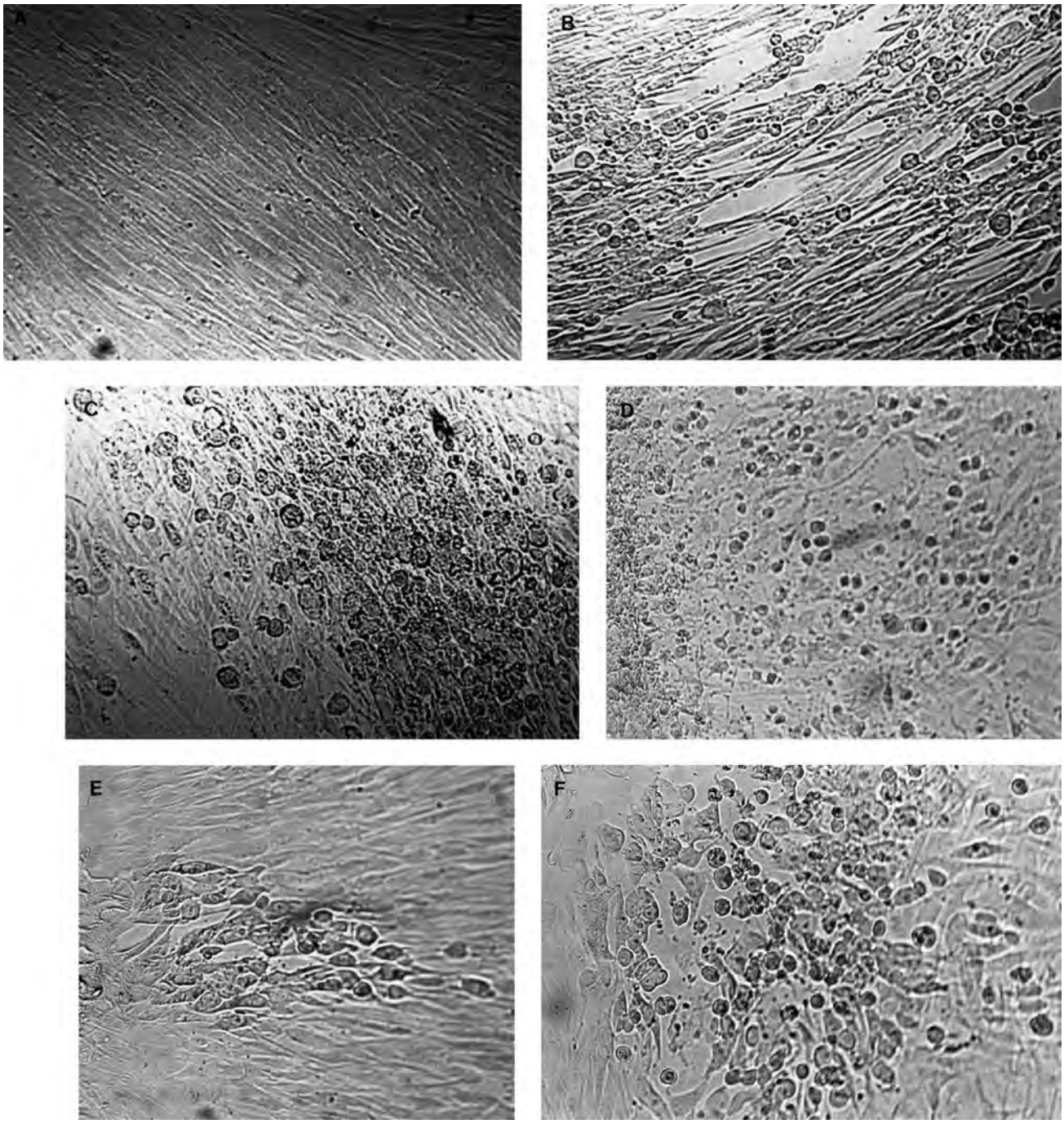

Figure 10.5–4 (part 6) Diploid human lung fibroblast cultures. Magnification, ×200. (A) Uninoculated confluent monolayer. (B) Advanced CPE produced by HSV type 2 (courtesy of CDC). (C) Focal area of CPE produced by VZV (courtesy of CDC). (D) Advanced CPE of VZV. (E) Focal area of CPE produced by CMV. This CPE usually progresses slowly. (F) Large focal area of late CPE produced by CMV.

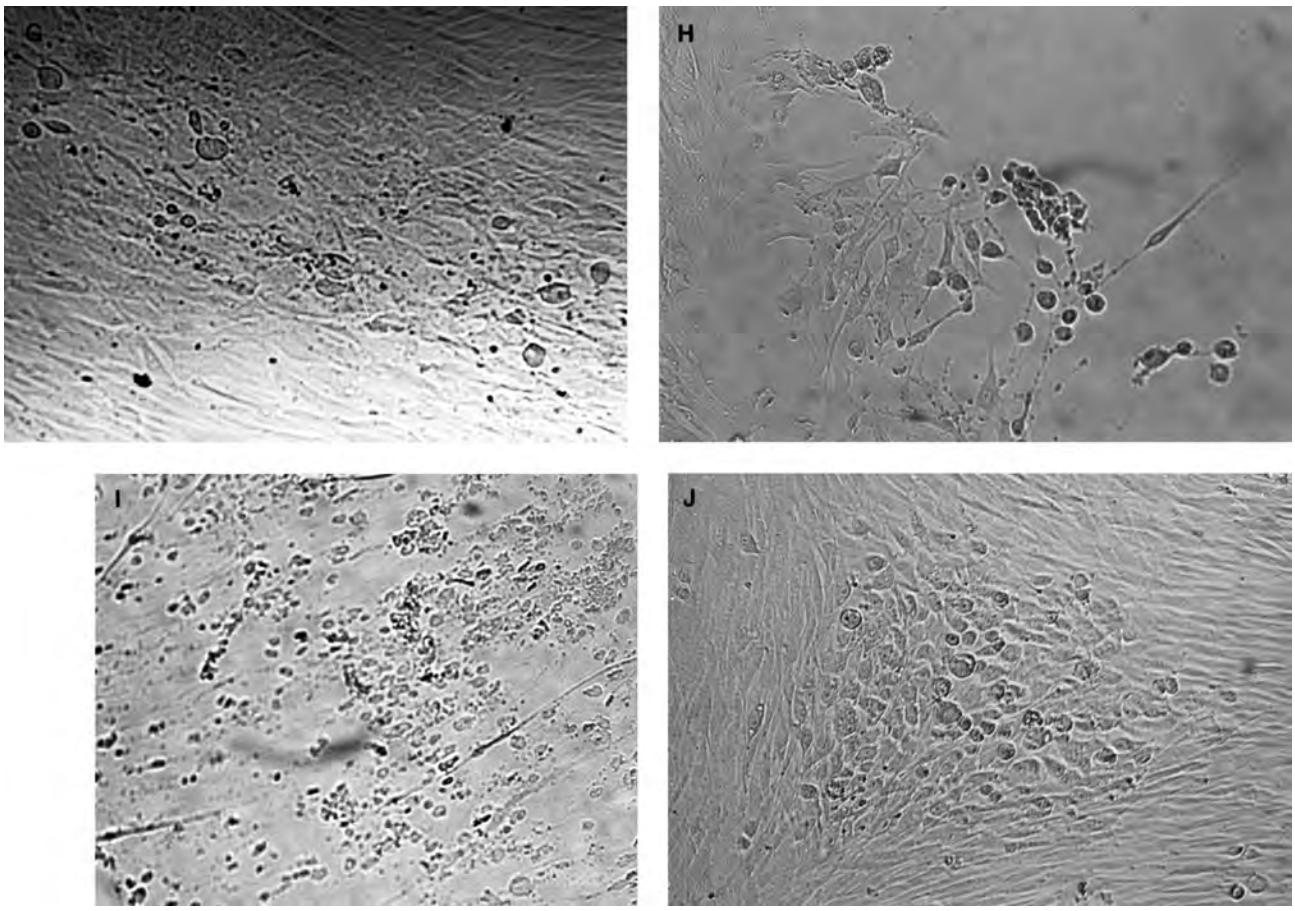

Figure 10.5–4 (part 6) *(continued)* (G) Late CPE produced by adenovirus (courtesy of CDC). (H) CPE of echovirus type 11 starting at the monolayer edge. (I) Late CPE of echovirus type 11 showing complete involvement of the monolayer. (J) Focal area of replicating A-549 cells that have inadvertently cross-contaminated an MRC-5 monolayer.

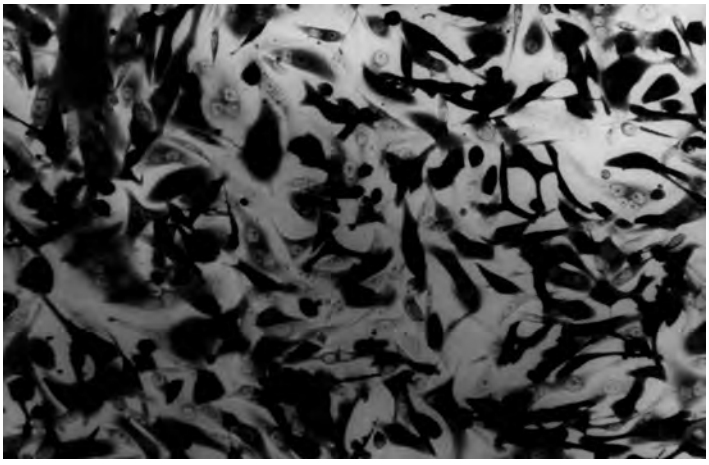

Figure 10.5–4 (part 7) HSV type 1-infected ELVIS HSV cells (black cells in this photo, $\times 200$) stained 16 h after infection. ELVIS (enzyme-linked virus-inducible system) HSV cells are genetically engineered BHK cells in which HSV proteins induce the intracellular accumulation of the reporter enzyme β-galactosidase, which is then histochemically detected. Provided by Diagnostic Hybrids, Inc. Reprinted from **H. D. Isenberg (ed.).** 1998. *Essential Procedures for Clinical Microbiology.* ASM Press, Washington, D.C.

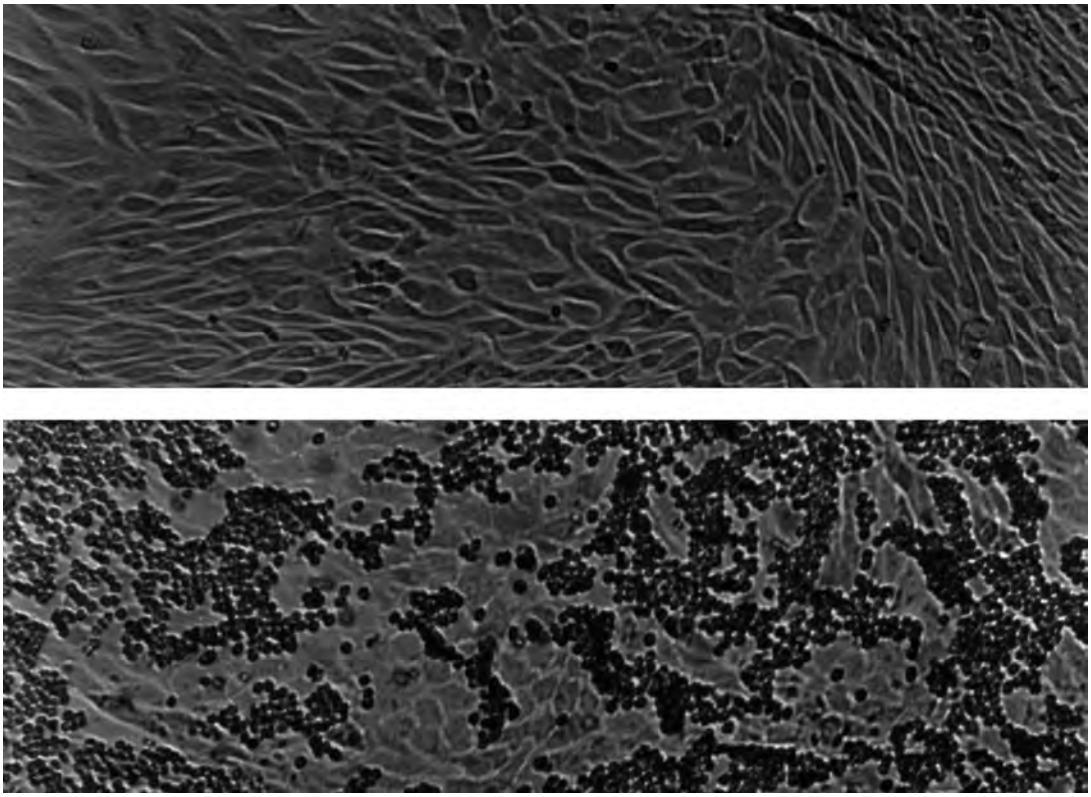

Figure 10.5–5 HAd with guinea pig RBCs. (Top) Uninoculated primary rhesus monkey kidney cells; (bottom) primary rhesus monkey kidney cells infected with parainfluenza virus type 3. Magnification, $\times 100$.

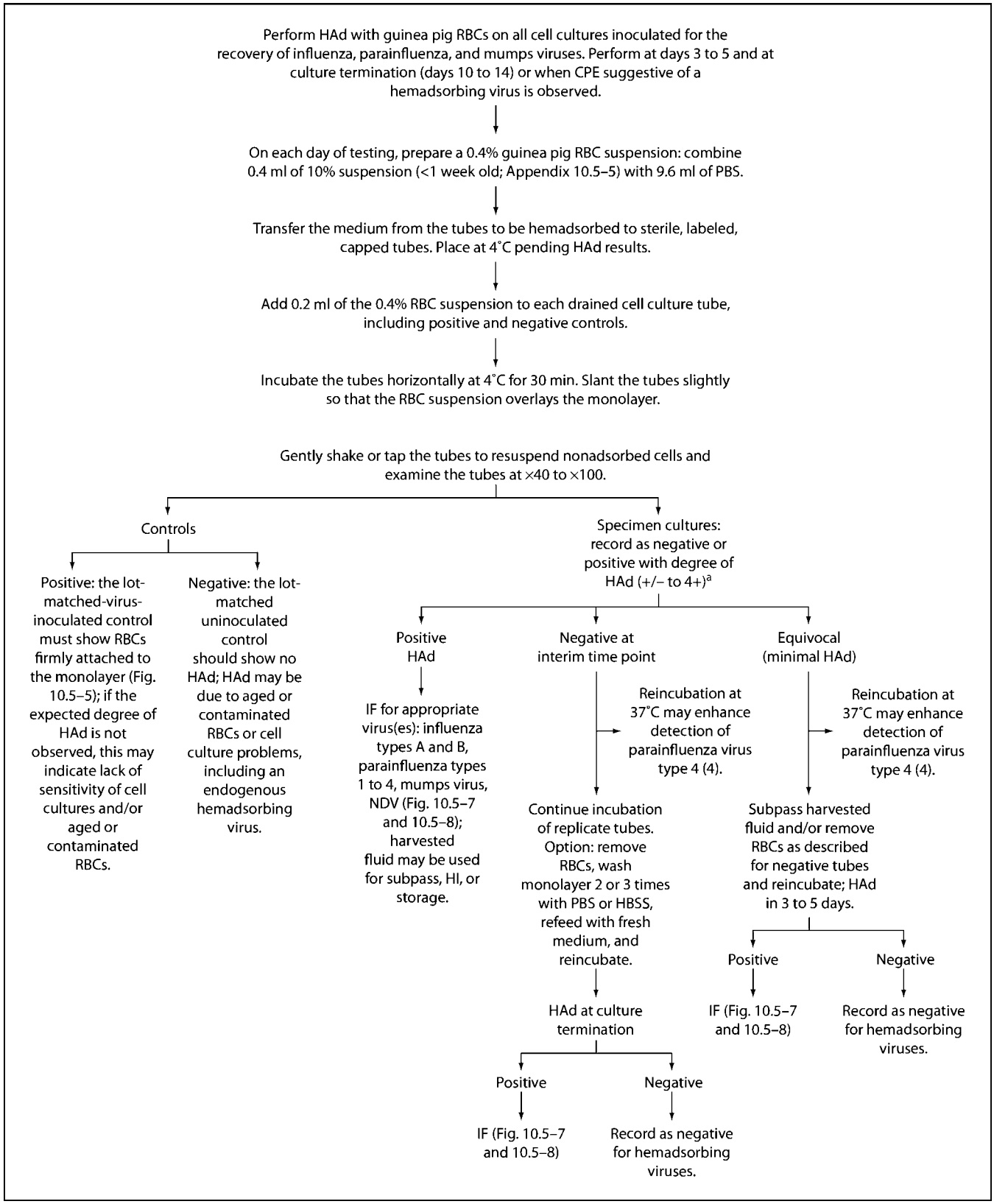
Perform HAd with guinea pig RBCs on all cell cultures inoculated for the recovery of influenza, parainfluenza, and mumps viruses. Perform at days 3 to 5 and at culture termination (days 10 to 14) or when CPE suggestive of a hemadsorbing virus is observed.
On each day of testing, prepare a 0.4% guinea pig RBC suspension: combine 0.4 ml of 10% suspension (<1 week old; Appendix 10.5–5) with 9.6 ml of PBS.
Transfer the medium from the tubes to be hemadsorbed to sterile, labeled, capped tubes. Place at 4°C pending HAd results.
Add 0.2 ml of the 0.4% RBC suspension to each drained cell culture tube, including positive and negative controls.
Incubate the tubes horizontally at 4°C for 30 min. Slant the tubes slightly so that the RBC suspension overlays the monolayer.
Gently shake or tap the tubes to resuspend nonadsorbed cells and examine the tubes at ×40 to ×100.
Controls
Positive: the lot-matched-virus-inoculated control must show RBCs firmly attached to the monolayer (Fig. 10.5–5); if the expected degree of HAd is not observed, this may indicate lack of sensitivity of cell cultures and/or aged or contaminated RBCs.
Negative: the lot-matched uninoculated control should show no HAd; HAd may be due to aged or contaminated RBCs or cell culture problems, including an endogenous hemadsorbing virus.
Specimen cultures: record as negative or positive with degree of HAd (+/– to 4+)[a]
Positive HAd
IF for appropriate virus(es): influenza types A and B, parainfluenza types 1 to 4, mumps virus, NDV (Fig. 10.5–7 and 10.5–8); harvested fluid may be used for subpass, HI, or storage.
Negative at interim time point
Reincubation at 37°C may enhance detection of parainfluenza virus type 4 (4).
Continue incubation of replicate tubes. Option: remove RBCs, wash monolayer 2 or 3 times with PBS or HBSS, refeed with fresh medium, and reincubate.
HAd at culture termination
Positive
IF (Fig. 10.5–7 and 10.5–8)
Negative
Record as negative for hemadsorbing viruses.
Equivocal (minimal HAd)
Reincubation at 37°C may enhance detection of parainfluenza virus type 4 (4).
Subpass harvested fluid and/or remove RBCs as described for negative tubes and reincubate; HAd in 3 to 5 days.
Positive
IF (Fig. 10.5–7 and 10.5–8)
Negative
Record as negative for hemadsorbing viruses.

Figure 10.5–6 HAd procedure. NDV, Newcastle disease virus.
[a] Agglutinated RBCs may be observed in the residual fluid (hemagglutination) or by incubating cell culture fluid with a suspension of appropriate RBCs (Appendix 10.5–5).

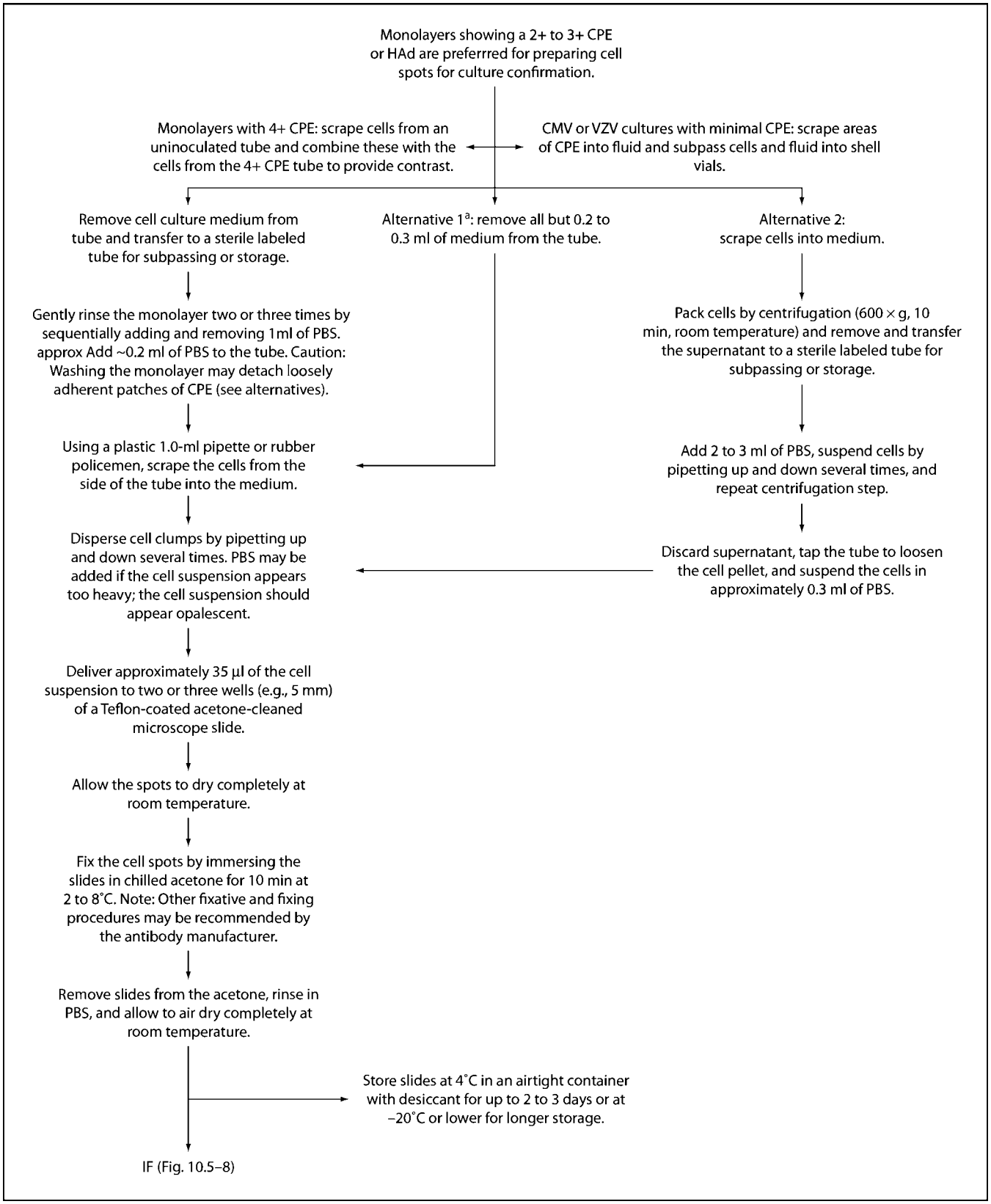
Monolayers showing a 2+ to 3+ CPE or HAd are preferrred for preparing cell spots for culture confirmation.
Monolayers with 4+ CPE: scrape cells from an uninoculated tube and combine these with the cells from the 4+ CPE tube to provide contrast.
CMV or VZV cultures with minimal CPE: scrape areas of CPE into fluid and subpass cells and fluid into shell vials.
Remove cell culture medium from tube and transfer to a sterile labeled tube for subpassing or storage.
Alternative 1[a]: remove all but 0.2 to 0.3 ml of medium from the tube.
Alternative 2: scrape cells into medium.
Gently rinse the monolayer two or three times by sequentially adding and removing 1ml of PBS. approx Add ~0.2 ml of PBS to the tube. Caution: Washing the monolayer may detach loosely adherent patches of CPE (see alternatives).
Pack cells by centrifugation (600 × g, 10 min, room temperature) and remove and transfer the supernatant to a sterile labeled tube for subpassing or storage.
Using a plastic 1.0-ml pipette or rubber policemen, scrape the cells from the side of the tube into the medium.
Add 2 to 3 ml of PBS, suspend cells by pipetting up and down several times, and repeat centrifugation step.
Disperse cell clumps by pipetting up and down several times. PBS may be added if the cell suspension appears too heavy; the cell suspension should appear opalescent.
Discard supernatant, tap the tube to loosen the cell pellet, and suspend the cells in approximately 0.3 ml of PBS.
Deliver approximately 35 µl of the cell suspension to two or three wells (e.g., 5 mm) of a Teflon-coated acetone-cleaned microscope slide.
Allow the spots to dry completely at room temperature.
Fix the cell spots by immersing the slides in chilled acetone for 10 min at 2 to 8°C. Note: Other fixative and fixing procedures may be recommended by the antibody manufacturer.
Remove slides from the acetone, rinse in PBS, and allow to air dry completely at room temperature.
Store slides at 4°C in an airtight container with desiccant for up to 2 to 3 days or at −20°C or lower for longer storage.
IF (Fig. 10.5–8)

Figure 10.5–7 Isolate identification: slide preparation.
[a] This method is rapid but may yield higher levels of nonspecific staining than observed when the monolayers or packed cells are washed before spotting.

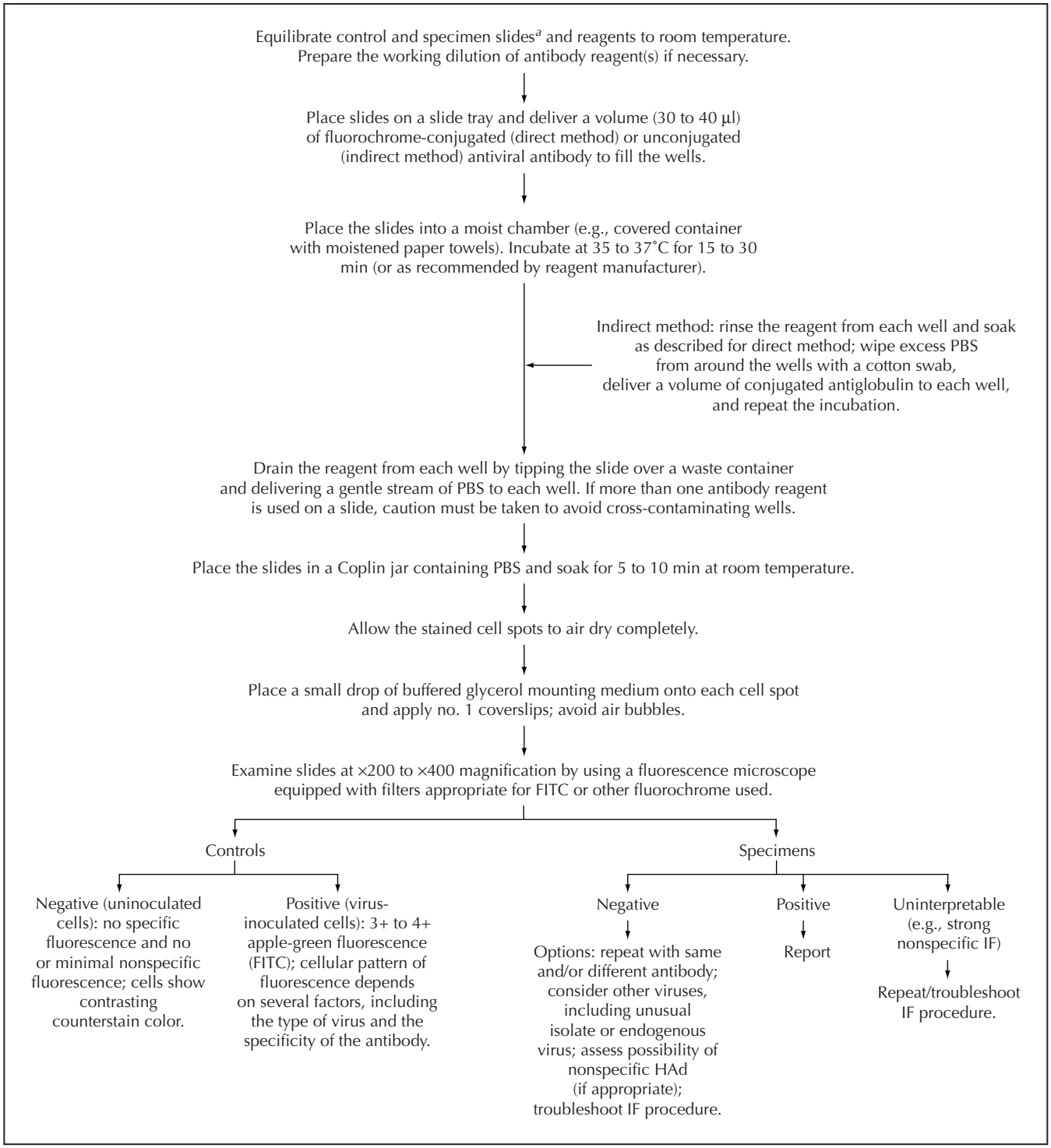
Equilibrate control and specimen slides[a] and reagents to room temperature. Prepare the working dilution of antibody reagent(s) if necessary.
Place slides on a slide tray and deliver a volume (30 to 40 µl) of fluorochrome-conjugated (direct method) or unconjugated (indirect method) antiviral antibody to fill the wells.
Place the slides into a moist chamber (e.g., covered container with moistened paper towels). Incubate at 35 to 37°C for 15 to 30 min (or as recommended by reagent manufacturer).
Indirect method: rinse the reagent from each well and soak as described for direct method; wipe excess PBS from around the wells with a cotton swab, deliver a volume of conjugated antiglobulin to each well, and repeat the incubation.
Drain the reagent from each well by tipping the slide over a waste container and delivering a gentle stream of PBS to each well. If more than one antibody reagent is used on a slide, caution must be taken to avoid cross-contaminating wells.
Place the slides in a Coplin jar containing PBS and soak for 5 to 10 min at room temperature.
Allow the stained cell spots to air dry completely.
Place a small drop of buffered glycerol mounting medium onto each cell spot and apply no. 1 coverslips; avoid air bubbles.
Examine slides at ×200 to ×400 magnification by using a fluorescence microscope equipped with filters appropriate for FITC or other fluorochrome used.
Controls
Specimens
Negative (uninoculated cells): no specific fluorescence and no or minimal nonspecific fluorescence; cells show contrasting counterstain color.
Positive (virus-inoculated cells): 3+ to 4+ apple-green fluorescence (FITC); cellular pattern of fluorescence depends on several factors, including the type of virus and the specificity of the antibody.
Negative
Positive
Uninterpretable (e.g., strong nonspecific IF)
Options: repeat with same and/or different antibody; consider other viruses, including unusual isolate or endogenous virus; assess possibility of nonspecific HAd (if appropriate); troubleshoot IF procedure.
Report
Repeat/troubleshoot IF procedure.

Figure 10.5–8 Isolate identification: IF.
[a] When removing slides from cold storage, allow them to equilibrate to room temperature before opening the storage container.

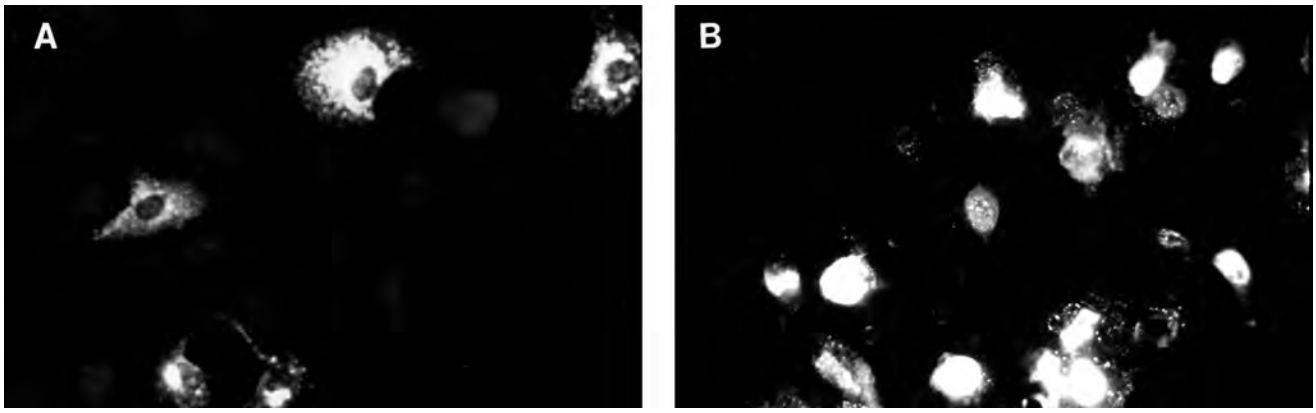

Figure 10.5–9 (A) LLC-MK_2 cells infected with parainfluenza virus (magnification, $\times 200$). (B) Adenovirus culture showing nuclear and cytoplasmic staining (magnification, $\times 400$).

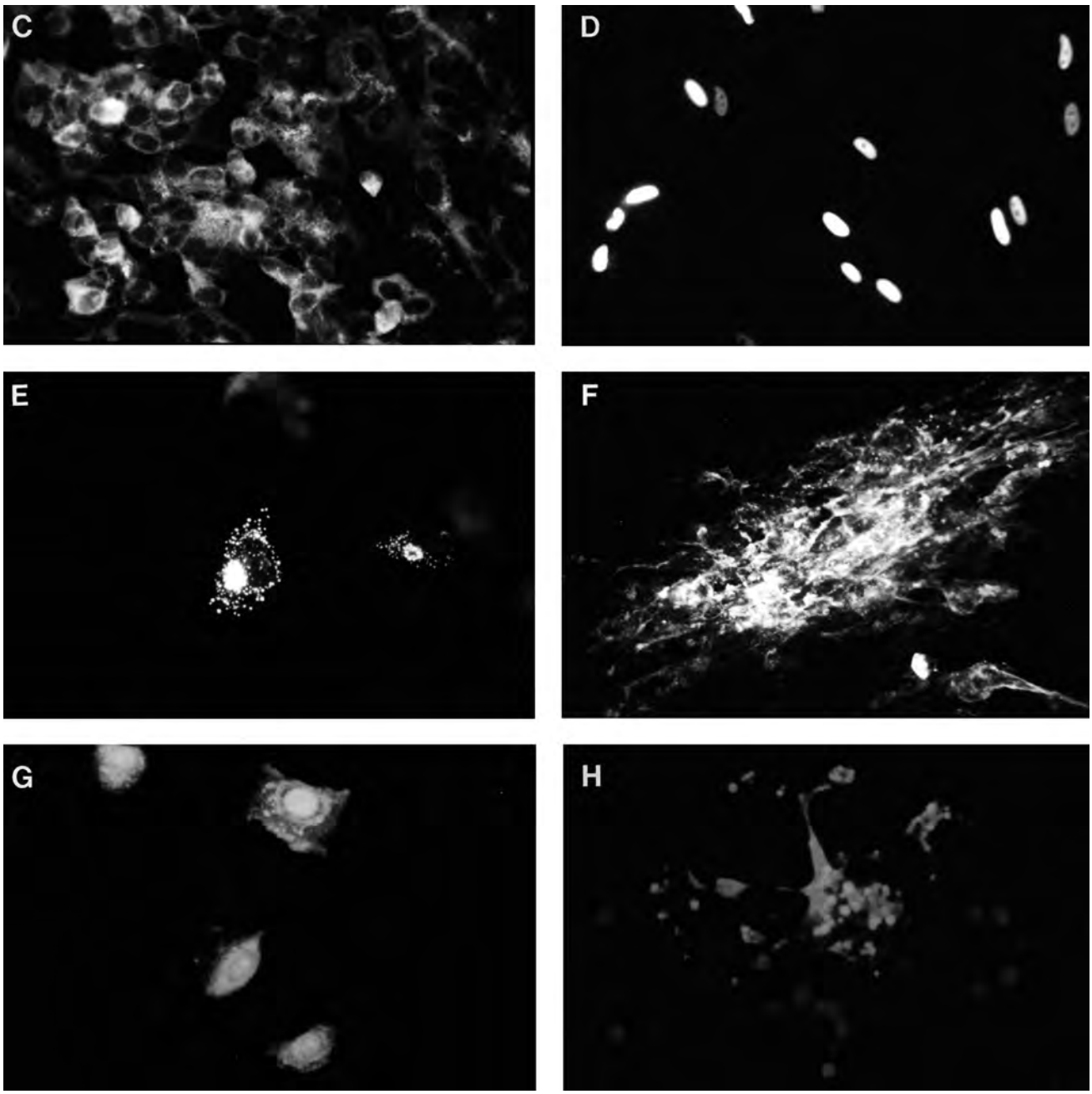

Figure 10.5–9 *(continued)* (C) Perinuclear and cytoplasmic staining characteristic of HSV-infected cells (magnification, ×400). (D) Brilliant oval nuclei of human fibroblasts infected with CMV after being stained with a MAb to CMV early nuclear protein (magnification, ×200). (E) MRC-5 cells stained after 72 h with a mixture of MAbs directed against intermediate-early nuclear and late cytoplasmic antigens (×200). (F) VZV in human fibroblast culture (magnification, ×400). (G) LLC-MK_2 cells infected with influenza A virus (×200). (H) HEp-2 cells infected with respiratory syncytial virus (×200).

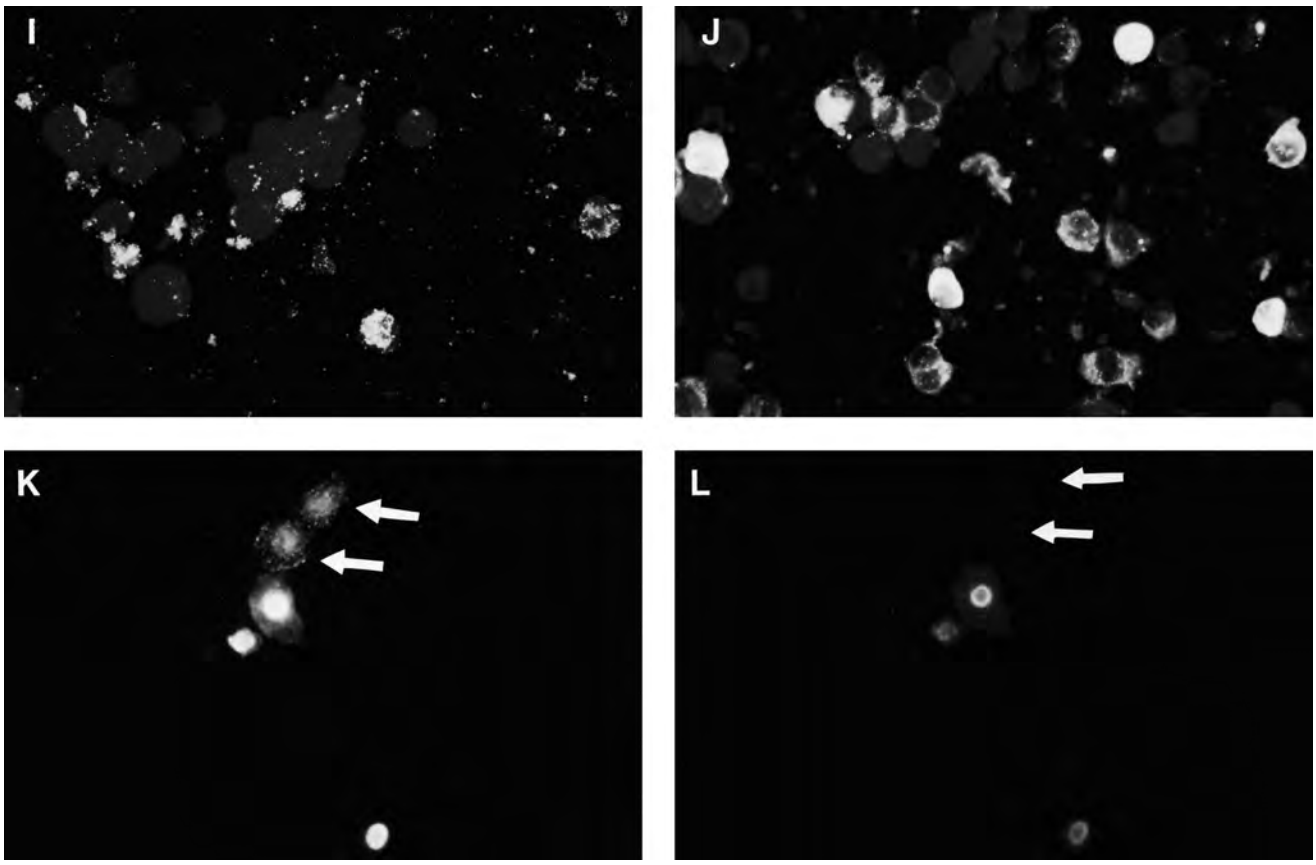

Figure 10.5–9 *(continued)* (I) Echovirus type 4-infected BGMK cells stained with panenterovirus blend of MAbs (×200). Provided by Chemicon International. (J) Echovirus type 4-infected BGMK cells stained using echovirus type 4 typing reagent (×200). Provided by Chemicon International. (K) Influenza A- and influenza B-infected LLC-MK_2 cells stained with SimulFluor influenza A/influenza B reagent and observed using filters for FITC. Influenza A-infected cells (arrows) stain apple-green; influenza B-infected cells are yellow-orange. Provided by Chemicon International. (L) Same field as panel K viewed with a tetramethyl rhodamine isocyanate/rhodamine filter. Influenza B-infected cells appear hot pink to red (×200); arrows indicate the location of influenza A-infected cells observed in panel K but which are not visible with this filter system. Provided by Chemicon International.

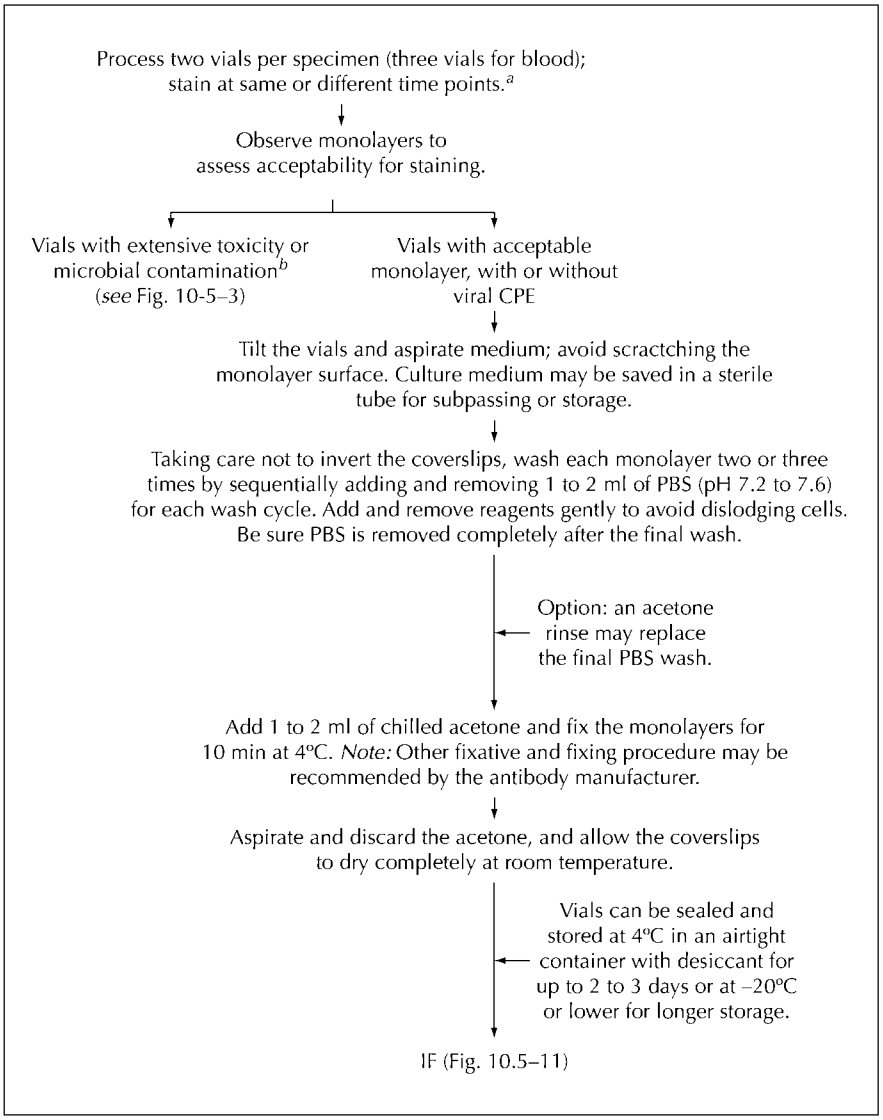
Process two vials per specimen (three vials for blood); stain at same or different time points.[a]
Observe monolayers to assess acceptability for staining.
Vials with extensive toxicity or microbial contamination[b] (see Fig. 10-5–3)
Vials with acceptable monolayer, with or without viral CPE
Tilt the vials and aspirate medium; avoid scractching the monolayer surface. Culture medium may be saved in a sterile tube for subpassing or storage.
Taking care not to invert the coverslips, wash each monolayer two or three times by sequentially adding and removing 1 to 2 ml of PBS (pH 7.2 to 7.6) for each wash cycle. Add and remove reagents gently to avoid dislodging cells. Be sure PBS is removed completely after the final wash.
Option: an acetone rinse may replace the final PBS wash.
Add 1 to 2 ml of chilled acetone and fix the monolayers for 10 min at 4°C. Note: Other fixative and fixing procedure may be recommended by the antibody manufacturer.
Aspirate and discard the acetone, and allow the coverslips to dry completely at room temperature.
Vials can be sealed and stored at 4°C in an airtight container with desiccant for up to 2 to 3 days or at –20°C or lower for longer storage.
IF (Fig. 10.5–11)

Figure 10.5–10 Shell vial processing.
[a] The sensitivity of shell vial cultures is influenced by the length of incubation, and each laboratory must determine appropriate staining timepoints. Typically, shell vial incubation times range from 18 to 36 h (e.g., CMV, HSV) to up to 5 days (e.g., VZV).
[b] Complete monolayer destruction may also be caused by a rapid CPE.

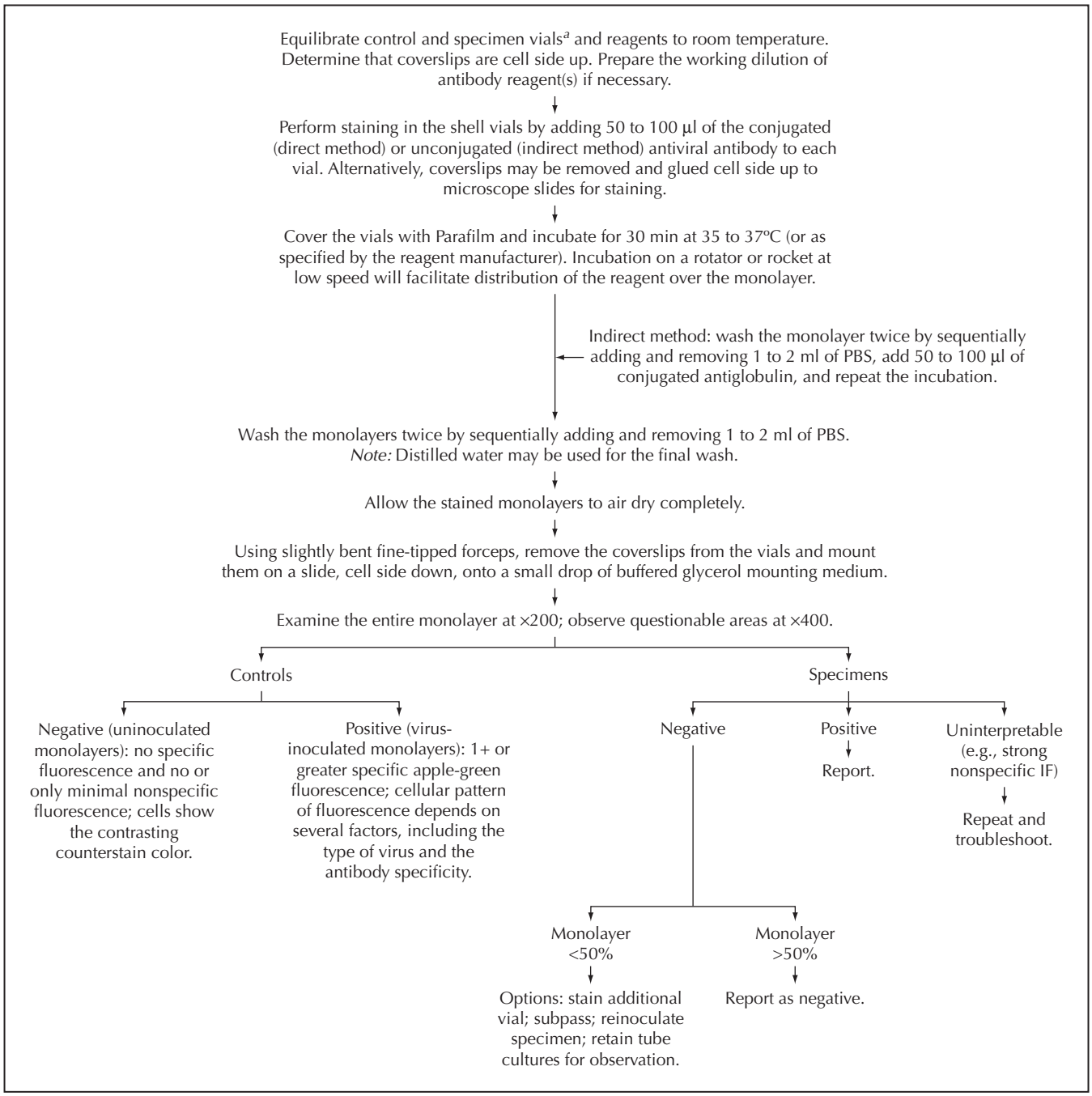
Equilibrate control and specimen vials[a] and reagents to room temperature. Determine that coverslips are cell side up. Prepare the working dilution of antibody reagent(s) if necessary.
Perform staining in the shell vials by adding 50 to 100 µl of the conjugated (direct method) or unconjugated (indirect method) antiviral antibody to each vial. Alternatively, coverslips may be removed and glued cell side up to microscope slides for staining.
Cover the vials with Parafilm and incubate for 30 min at 35 to 37ºC (or as specified by the reagent manufacturer). Incubation on a rotator or rocket at low speed will facilitate distribution of the reagent over the monolayer.
Indirect method: wash the monolayer twice by sequentially adding and removing 1 to 2 ml of PBS, add 50 to 100 µl of conjugated antiglobulin, and repeat the incubation.
Wash the monolayers twice by sequentially adding and removing 1 to 2 ml of PBS. *Note:* Distilled water may be used for the final wash.
Allow the stained monolayers to air dry completely.
Using slightly bent fine-tipped forceps, remove the coverslips from the vials and mount them on a slide, cell side down, onto a small drop of buffered glycerol mounting medium.
Examine the entire monolayer at ×200; observe questionable areas at ×400.
Controls
Specimens
Negative (uninoculated monolayers): no specific fluorescence and no or only minimal nonspecific fluorescence; cells show the contrasting counterstain color.
Positive (virus-inoculated monolayers): 1+ or greater specific apple-green fluorescence; cellular pattern of fluorescence depends on several factors, including the type of virus and the antibody specificity.
Negative
Positive
Report.
Uninterpretable (e.g., strong nonspecific IF)
Repeat and troubleshoot.
Monolayer <50%
Monolayer >50%
Options: stain additional vial; subpass; reinoculate specimen; retain tube cultures for observation.
Report as negative.

Figure 10.5–11 Shell vial cultures, IF.
[a] When removing vials from storage, allow them to equilibrate to room temperature before opening the storage container.

V. PROCEDURES *(continued)*

G. Refeeding inoculated cultures

1. Pipette or aspirate the medium from the culture tubes or vials.
   ◩ **NOTE:** Be careful not to scrape the monolayers.
2. Using a separate pipette for each specimen, add 1 to 2 ml of cell culture maintenance medium to each tube.
   ◩ **NOTE:** To reduce cross-contamination with detached cells or endogenous viruses, aspirate and refeed in the following sequence: fibroblast, primary nonsimian, transformed, and primary simian.

H. Harvesting and subpassing inoculated cultures

1. Isolates are frequently harvested and stored for many reasons, such as to provide teaching and QC material and to provide material for additional identification procedures (e.g., neutralization, acid lability, or HI). The extent to which viruses are shed or released into culture medium depends on the particular virus. For example, while most enteroviruses are readily released into the culture fluid, CMV and varicella-zoster virus (VZV) are cell associated, and successful harvesting and subpassing require infected cells; subpassage of CMV or VZV cultures with a CPE less than 2+ may not be efficient.
2. To harvest isolates, use a plastic 1-ml pipette or sterile rubber policeman to dislodge cells from the tube surface, and mix cells and fluid by pipetting up and down several times.
   ◩ **NOTE:** Successful subpassage of isolates may generally be accomplished by using the fluid only for isolates other than CMV, VZV, or adenoviruses.
3. To subpass cultures, deliver 0.2 to 0.3 ml of the harvested material to each new tube to be inoculated.
   ◩ **NOTE:** Isolates exhibiting very strong and rapid CPE may first be diluted (e.g., 1:10, 1:100, or greater) with HBSS.
4. To store isolates, transfer harvested material to a tube suitable for low-temperature storage (e.g., Nunc cryotube) and store at −70°C or lower.
   ◩ **NOTE:** Rapid freezing in a dry ice-acetone slurry is recommended; adding a cryopreservative is recommended for CMV and VZV harvests (add dimethyl sulfoxide [DMSO] to a final concentration of 10%).

I. Culture decontamination

1. Harvest cells and fluids as described above.
2. Draw the harvested material into a 3- to 5-ml disposable plastic syringe, attach a disposable 0.2-μm-pore-size filter (e.g., Millipore), and expel the material into a sterile tube.
   ◩ **NOTE:** Filtration of CMV and VZV cultures may markedly reduce the viral titer of recovered material, particularly with cultures exhibiting less than a 3+ CPE. Cell disruption by addition of glass beads and vortexing the material for 60 s before filtration will facilitate viral release. Viruses not adversely affected by freezing and thawing (e.g., unenveloped viruses such as adenoviruses and enteroviruses) may be subjected to three rapid freeze-thaw cycles to facilitate viral release.

POSTANALYTICAL CONSIDERATIONS

VI. PROCEDURE NOTES

Observe standard precautions.

A. Observe standard precautions.

B. Cell culture fluids may contain large concentrations of virus capable of aerosolization. Place a pipette discard jar or bucket containing 2 to 3 in. (ca. 5 to 7.5 cm) of liquid disinfectant (e.g., Wescodyne) inside the biological safety cabinet; to discard contaminated pipettes, immerse the tip in the disinfectant and then disengage it from the pipetting device. If the discard container is to be reused, seal it or place it into a sealable container and autoclave. If a suction apparatus is used, include an aerosol trap containing disinfectant between the collection flask and the vacuum source.

C. Use aseptic techniques for all cell culture manipulations (*see* Appendix 10.2–2).

D. Use appropriate measures to minimize the potential risk of cross-contamination of cultures and infection of personnel from manipulations that generate aerosols or may contaminate gloved hands (e.g., disengaging pipettes from pipetting devices, removing shell vial caps or stoppers, pouring).

E. The sensitivity of viral culture is affected by a number of variables, including the viral concentration; specimen type and quality; cell culture type, age, density, and quality; number of tubes or vials inoculated; incubation conditions; and choice of detection or identification reagent.

F. Blind subpassage of negative tube cultures midway through the incubation-observation period may slightly increase the number of positive cultures. However, this practice has been largely discontinued because of the expense and time involved. Subpassage is now generally limited to specimens showing toxicity or contamination or to prepare larger volumes of isolates for additional studies (e.g., antiviral susceptibility testing).

G. Vaccinia virus, simian B virus, arboviruses, arenaviruses, and filoviruses replicate in some heteroploid cell lines that may be in use in the routine diagnostic setting. While not frequently encountered, the symptoms and travel or exposure history should alert the laboratorian to the special safety considerations required for processing these specimens and the need to contact the appropriate public health department laboratory, CDC, or specialized reference laboratory.

H. Monolayers exhibiting CPE or HAd but not needed for identification or harvesting may be preserved indefinitely for teaching purposes (Appendix 10.5–7).

VII. CULTURE RESULTS

A. A positive viral culture is one exhibiting evidence of viral replication by the development of CPE, positive HAd, and/or the presence of a viral antigen or nucleic acid.

◩ **NOTE:** Identification on the basis of CPE or HAd is tentative, and reports should not be based on these findings.

B. A negative viral culture is one that fails to develop CPE and/or HAd or one in which no viral antigen or nucleic acid is detected after incubation of an acceptable monolayer for the prescribed period of time.

C. An incomplete or unsatisfactory culture is one that cannot be completed or processed in the appropriate manner for reasons that include extensive microbial contamination.

VIII. LIMITATION OF THE PROCEDURE

A negative viral culture does not rule out the possibility of a viral etiology. Reasons include a low viral titer, nonviable virus, insensitive cell cultures, or the agent may be noncultivatable or require specialized techniques.

REFERENCES

1. **Bankowski, M. J., K. Erickson, A. Alvarez, P. Horton, J. Rogers, S. Maxwell, and M. A. Neumann.** 1997. The SimulFluor™ (Chemicon) assay compared to Bartels IFA reagents for direct testing and viral culture confirmation of influenza types A and B. Thirteenth Annual Clinical Virology Symposium, Clearwater, Fla.
2. **Buller, R. S., T. C. Bailey, N. A. Ettinger, M. Keener, T. Langlois, J. P. Miller, and G. A. Storch.** 1992. Use of a modified shell vial technique to quantitate cytomegalovirus viremia in a population of solid-organ transplant recipients. *J. Clin. Microbiol.* **30:**2620–2624.
3. **Canas, L., T. Ottensmeier, C. Douglas, P. Asbury, and J. Treadwell.** 1997. Evaluation of the single reagent Simulfluor™ influenza A/influenza B fluorescence assay (Light Diagnostics) for the simultaneous detection and identification of influenza A and/or influenza B for use in a world wide influenza surveillance program. Thirteenth Annual Clinical Virology Symposium, Clearwater, Fla.
4. **Canchola, J., A. J. Vargosko, H. W. Kim, et al.** 1964. Antigenic variation among newly isolated strains of parainfluenza type 4 virus. *Am. J. Hyg.* **79:**357–364.

4a. **Chan, P. K. S., J. S. Tam, C. W. Lam, E. Chan, A. Wu, et al.** 2003. Human metapneumovirus detection in patients with severe acute respiratory syndrome. *Emerg. Infect. Dis.* **9:**1058–1063.

5. **Engler, H. D., and S. T. Selepak.** 1994. Effect of centrifuging shell vials at 3,500 × *g* on detection of viruses in clinical specimens. *J. Clin. Microbiol.* **32:**1580–1582.
6. **Espy, M. J., T. F. Smith, M. W. Harmon, and A. P. Kendal.** 1986. Rapid detection of influenza virus by shell vial assay with monoclonal antibodies. *J. Clin. Microbiol.* **24:**677–679.

6a. **Fouchier, R. A., N. G. Hartwig, T. M. Bestebroer, B. Niemeyer, J. C. de Jong, J. H. Simon, and A. D. Osterhaus.** 2004. A previously undescribed coronavirus associated with respiratory disease in humans. *Proc. Natl. Acad. Sci. USA* **101:**6212–6216.

7. **Frank, A. L., R. B. Couch, C. A. Griffis, and B. D. Baxter.** 1979. Comparison of different tissue cultures for isolation and quantitation of influenza and parainfluenza viruses. *J. Clin. Microbiol.* **10:**32–36.

7a. **Gillim-Ross, L., J. Taylor, D. R. Scholl, J. Ridenour, P. S. Masters, and D. E. Wentworth.** 2004. Discovery of novel human and animal cells infected by the severe acute respiratory syndrome coronavirus by replication-specific multiplex reverse transcription-PCR. *J. Clin. Microbiol.* **42:**3196–3206.

8. **Gleaves, C. A., D. A. Hursh, D. H. Rice, and J. D. Meyers.** 1989. Detection of cytomegalovirus from clinical specimens in centrifugation culture by in situ DNA hybridization and monoclonal antibody staining. *J. Clin. Microbiol.* **27:**21–23.
9. **Gleaves, C. A., C. F. Lee, C. I. Bustamante, and J. D. Myers.** 1988. Use of murine monoclonal antibodies for laboratory diagnosis of varicella-zoster virus infection. *J. Clin. Microbiol.* **26:**1623–1625.
10. **Gleaves, C. A., T. F. Smith, E. A. Shuster, and G. R. Pearson.** 1984. Rapid detection of cytomegalovirus in MRC-5 cells inoculated with urine specimens by using low-speed centrifugation and monoclonal antibody to an early antigen. *J. Clin. Microbiol.* **19:**917–919.

10a. **Hamelin, M. E., and G. Boivin.** 2005. Human metapneumovirus: a ubiquitous and long-standing respiratory pathogen. *Pediatr. Infect. Dis. J.* **24:**S203–207.

11. **Hughes, J. H.** 1993. Physical and chemical methods for enhancing rapid detection of viruses and other agents. *Clin. Microbiol. Rev.* **6:**150–175.

11a. **Ksiazek, T. G., D. Erdman, C. S. Goldsmith, et al.** 2003. A novel coronavirus associated with severe acute respiratory syndrome. *N. Engl. J. Med.* **348:**1953–1966.

12. **Lee, S. H. S., J. E. Boutilier, M. A. MacDonald, and K. R. Forward.** 1992. Enhanced detection of respiratory viruses using the shell vial technique and monoclonal antibodies. *J. Virol. Methods* **39:**39–46.

12a. **MacPhail, M., J. H. Schickli, R. S. Tang, J. Kaur, C. Robinson, et al.** 2004. Identification of small-animal and primate models for evaluation of vaccine candidates for human metapneumovirus (hMPV) and implications for hMPV vaccine design. *J. Gen. Virol.* **85:**1855–1863.

13. **Matthey, S., D. Nicholson, S. Ruhs, B. Alden, M. Knock, K. Schultz, and A. Schmuecker.** 1992. Rapid detection of respiratory viruses by shell vial culture and direct staining by using pooled and individual monoclonal antibodies. *J. Clin. Microbiol.* **30:**540–544.
14. **Minnich, L. L., F. Goodenough, and C. G. Ray.** 1991. Use of immunofluorescence to identify measles virus infections. *J. Clin. Microbiol.* **29:**1148–1150.
15. **Olsen, M. A., K. M. Shuck, A. R. Sambol, S. M. Flor, J. O'Brien, and B. J. Cabrera.** 1993. Isolation of seven respiratory viruses in shell vials: a practical and highly sensitive method. *J. Clin. Microbiol.* **31:**422–425.

15a. **Percivalle, E., A. Sarasini, L. Visai, M. Grazia Revello, and G. Gerna.** 2005. Rapid detection of human metapneumovirus strains in nasopharyngeal aspirates and shell vial culture by monoclonal antibodies. *J. Clin. Microbiol.* **43:**3443–3446.

16. **Rabalais, G. P., G. G. Stout, K. L. Ladd, and K. M. Cost.** 1992. Rapid diagnosis of respiratory viral infections by using a shell vial assay and monoclonal antibody pool. *J. Clin. Microbiol.* **30:**1505–1508.
17. **Salmon, V. C., B. R. Kenyon, and J. C. Overall, Jr.** 1990. Cross contamination of viral specimens related to shell vial caps. *J. Clin. Microbiol.* **28:**2820–2822.
18. **Shuster, E. A., J. S. Beneke, G. E. Tegtmeier, G. R. Pearson, C. A. Gleaves, A. D. Wold, and T. F. Smith.** 1985. Monoclonal antibody for rapid laboratory detection of cytomegalovirus infections: characterization and diagnostic application. *Mayo Clin. Proc.* **60:**577–585.
19. **Stout, C., M. D. Murphy, S. Lawrence, and S. Julian.** 1989. Evaluation of a monoclonal

REFERENCES *(continued)*

antibody pool for rapid diagnosis of respiratory viral infections. *J. Clin. Microbiol.* **27:**448–452.

20. **Tobita, K., A. Sugiura, C. Enomoto, and M. Furuyama.** 1975. Plaque assay and primary isolation of influenza A viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. *Med. Microbiol. Immunol.* **162:**9–14.

20a. **Van den Hoogen, B. G., J. C. de Jong, J. Groen, T. Kuiken, R. de Groot, et al.** 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *Nat. Med.* **7:**719–724.

21. **Wiedbrauk, D. L., J. P. Gibson, R. Bollinger, and E. Ostler.** 1993. Centrifugation-enhanced (shell vial) method for detecting rubella virus. Ninth Annual Clinical Virology Symposium, Clearwater, Fla.

22. **Zhao, L., M. L. Landry, E. S. Balkovic, and G. D. Hsiung.** 1987. Impact of cell culture sensitivity and virus concentration on rapid detection of herpes simplex virus by cytopathic effects and immunoperoxidase staining. *J. Clin. Microbiol.* **25:**1401–1405.

APPENDIX 10.5–1

Viral Titration and Determination of $TCID_{50}$

Reprinted from **S. M. Lipson.** 1992. Neutralization test for the identification and typing of viral isolates, p. 8.14.1–8.14.8. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook,* vol. 2. American Society for Microbiology, Washington, D.C.

A. Viral quantitation

The viral isolate to be identified in the neutralization assay must be quantitated to ensure optimal performance of the assay. Viral quantitation is achieved by preparing a series of dilutions of the material and assaying this material by using either quantitative or quantal assay systems. In the quantitative assay, the number of infectious viral particles is established by using a plaque or infectious-focus assay (2). Although this type of assay is more precise, it is, like the plaque reduction neutralization assay, extremely cumbersome, costly, and labor-intensive. Thus, these quantitative procedures are reserved for the research setting or are limited to special procedures.

The viral quantitation method used in the diagnostic laboratory is the endpoint dilution method. This is a quantal assay, since it measures an "all-or-none" effect and does not determine the number of infectious particles (1). Thus, the quantal assay determines only that the material contains a sufficient dose of infectious virus to produce infection in a susceptible host system. With the endpoint dilution method, the viral titer is expressed as the 50% tissue culture infective dose ($TCID_{50}$) or, in animals, as the 50% lethal dose.

B. Preparation of the viral titration (Fig. 10.5–A1)

1. Harvest the viral isolate to be titrated (*see* item V.H.1 to 4 above) or thaw a frozen aliquot.
2. Determine the number of serial 10-fold dilutions to be inoculated.
   a. With slower-growing low-titered viruses (e.g., adenoviruses), prepare dilutions ranging from 10^{-1} through 10^{-5}, and inoculate tubes starting with the undiluted material.
   b. With high-titered material (e.g., many enteroviruses), prepare dilutions ranging from 10^{-1} through 10^{-7}, and inoculate tubes starting with 10^{-1} or 10^{-2}.
3. Label sterile dilution tubes for each virus to be titrated.
4. Add 1.8 ml of HBSS to each dilution tube.

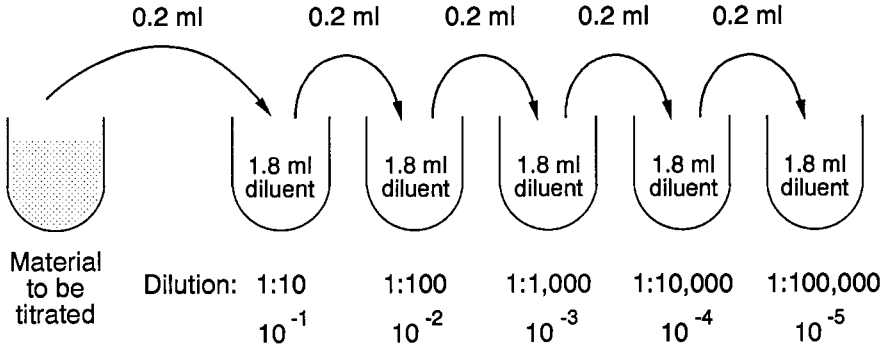

Figure 10.5–A1 Viral titration with serial 10-fold dilutions.

APPENDIX 10.5–1 *(continued)*

Table 10.5–A1 Calculation of $TCID_{50}$ by the Reed-Muench method (1)

Dilution	Observed value[a]		Cumulative value			
	(1) Pos	(2) Neg	(3) Pos[b]	(4) Neg[c]	(5) Ratio[d]	(6) % Pos[e]
10^{-5}	5	0	10	0	10/10	100.0
10^{-6}	4	1	5	1	5/6	83.3
10^{-7}	1	4	1	5	1/6	16.7
10^{-8}	0	5	0	10	0/10	0.0

[a] Pos, tube showing CPE; Neg, tube not showing CPE.

[b] Obtained by adding numbers in column 1, starting at the bottom.

[c] Obtained by adding numbers in column 2, starting at the top.

[d] Value in column 3/(value in column 3 + value in column 4).

[e] $\frac{\%\text{ CPE next above 50\% } - \text{ 50}}{\%\text{ CPE next above 50\% } - \%\text{ CPE next below 50\%}}$ = proportional distance between the two dilutions at which 50% CPE occurs

From the above example: $\frac{83.3 - 50}{83.3 - 16.7} = 0.5$

One $TCID_{50}$ in this case is $10^{-6.5}$, and therefore 100 $TCID_{50}$s equal $10^{-4.5}$.

5. Add 0.2 ml of each virus to be titrated to the corresponding tube labeled 10^{-1}, and mix by pipetting up and down several times.
6. With a new pipette, remove 0.2 ml from the 10^{-1} dilution, transfer it to the tube labeled 10^{-2}, and mix.
7. Continue preparing dilutions through the highest dilution, using a new pipette for each dilution.
8. Label four to six cell culture tubes for each dilution to be inoculated; select two tubes as negative (uninoculated controls).
9. Refeed each tube with 1.0 ml of maintenance medium.
10. Inoculate 0.1 ml of each virus dilution into the corresponding culture tubes.
11. Incubate the culture tubes at 35 to 37°C for 7 to 10 days. Observe the cell culture monolayers daily for the development of CPE, and record the presence or absence of CPE (*see* Fig. 10.5–2).
12. *Note:* some viruses grow more slowly than others. For example, culture readings should be terminated after 10 to 12 days with the slower-growing adenoviruses, while a 7-day incubation period would be appropriate for the faster-growing enteroviruses.

C. Calculation of $TCID_{50}$ by the Reed-Muench method (1)

The $TCID_{50}$ is that dilution that produces CPE in one-half of the cumulative number of cell cultures (Table 10.5–A1).

1. Record the number of positive (column 1) and negative (column 2) values.
2. Calculate the cumulative numbers of positive (column 3) and negative (column 4) tubes.
3. Calculate the percentage of positive tubes (column 5) by using the cumulative values.
4. Calculate the proportionate distance between the dilution showing >50% CPE and the dilution showing <50% CPE.
5. Calculate the $TCID_{50}$ by adding the proportionate distance factor (as a negative value) to the dilution (negative logarithm) showing >50% CPE.
6. Since each tube was inoculated with 0.1 ml of each virus dilution, the $TCID_{50}$ is expressed as $TCID_{50}$/0.1 ml.
7. *Note:* the proportionate distance must be multiplied by a correction factor, which is the logarithm of the dilution factor. In this procedure, with 10-fold dilutions, that factor is equal to 1, and the step can be disregarded. However, if other dilution series are used, the factor must be considered. For example, the correction factor for a twofold dilution series would be 0.3, and that for a fivefold dilution series would be 0.7.

References

1. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* **27:**493–497.
2. **Schmidt, N. J.** 1989. Cell culture procedures for diagnostic virology, p. 51–100. *In* N. J. Schmidt and R. W. Emmons (ed.), *Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections*, 6th ed. American Public Health Association, Washington, D.C.

APPENDIX 10.5–2

Viral Neutralization Assay

Reprinted from **S. M. Lipson.** 1992. Neutralization test for the identification and typing of viral isolates, p. 8.14.1–8.14.8. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook,* vol. 2. American Society for Microbiology, Washington, D.C.

I. PRINCIPLE

The neutralization test is a highly sensitive assay applicable to the identification of all viral isolates. However, it is costly and labor-intensive to perform and has been largely supplanted by more rapid and convenient methods. Currently, the major applications of the neutralization test in the diagnostic virology laboratory are for the identification of enteroviruses and the typing of adenoviruses (1, 3, 4).

The neutralization test is a biphasic system consisting of (i) an antigen-antibody reaction step, in which the virus to be identified is mixed and incubated with the appropriate antibody reagent(s), and (ii) an inoculation step, in which the mixture is inoculated into the appropriate host system (e.g., cell cultures, animals). The absence of infectivity, for example, the failure of CPE or HAd to develop in cell cultures, constitutes a positive neutralization reaction and indicates the identity of the virus.

II. SPECIMEN

Cell culture harvest of isolate to be identified (*see* item V.H.1 to 4 above).

A. Titrate the viral isolate and determine the 50% tissue culture infective dose ($TCID_{50}$) as described in Appendix 10.5–1.

B. Alternatively, select the test dilution empirically. For example, use a 1:10 or 1:50 dilution for isolates with a slowly progressing CPE (e.g., adenovirus) and a 1:500 or 1:1,000 dilution for viruses with a rapidly progressing CPE (e.g., enteroviruses).

Include QC information on reagent container and in QC records.

III. MATERIALS

A. Cell cultures and reagents

1. Cell culture monolayers
   a. Perform the neutralization test with the same type of cell culture as used for determination of the test dose ($TCID_{50}$).
   b. Refeed the cell cultures with maintenance medium prior to performing the assay.
2. Cell culture maintenance medium. For example, use Eagle minimum essential medium supplemented with 50 μg of gentamicin per ml, 1.25 μg of amphotericin B per ml, and 2% heat-inactivated fetal bovine serum.
3. Antiviral neutralizing antisera
   a. Availability

      Antisera suitable for neutralization tests for many viruses are available from numerous commercial sources and reference laboratories. Pools for the identification of enterovirus isolates are available on a more limited basis. There are differences in the quality of products obtained from different sources or different lots. It is therefore the responsibility of the individual laboratory to monitor reagent quality by using homologous and heterologous viruses.

   b. Preparation and storage
      (1) Reconstitute lyophilized antiserum according to the recommendation of the manufacturer. Product data sheets should include information on reagent potency as well as storage and shelf life guidelines. Label containers with expiration dates.
      (2) Heat inactivate the serum in a 56°C water bath for 30 min to inactivate nonspecific heat-labile inhibitors of infectivity. Alternatively, heat inactivate the serum just prior to use.
      (3) Dispense antiserum into aliquot volumes consistent with the needs of the laboratory. Freeze and store the aliquots at −70°C. Avoid repeated freeze-thaw cycles. Label containers with expiration dates.

APPENDIX 10.5–2 *(continued)*

(4) Record all pertinent information, including source, viral specificity and cross-reactivity, donor species, lot number, potency (neutralizing units), aliquot volume and quantity, recommended test dilution and suggested diluent, lot number of matching normal (nonimmune) serum (if applicable), and dates of receipt, opening, use, heat inactivation, and expiration.

(5) Detailed step-by-step instructions for the reconstitution, storage, and use of the Lim Benyesh-Melnick (LBM) intersecting serum pools for the identification of enteroviruses are provided with the reagents.

(6) Handle all sera aseptically to maintain sterility.

(7) Monitor antisera for deterioration by performing neutralization assays with the matching (homologous) viruses. Perform these assays periodically (e.g., annually) on frozen stock, whenever the material has been subjected to improper storage conditions, or if deterioration is suspected based on performance.

4. HBSS, pH 7.2 to 7.6

B. Supplies
1. Sterile capped tubes (12 by 75 mm)
2. Assorted sterile pipettes and pipetting device
3. Autoclavable containers for discarding cultures

C. Equipment
1. Class II biological safety cabinet
2. Water bath, 56°C
3. Incubator, 35 to 37°C
4. Inverted microscope or standard microscope fitted with tracks for the observation of culture tubes; 10× to 20× objectives
5. Freezer, −70°C

IV. QUALITY CONTROL

A. Test each lot of antiserum at the working dilution prior to use to determine whether the reagent has a toxic effect on the cell cultures used for neutralization tests.
1. Inoculate 0.1 ml of each working dilution of serum into two tubes of the appropriate cell culture type.
2. Observe the cultures for 7 to 10 days for evidence of toxicity (cell rounding and degeneration of the monolayer).

B. Include a normal serum control, a positive serum control, a virus check titration, and a negative cell control with each assay.
1. Normal serum control
Include a normal (nonimmune, negative) serum to determine whether the virus is nonspecifically inactivated by antiserum components.
a. Include a nonimmune serum obtained from the same animal species as used to produce the antiserum. Ideally, normal serum should be obtained as a preimmune serum from the animal prior to inoculation.
b. Use the normal serum at the same dilution as the matching viral antiserum.
c. If more than one antiserum from the same animal species is used in a neutralization test (i.e., an antiserum with a different [heterologous] viral specificity), the heterologous serum may substitute for the normal serum.
2. Positive serum control
Include a reference virus incubated (neutralized) with its matching (homologous) serum as a positive control. Since this is not practical for each antiserum when multiple antisera are used in a neutralization test, select the antiserum to the prevalent or suspected virus.
3. Virus check titration
Include a confirmatory (check) titration of the virus consisting of 3 or 4 serial $\log_{10}$ dilutions, starting with the test dilution of virus.
4. Negative cell control
Select two uninoculated lot-matched cell cultures to be incubated and observed in parallel with the neutralization test.

APPENDIX 10.5–2 *(continued)*

V. PROCEDURE

A. Preparation of antiserum working dilution

1. Dilute each type-specific and normal antiserum 1:10 (or other predetermined dilution) with HBSS. The final working dilution of the type-specific antiserum should contain 20 (or more) neutralization units per 0.1 ml of antiserum. Determine the appropriate dose on the basis of the manufacturer's specifications and in-house QC results.
2. If the serum has not been heat inactivated, place the diluted serum in a 56°C water bath for 30 min.
3. The working dilutions of antisera may be stored at 4°C for up to 5 days. Diluted antiserum may be stored at −70°C for extended periods (i.e., months to years).
4. Do not repeatedly freeze-thaw undiluted or working dilution sera. Aliquot in single-use volumes, and freeze at −70°C.

B. Preparation of virus test dilution and check titration

1. Determine the dilution of each unknown and control virus to be used.
   a. Prepare a dilution of virus in HBSS that contains 100 $TCID_{50}$s/0.1-ml volume (Appendix 10.5–1). For example, if the $TCID_{50}$ of a viral harvest is 10^{-3}/0.1 ml (1:1,000 dilution), use the material at 10^{-1} (1:10 dilution) in the neutralization test by combining 1 part undiluted material with 9 parts diluent.
   b. Alternatively, prepare a dilution of virus based on culture characteristics (item II.B).
2. Check titration
   Starting with the virus test dilution, prepare three additional serial $\log_{10}$ dilutions with HBSS as diluent. For example, if the test dilution is 10^{-1}, prepare dilutions through 10^{-4} (Appendix 10.5–1).

C. Performance of the neutralization test

1. For each virus to be neutralized, add 0.3 ml of the diluted normal serum to a labeled tube.
2. For each virus to be neutralized, add 0.3 ml of the appropriate diluted immune antiserum to a labeled tube.
3. Add 0.3 ml of the viral dilution containing 100 $TCID_{50}$s/0.1 ml to the corresponding serum tubes.
4. Gently agitate the virus-serum mixtures, and incubate them and the virus check titration at room temperature for 1 h or as specified by the antiserum manufacturer.
5. Inoculate 0.2 ml of each virus-serum mixture into two cell culture tubes, each containing 1 to 2 ml of maintenance medium.
6. Inoculate 0.1 ml of the test dose and each dilution of the check titration into two or three cell culture tubes, each containing 1 to 2 ml of maintenance medium.
7. Incubate the cell culture tubes at 35 to 37°C. Observe the monolayers daily for the appearance of CPE. Record the presence or absence of CPE for each tube (*see* Fig. 10.5–2).

VI. RESULTS

A. The neutralization test is completed when the anticipated endpoint of the check titration is achieved. The endpoint should be attained after an incubation period of 7 to 10 days, depending on the virus.

B. Controls

1. The degree of CPE in the normal serum control should be similar to that observed in the virus test dilution of the check titration.
2. The positive control should show no or only minimal (breakthrough) CPE compared with CPE in the virus control.
3. The virus test dose of 100 $TCID_{50}$s is confirmed by the development of CPE in the virus test dilution and in the two subsequent serial dilutions of the check titration. In most cases, a test dose of 3 to 300 $TCID_{50}$s is acceptable.
4. The negative cell control should not show any CPE, and the monolayer quality should be acceptable.

Table 10.5–A2 Problems associated with interpretation of the neutralization test

Problem	Definition	Possible cause(s)	Solution
No neutralization	CPE develops at same rate with all or most antisera.	Test dose of virus too strong	Redetermine endpoint titers.
		Serum deteriorated	Obtain new antisera. Check storage conditions.
Breakthrough	Neutralized virus produces delayed and reduced CPE.	Test dose of virus too strong	Redetermine endpoint titers.
		Cross-reactivity	Check product specifications for cross-reactive properties; obtain non-cross-reactive serum if possible.
		Antiserum deteriorated or failed to attain expected efficacy	Check against reference virus; use lower dilution.
			Other methods, such as kinetic neutralization assay (2), required.
		Clumps or aggregates in virus suspension	Remove by ultrafiltration.
Neutralization by more than one antiserum	CPE is inhibited by two or more antisera.	Cross-reactivity	Check product specifications for cross-reactive properties.
		Test dose of virus too weak	Redetermine endpoint titers.
		Antiserum dose too strong	Check suggested working dilution.
			Heat inactivate serum (56°C, 30 min).
			Obtain new antiserum.
Nonspecific virus inactivation	Virus is neutralized (no CPE) by normal serum.	Serum not heat inactivated or contains nonspecific viral inhibitors	
		Virus inactivated during incubation step	Check recommended temp.
			Check incubator-water bath temp.
Monolayer toxicity	Nonspecific degeneration of culture monolayer	Serum not heat inactivated or is toxic to cells	Heat inactivate serum (56°C, 30 min).
			Obtain new antiserum.

APPENDIX 10.5–2 *(continued)*

C. Specimens

Compare the development and degree of CPE observed in the neutralized tubes with those observed in the normal serum controls and in the test dilution of the check titration.

1. The absence of CPE in tubes inoculated with a particular virus-antiserum mixture indicates a positive neutralization reaction and thus identifies the viral isolate.
2. The simultaneous development of CPE in the unknown virus-antiserum mixture, the normal serum control, and the test dilution indicates that there is no relationship between the unknown virus and the antiserum.
3. Occasionally, the neutralization test may be difficult to interpret. In such cases, consider the factors presented in Table 10.5–A2 or the possibility of a mixed infection.

References

1. **Horstmann, D. M., and G. D. Hsiung.** 1965. Principles of diagnostic virology, p. 405–429. *In* F. L. Horsfall, Jr., and I. Tamm (ed.), *Viral and Rickettsial Infections of Man.* J. B. Lippincott Co., Philadelphia, Pa.
2. **McBride, W. D.** 1959. Antigenic analysis of poliovirus by kinetic studies of serum neutralization. *Virology* **7:**45–48.
3. **Melnick, J. L., H. A. Wenner, and C. A. Phillips.** 1979. Enteroviruses, p. 471–534. *In* E. H. Lennette and N. J. Schmidt (ed.), *Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections,* 5th ed. American Public Health Association, Washington, D.C.
4. **Schmidt, N. J.** 1989. Cell culture procedures for diagnostic virology, p. 51–100. *In* N. J. Schmidt and R. W. Emmons (ed.), *Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections,* 6th ed. American Public Health Association, Washington, D.C.

APPENDIX 10.5–3

Acid Lability Assay

Reprinted from **S. Aarnaes.** 1992. Differentiation of rhinoviruses from enteroviruses: acid lability test, 8.13.1–8.13.3. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook*, vol. 2. American Society for Microbiology, Washington, D.C.

I. PRINCIPLE

The differentiation of rhinoviruses from enteroviruses cannot be made on the basis of routine cultural characteristics, since both groups may be isolated from respiratory samples and produce similar CPEs. Furthermore, routine confirmation of a suspected rhinovirus isolate is not feasible because of the very numerous rhinovirus types. While extreme lability, short-term shedding, and preference for cultivation at 33°C limit the number of rhinovirus isolates, the possibility that a rhinovirus may be isolated must be considered. The suspected rhinovirus isolate can be conveniently differentiated from an enterovirus on the basis of acid lability (1, 2). Enteroviruses have been shown to be stable at pHs 3.0 to 5.0. Rhinoviruses, by contrast, are inactivated at a low pH.

II. SPECIMEN

Cell culture supernatants from cultures showing a 3 to 4+ CPE are suitable specimens (*see* Fig. 10.5–2).

Include QC information on reagent container and in QC records.

III. MATERIALS

A. Reagents and supplies

1. Human diploid fibroblast cultures
2. Citrate buffer, 0.1 M, pH 4.0
   a. Stock solutions
      (1) Citric acid solution, 0.1 M

      citric acid ……21.01 g
      deionized water ……to 1,000.00 ml

      (2) Sodium citrate solution, 0.1 M

      sodium citrate ……29.41 g
      deionized water ……to 1,000.00 ml

      (3) Sterilize by filtration through a 0.2-μm-pore-size filter. Store at 2 to 8°C. Shelf life is 6 months or longer.
   b. Citrate buffer, pH 4.0
      (1) Ingredients

      citric acid, 0.1 M …… 33.0 ml
      sodium citrate, 0.1 M …… 17.0 ml

      (2) Aseptically combine sterile stock solutions. Prepare fresh on day of use.
3. Phosphate buffer, 0.1 M, pH 7.0
   a. Ingredients

      Na_2HPO_4 ……8.7 g
      KH_2PO_4 ……5.3 g
      deionized water …… to 1,000.0 ml

   b. Sterilize by filtration through a 0.2-μm-pore-size filter. Store at 2 to 8°C. Shelf life is 1 year.
4. Phosphate buffer, 0.5 M, pH 7.2
   a. Ingredients

      Na_2HPO_4 …… 51.1 g
      KH_2PO_4 …… 19.0 g
      deionized water …… to 1,000.0 ml

   b. Sterilize by filtration through a 0.2-μm-pore-size filter. Store at 2 to 8°C. Shelf life is 1 year.
5. Cell culture maintenance medium: Eagle's minimal essential medium supplemented with 2% heat-inactivated fetal bovine serum

APPENDIX 10.5–3 *(continued)*

6. Sterile disposable capped tubes, 12 by 75 mm and 13 by 100 mm
7. Sterile pipettes (assorted) and pipetting device
8. Cell culture racks

B. Equipment
1. Incubator, 33°C
2. Inverted microscope or standard microscope fitted with stage tracks to accommodate cell culture tube

IV. QUALITY CONTROL

A. Positive control
1. Determine the 50% tissue culture infective dose ($TCID_{50}$) of a reference rhinovirus (*see* Appendix 10.5–1).
2. Treat the reference virus with the buffer solutions in the same manner as the unknown virus. Use the reference virus at approximately 1,000 $TCID_{50}$s/ml.

B. Negative control
Treat supernatant fluid from negative (uninoculated) cell culture controls with buffer solutions in the same manner as the unknown virus.

V. PROCEDURE

A. Add 0.5 ml of each unknown virus, the reference rhinovirus, and the negative control supernatant to a labeled tube (13 by 100 mm) containing 0.5 ml of citrate buffer (pH 4.0).

B. Add 0.5 ml of each unknown virus, the reference rhinovirus, and the negative control supernatant to a labeled tube (13 by 100 mm) containing 0.5 ml of phosphate buffer (pH 7.0).

C. Incubate the tubes containing the virus and control mixtures at 37°C for 1 h.

D. Following the incubation, add 4.0 ml of 0.5 M phosphate buffer (pH 7.2) to each mixture of virus and control. This represents a 1:10 (10^{-1}) dilution of the original material.

E. Prepare serial 10-fold dilutions (10^{-2} to 10^{-5}) of each unknown and reference virus mixture. Use cell culture maintenance medium as diluent. Serial titration of the negative control is not necessary.
1. Add 0.9 ml of diluent to four labeled tubes (12 by 75 mm) for each mixture to be titrated.
2. Transfer 0.1 ml of the 1:10 dilution of each material to the appropriate 10^{-2} dilution tube, and mix by pipetting up and down. This represents a 1:100 (10^{-2}) dilution of the original material.
3. Prepare the remaining dilutions in the same manner. Use a separate pipette for each dilution.

F. Inoculate two or three diploid fibroblast cultures containing 1.0 ml of maintenance medium with 0.2 ml of each of the unknown and reference virus dilutions.

G. Inoculate two diploid fibroblast cultures containing 1.0 ml of maintenance medium with 0.2 ml of the negative control.

H. Incubate the cell cultures at 33°C in a stationary rack, and examine them daily for CPE. Record the titers.

VI. RESULTS

A. Rhinoviruses are acid labile and will show either a total inactivation or a reduction in titer in the material treated with acid (pH 4.0) compared with the activity and titer of viruses treated with the pH 7.0 buffer. High-titered isolates may not be completely inactivated, and in such cases, a ≥100-fold reduction in titer would be interpreted as acid lability. Although definitive confirmation requires a neutralization test, the combination of enterovirus-type CPE and acid lability is considered sufficient to designate an isolate a rhinovirus.

B. An enterovirus will show no reduction in titer after treatment at pH 4.0 compared with the titer after treatment at pH 7.0.

C. The rhinovirus control should show either total or a ≥100-fold reduction in titer after treatment at pH 4.0.

D. Negative controls should show no toxicity or CPE.

APPENDIX 10.5–3 *(continued)*

References

1. **Ballew, H., F. T. Forrester, H. C. Lyerla, W. M. Velleca, and B. R. Bird.** 1977. *Differentiation of Enteroviruses from Rhinoviruses,* p. 112–113. U.S. Department of Health, Education and Welfare. Center for Disease Control, Atlanta, Ga.
2. **Gwaltney, J. M., Jr., R. J. Colonno, V. V. Hamparian, and R. B. Turner.** 1989. Rhinovirus, p. 579–614. *In* N. J. Schmidt and R. W. Emmons (ed.), *Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections,* 6th ed. American Public Health Association, Washington, D.C.

APPENDIX 10.5–4

Rubella Interference Assay

Reprinted from **S. Aarnaes and B. J. Daidone.** 1992. Observation and maintenance of inoculated cell cultures, p. 8.7.1–8.7.16. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook,* vol. 2. American Society for Microbiology, Washington, D.C.

A. Cells

Primary African green monkey kidney (AGMK) cells are the cells of choice for isolation of rubella virus from clinical specimens. A CPE is not produced, and a positive rubella culture is detected by interference with the growth of a superinfected challenge virus.

B. Materials (in addition to materials used for routine viral culture)
   1. Five specimen-inoculated tubes and four each of lot-matched negative and positive (rubella-inoculated) controls
   2. Challenge virus (echovirus type 11 or coxsackievirus A9) diluted with maintenance medium or HBSS to contain 100 50% tissue culture infective doses ($TCID_{50}$s) per 0.1 ml. Determine endpoint titer and appropriate dilution as described in Appendix 10.5–1. Do not inoculate specimens until a titered challenge virus is available.
   3. Incubator, 35 to 37°C

C. Interference assay
   1. Procedure
      a. Remove the culture medium from the tubes to be challenged, and save it. Store the harvested fluid at −70°C; discard negative harvests upon completion of the interference assay.
      b. Refeed tubes to be challenged with 1 ml of maintenance medium.
      c. Deliver 0.1 ml of the challenge virus dilution containing 100 $TCID_{50}$s to each specimen and control tube to be challenged.
      d. Refeed unchallenged tubes weekly with 1 ml of culture maintenance medium.
      e. Incubate challenged and unchallenged cultures at 35°C. Observe challenged cultures daily for 7 days for the development of enterovirus CPE.
   2. Schedule summary
      a. At 1 week
         (1) Challenge one specimen tube, one lot-matched positive control, and one lot-matched negative control.
         (2) Refeed unchallenged tubes with maintenance medium.
      b. At 2 weeks
         (1) Challenge one specimen tube, one lot-matched positive control, and one lot-matched negative control.
         (2) Subpassage harvested cells and fluid from one specimen tube into two AGMK cell tubes. Prepare two lot-matched positive and negative controls.
         (3) Refeed unchallenged tubes with maintenance medium.
      c. At 3 weeks
         (1) Challenge two specimen tubes (one from primary inoculation and one subpassage) and the lot-matched positive and negative controls.
         (2) Refeed unchallenged tubes with maintenance medium.

APPENDIX 10.5–4 *(continued)*

d. At 4 weeks
Challenge two specimen tubes (one from primary inoculation and one subpassage) and the lot-matched positive and negative controls.

D. Interpretation of interference assay
1. Controls
   a. The challenged positive control should show no enterovirus CPE.
   b. The challenged negative control should show enterovirus CPE.
   c. The unchallenged negative control should show no CPE.
2. Specimens
   a. Cultures that exhibit enterovirus CPE are negative for rubella virus.
   b. Cultures that do not exhibit enterovirus CPE are considered positive for rubella virus and may be confirmed by neutralization (*see* Appendix 10.5–2) or other immunoassay.
   c. Subpassage cultures exhibiting partial interference.

◩ **NOTE:** Infection of AGMK cells by simian viruses may be associated with reduced sensitivity for the recovery of rubella virus (1). Therefore, in addition to the positive and negative controls included in the interference assay, select 8 to 10 uninoculated AGMK cell tubes from each lot, and observe these in parallel with specimen-inoculated tubes. Observe for the development of spontaneous CPE, and use guinea pig RBCs to test for HAd in two tubes each week (*see* Fig. 10.5–6).

Reference

1. **Herrmann, K. L.** 1979. Rubella virus, p. 725–766. *In* E. H. Lennette and N. J. Schmidt (ed.), *Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections*, 5th ed. American Public Health Association, Washington, D.C.

APPENDIX 10.5–5

Preparation of 10% RBC Suspension

◩ **NOTE:** Use aseptic technique.

1. Obtain guinea pig RBCs collected aseptically in Alsever's solution. Store at 4°C and use within 7 days of receipt.
2. Transfer 5 to 7 ml of suspended blood to a sterile, graduated, 15-ml conical centrifuge tube.
3. Add an equal volume of phosphate-buffered saline (PBS) and centrifuge at 700 to 900 $\times$ *g* for 5 min at room temperature.
4. Discard the supernatant, suspend the RBC pellet in 10 ml of PBS by gently pipetting up and down several times, and centrifuge the suspension at 700 to 900 $\times$ *g* for 5 min at room temperature.
5. Repeat step 4 twice.
6. Discard the supernatant, and measure the packed cell volume.
7. Add a volume of PBS equal to nine times the packed cell volume.
8. Store at 4°C and use within 7 days. Discard if evidence of hemolysis or contamination occurs.

APPENDIX 10.5–6

Hemagglutination Procedure

Several viruses, including those identified in Table 10.5–A3, agglutinate RBCs of various avian and mammalian species.

◩ **NOTE:** The procedure can be performed using microtiter trays, adjusting volumes accordingly.

Procedure

1. Prepare a 10% suspension of RBCs by using as described in Appendix 10.5–5. Store at 4°C and use within 1 week of preparation.
2. On the same day as performing the hemagglutination procedure, dilute an aliquot of the 10% stock suspension with phosphate-buffered saline (PBS) (pH 7.2 to 7.4) or physiologic saline to yield a 0.5% RBC suspension.

APPENDIX 10.5–6 *(continued)*

Table 10.5–A3 Hemagglutination characteristics of representative viruses

Virus	RBC type	Incubation temp (°C)[a]
Adenovirus	Monkey or rat[b]	37
Enteroviruses (some)	Human O	4, 37, RT
Influenza, mumps, parainfluenza, Newcastle disease viruses	Chicken or guinea pig	RT, 4
Measles virus	Monkey	37
Reoviruses	Human O	RT

[a] RT, room temperature.

[b] Depends on virus type.

3. Label 12- by 75-mm tubes starting with 1:10 through 1:160.
4. Label one tube as an RBC control and one tube as a monolayer supernatant control.
5. Add 0.9 ml of PBS or saline to the 1:10 tube and 0.5 ml to the other tubes, including the control tubes.
6. Add 0.1 ml of viral culture supernatant to the 1:10 tube and mix well by pipetting up and down several times.
7. Transfer 0.5 ml from the 1:10 viral supernatant dilution to the 1:20 dilution tube and mix as before. Continue preparing serial dilutions in the same manner, discarding 0.5 ml from the final dilution (1:160).
8. Add 0.5 ml of cell culture supernatant from a lot-matched uninoculated control monolayer to the tube serving as the monolayer supernatant control. Mix by pipetting up and down several times and discard 0.5 ml of material from the tube.
9. Add 0.5 ml of RBC suspension to each tube, including the controls.
10. Shake to mix and incubate at the appropriate temperature (refer to Table 10.5–A3) for 30 to 45 min.
11. Read the agglutination patterns in each tube. The RBC and uninoculated supernatant controls should exhibit a button of settled RBCs at the tube bottom. Positive agglutination is recorded for tubes exhibiting a diffuse carpet of RBCs, while intermediate patterns are recorded as +/−. The hemagglutination endpoint is the highest dilution to show a + (complete) agglutination pattern.

   ◩ **NOTE:** Incubation of a viral supernatant with homologous antibody prior to the addition of the RBCs will result in inhibition of the agglutination reaction. This forms the basis for the HI assay which has been used primarily to identify influenza virus subtypes but is not typically performed in the routine viral diagnostic setting. Prior to using an antiserum in the HI test, nonspecific inhibitors of agglutination must be removed. Methods vary with the species of antiserum used, and the procedure should be performed as indicated by the reagent source.

APPENDIX 10.5–7

Preservation of Cell Culture Monolayers (1)

1. Prepare buffered formaldehyde preservative medium by combining the following ingredients. Store at room temperature.

   formaldehyde solution (37 to 40%) ..... 100 ml
   distilled water ..... 900 ml
   sodium phosphate, monobasic ($NaH_2PO_4 \cdot H_2O$) ..... 4 g
   sodium phosphate, dibasic (Na_2HPO_4) ..... 6.5 g

2. Aspirate the medium from the cell culture tubes to be preserved.
3. Add 2 to 4 ml of preservative to each tube.
4. Store tubes indefinitely at room temperature.

Reference

1. **Gurtler, J. H., C. Ballew, C. M. Preissner, and T. F. Smith.** 1982. Cell culture medium for preserving cytopathic effects in cell cultures. *Lab. Med.* **13:**244–245.

10.6 Isolation of *Chlamydia* spp. in Cell Culture

Chlamydiae are obligate intracellular bacteria that contain RNA and DNA, have a cell wall resembling those of gram-negative bacteria, and multiply by binary fission in a manner distinct from those of other bacteria. The 300- to 400-nm spherical elementary body (EB) is the infectious form of the organism. Following cellular infection, the EB reorganizes into a larger, metabolically active reticulate body (RB), which divides repeatedly by binary fission for 24 to 48 h and eventually develops into the characteristic intracytoplasmic inclusion. Human infections associated with the genus *Chlamydia* are summarized in Table 10.6–1. Despite the introduction of numerous nonculture assays, including amplified assays, culture remains an important assay for the detection of *Chlamydia trachomatis* infections and, because of its specificity, is recommended for laboratory testing in cases of sexual abuse and medicolegal situations.

I. PRINCIPLE

Chlamydiae are obligate intracellular bacteria, and the propagation of these organisms requires the use of cell culture techniques as used in culturing viruses. Although the growth cycles of the species *C. trachomatis, Chlamydia psittaci,* and *Chlamydia pneumoniae* vary to some extent, in general, a complete cycle takes from 36 to 72 h. The infectious EB enters the host cell and then, inside a cytoplasmic inclusion during the initial 8 to 12 h, reorganizes into the noninfectious RB (Fig. 10.6–1). After this latent phase, there is rapid growth of the inclusion, during which time the RB divides by binary fission. The RB then begins to reorganize into EBs within 24 to 72 h and forms an inclusion that often displaces the nucleus to one side of the host cell. The cell may rupture, releasing the infectious EBs, which, for some strains, are capable of initiating another round of replication in vitro. (For further discussion, see Appendixes 10.6–3 and 10.6–4).

II. SPECIMEN

A. Collection

Chlamydiae replicate primarily in columnar epithelium, and specimens should be collected in a manner that optimizes obtaining these cells.

1. Obtain samples as described in Table 10.6–2.

Table 10.6–1 Human chlamydial infections

Organism	Disease(s)
C. trachomatis	
Serotypes A, B, Ba, and C	Trachoma
Serotypes D through K	Cervicitis, epididymitis, inclusion conjunctivitis, nongonococcal disease, pelvic inflammatory disease, neonatal pneumonitis, proctitis
Serotypes L-1, L-2, and L-3	Lymphogranuloma venereum
C. psittaci	Psittacosis, ornithosis following contact with infected birds
C. pneumoniae	Upper and lower respiratory tract disease

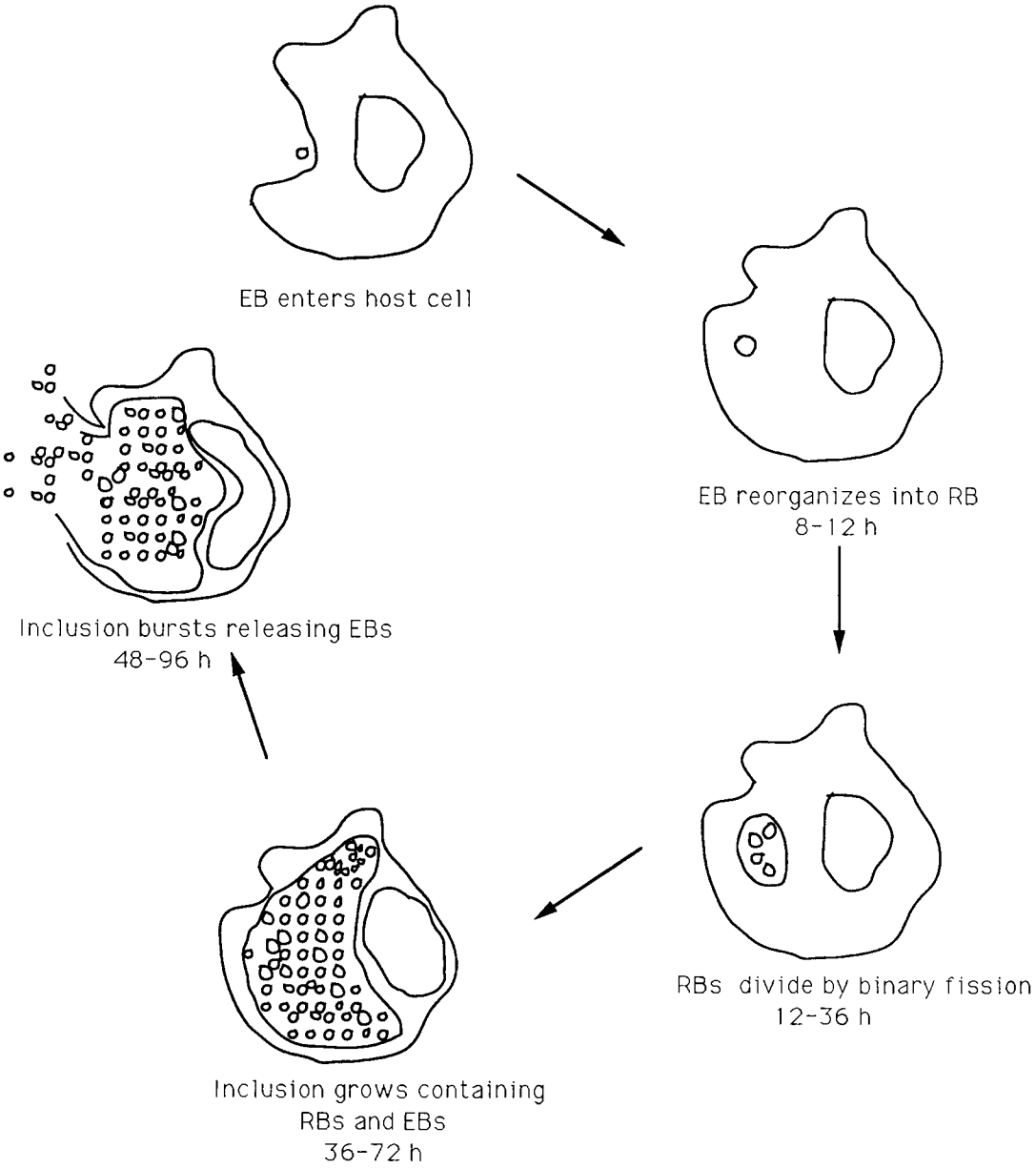

Figure 10.6–1 Developmental cycle of *Chlamydia* spp.

II. SPECIMEN *(continued)*

2. Specimens that consist solely of a purulent discharge are not appropriate. Using a sterile swab, clean the discharge from the area to be sampled (e.g., cervix, urethra, conjunctiva), and use a new swab to obtain a sample of the epithelial cells.
3. Immediately place swabs, scrapings, tissue biopsy samples, and small-volume aspirates into sterile leakproof screw-cap vials containing 1 to 2 ml of a chlamydial transport medium (CTM) such as 2-SP.
4. Collect nasopharyngeal aspirates and sputum specimens into sterile screw-cap containers, and mix with an equal volume of CTM.

B. Transport and storage

1. Place the specimen at 4°C, and transport it to the laboratory as soon as possible. Transport on wet ice or with a cold pack (2 to 8°C) if transport

Table 10.6–2 Collection of specimens

Type of specimen	Method of collection	Comment(s)
Swabs		
Cervical	Remove exocervical mucus with swab, and discard. Insert fresh swab at least 1 cm into endocervical canal, and rotate swab for 10 s.	Additional collection of urethral swab may increase number of positive individuals.
Conjunctival	Remove purulent exudate if present. Then, using flexible fine-shafted swab, rotate swab tip (premoistened with saline) over conjunctival surface.	
Nasopharyngeal	Insert dry, fine-shafted swab through nostril into nasopharynx, and rotate swab.	Preferred to throat swab
Rectal	Insert premoistened swab into rectum, and roll swab against mucosal surface.	Collected primarily in cases of proctitis
Urethral	Express and discard any exudate. Insert fine, flexible-shafted swab 2–4 cm into urethra, rotate swab gently two or three times, and withdraw swab. Patient should not urinate for at least 1 h before specimen collection.	
Vaginal	Rotate swab against mucosal surface for 10 s.	*C. trachomatis* is not associated with vaginitis but can infect anestrogenic vagina.
Fluids and aspirates		
Epididymal aspirate	Mix with equal volume of CTM.	
Fallopian tube aspirate	Same as above	
Nasopharyngeal aspirates and other respiratory tract fluids or washes	Place into sterile screw-cap container. May be mixed with equal volume of CTM if transport to laboratory is not immediate.	
Semen, seminal fluid	Mix with equal volume of CTM.	These specimens are usually toxic to cell cultures.
Tissues		
Endometrial or tubal biopsy samples; lung, surgical, and autopsy tissues	Place into sterile screw-cap container. Add CTM to prevent drying.	

II. SPECIMEN *(continued)*

time is more than a few minutes. Ideally, specimens should be transported to the laboratory within 4 h of collection.

2. Upon receipt in the laboratory, place the specimen at 4°C. If specimens cannot be cultured within 48 h, freeze them at −70°C. The loss in inclusion-forming units (IFU) is greater if the specimen is frozen rather than refrigerated during the initial 24 h and is comparable to the loss when specimens are held for 48 h at 4°C (1). After 48 h, the viability of *C. trachomatis* at 4°C drops below that which would be obtained if the specimen were frozen at −70°C.

III. MATERIALS

A. Reagents

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

1. CTM: 2-SP (0.2 M sucrose, 0.02 M phosphate)

a. Dissolve the following ingredients separately in approximately 300 ml of deionized water.

K_2HPO_4 …… 2.01 g
KH_2PO_4 …… 1.01 g
sucrose ….. 68.46 g

b. Combine the solutions, and add deionized water to 1,000.0 ml.

c. Adjust the pH to 7.2 to 7.4.

d. Sterilize by Millipore filtration through a 0.2-μm-pore-size filter.

e. Aseptically add sterile antimicrobial solutions. The choice and concentration of antimicrobial agents may vary depending on individual laboratory experience with contamination. Antimicrobial agents (and final concentrations) frequently used include gentamicin (50 μg/ml), vancomycin (100 μg/ml), and amphotericin B (25 μg/ml). Nystatin (25 U/ml) may be used in place of amphotericin B; nystatin is not soluble in aqueous form, and care must be taken to keep this material in suspension while dispensing. Do not include penicillin in CTM.

f. Dispense 1- to 2-ml volumes into sterile screw-cap vials. Three or four sterile 5-mm-diameter glass beads added to each vial will facilitate disruption of cells prior to inoculation. Glass beads may be sterilized by autoclaving or by dry heat (160°C, 60 min).

g. Store at −20°C for up to 6 months or at 4°C for 1 week.

h. *Note:* Some laboratories include 3 to 10% heat-inactivated fetal bovine serum in CTM. This may help retain viability of cells during freezing. However, each lot of serum must be shown to be free of chlamydial inhibitors and antibodies.

2. Chlamydial isolation medium

a. Aseptically combine the following sterile stock solutions.

Eagle's minimal essential medium (EMEM), 10×, in Earle's salts without glutamine …… 50.0 ml
glutamine (200 mM) ……5.0 ml
fetal bovine serum (heat inactivated [56°C, 30 min]) …… 50.0 ml
cycloheximide (100× stock) ..5.0 ml
gentamicin, sufficient volume to yield …… 50 μg/ml

HEPES may be included as an additional buffer to yield a final concentration of 20 mM.

b. Add sterile deionized water to a final volume of 500.0 ml. The medium should be reddish orange to cherry red, indicating a pH of 7.2 to 7.4.

c. Dispense 100- to 500-ml volumes into sterile screw-cap bottles.

d. Store at 4°C for up to 2 weeks once glutamine and cycloheximide have been added. Without these components, medium may be stored for up to 3 months at 4°C, with glutamine and cycloheximide added when the medium is used.

e. If 1 × EMEM is used, eliminate the deionized water and bring the final volume to 500 ml with the 1× EMEM.

3. Cycloheximide (100× stock)

a. Ingredients

cycloheximide …….. 10.0 mg
deionized water …. 100.0 ml

b. Dissolve cycloheximide in water, and sterilize by filtration through a 0.2-μm-pore-size filter.

c. Aliquot in small volumes, and store frozen in the dark. The material may be stored at −20°C or lower for up to 1 year.

d. The final concentration of cycloheximide in the chlamydial isolation medium is usually 1 μg/ml; however, this may vary with each new lot or batch of cycloheximide. Using the chlamydial control stock (Appendix 10.6–1), assay each new

III. MATERIALS *(continued)*

batch at several concentrations ranging from 0.5 to 2.5 μg/ml. The optimal concentration is that which yields an inclusion number equal to or greater than the count obtained with the cycloheximide currently in use.

e. Cycloheximide is toxic. Avoid inhalation and exposure to skin.

4. Streptomycin-vancomycin stock (20×)
A stock solution of 2 mg/ml can be prepared, aliquoted, and frozen at −20°C or lower. To use, add 0.1 ml to each 2.0 ml of specimen to be decontaminated.

5. 0.01 M phosphate-buffered saline (PBS), pH 7.2

a. Ingredients

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.20 g
deionized water	to 1,000.0 ml

b. Dissolve the components completely in distilled water.

c. Sterilize by filtration through a 0.2-μm-pore-size filter, and dispense into sterile screw-cap bottles.

d. Store at 4°C. Do not use if cloudiness or a precipitate develops.

6. Hanks' balanced salt solution (HBSS), pH 7.2 to 7.6

7. Cell culture medium
Isolation medium is EMEM with Earle's salts and 10% heat-inactivated fetal bovine serum, 2 mM glutamine, gentamicin (50 μg/ml), and cycloheximide (1 μg/ml). The optimal cycloheximide concentration may range from 0.5 to 2.0 μg/ml; determine the appropriate concentration for each batch of cycloheximide. While a high-glucose medium (e.g., 30 μM) is frequently used, its efficacy in detecting inclusions when immunostaining rather than iodine staining is used has not been determined. Cycloheximide is not included in culture medium used to maintain cell cultures prior to inoculation.

8. Acetone or methanol to fix monolayers: choice depends on fixative specified by the immunostaining reagent supplier.

9. Immunostaining reagents (monoclonal or polyclonal antibodies)
Fluorescein- or enzyme-conjugated antibodies suitable for culture confirmation by immunostaining procedures are commercially available. Direct immunofluorescence is the assay most frequently used for culture confirmation. Immunoreagents may be obtained at the working dilution and containing a counterstain. In all cases, the manufacturer's instructions must be followed when these products are used.

10. Buffered-glycerol mounting medium, pH ≥8.0

B. Supplies

1. Screw-cap vials containing 1 to 2 ml of CTM

2. Collection swabs
Test each lot of swabs for inactivation of chlamydial replication and toxicity (Appendix 10.6–2). Use cotton, Dacron, or rayon swabs with metal or plastic shafts; do not use calcium alginate or wooden-shafted swabs. Cytobrushes may also be used to obtain cervical specimens. Both collection devices yield comparable results (7).

3. Cell culture vials or plates (Appendix 10.6–3)
Shell vial cultures can be obtained from several sources. Alternatively, 1-dram shell vials (12 by 45 mm) can be purchased for seeding in the laboratory. McCoy cells, a continuous mouse fibroblast line, are the cells most commonly used to culture *C. trachomatis* from clinical material (12), while HL (5) and HEp-2 (10) cells are preferred for propagating *C. pneumoniae* (5, 9).

a. Use cell culture monolayers within 1 week of receipt.

b. See procedure 10.2 for assessment and storage of cell culture monolayers.

c. Incubate cultures at 35 to 37°C prior to inoculation. Confluent monolayers may be incubated at room temperature to retard monolayer overgrowth.

4. Sterile pipettes and safety pipetting device

5. Protective clothing and gloves

III. MATERIALS *(continued)*

6. Sterile disposable tissue grinder, scalpels, small sterile petri plates, and CTM for processing tissue samples
7. Stock strains to be used as positive controls (Appendix 10.6–1)
8. Glass slides, 1 by 3 in.

C. Equipment

1. Vortex mixer
2. Incubator, 36 ± 1°C with 5 to 8% CO_2
   CO_2 is not needed for confluent monolayers in tightly capped vials.
3. Class II biological safety cabinet
4. Centrifuge and carriers to accommodate shell vials
5. Refrigerator
6. Freezer, −70°C for storage of specimens and isolates; −20°C or lower for storage of CTM and reagents requiring frozen storage
7. Inverted light microscope, ×100 to ×200
8. Microscope (×200 to ×400), fluorescence or light, depending on whether fluorescein- or enzyme-conjugated immunostaining reagents are used

IV. QUALITY CONTROL

A. Include a positive control with each batch of chlamydial cultures. Ideally, the control used when genital specimens are cultured should be a *C. trachomatis* serovar that is commonly isolated from the genital tract (e.g., serovar E or D). Prepare control stock as described in Appendix 10.6–1.

B. Include a negative control with each run.

C. Studies have shown variation in the abilities of different types and lots of swabs to affect chlamydial recovery (2, 8). Test each lot of swabs for chlamydial inactivation (Appendix 10.6–2).

V. PROCEDURE

A. Specimen preparation

The principles of specimen preparation for chlamydial cultures are similar to those applied to preparation of specimens for viral culture (*see* procedure 10.4). Do not include penicillin in reagents used for specimen preparation.

1. Swabs or scrapings
   Vortex the sample for 2 min, and discard the swabs.
2. Fluids
   a. Mix with an equal volume of CTM.
   b. Vortex for 2 min.
3. Tissues
   Vortex 1- to 2-mm fragments in 1 to 2 ml of CTM with three or four sterile glass beads. For larger pieces, process in a sterile tissue grinder using 2 to 6 ml of CTM.

B. Inoculation and incubation of cell cultures

1. Inoculate each specimen into two shell vials. Examine each monolayer prior to inoculation to ensure that the monolayer is healthy and confluent (*see* procedure 10.5).
2. Revortex specimens prior to inoculation.
3. Remove the culture medium from the vials to be inoculated, and discard it.
4. Add 0.2 ml of vortexed specimen to each of two labeled vials. Inoculate positive control vials with stock material diluted to contain 50 to 100 IFU/0.2 ml (Appendix 10.6–1). Mock-inoculate negative control vials with 0.2 ml of CTM.
5. Centrifuge the inoculated vials in a swinging bucket rotor at 1,500 × *g* for 45 to 60 min at 22 to 37°C.
6. After centrifugation, remove vials from the centrifuge, and feed the cultures with chlamydial isolation medium containing cycloheximide. Alternatively, remove the vials from the centrifuge and incubate them in a stationary position at 37°C for an additional 60 min prior to adding the medium. With

V. PROCEDURE *(continued)*

potentially toxic specimens (e.g., tissue homogenates), remove the inoculum and rinse the monolayer once with HBSS prior to adding the isolation medium.

7. Incubate the cultures at 37°C for 48 to 72 h. A CO_2 incubator (5 to 8%) is not necessary when tightly stoppered vials are used.

C. Fixation of monolayers

1. After incubation is completed, examine cultures with an inverted microscope to identify those that are unsatisfactory because of contamination or excessive toxicity. Reinoculate these samples, or obtain new specimens.
2. If blind subpassage is to be performed, select one vial for fixation and immunostaining, and put aside the second vial for harvesting and subpassage as described below.
3. Remove medium from the cultures to be stained.
4. Rinse each monolayer by adding and removing 1 ml of PBS.
5. Add 1 ml of fixative (as specified for the staining reagent), and fix the monolayer for 10 min.
6. Decant the fixative, and rinse the monolayer with 1 ml of PBS as described above.
7. The monolayer is now ready to be stained.

D. Immunostaining of monolayers (direct immunofluorescence)

1. Include positive and negative controls.
2. Add approximately 0.05 ml of immunoreagent to each vial so that the monolayer is covered. Do not add immunoreagent to dry monolayers. If necessary, premoisten the monolayers by rinsing them with distilled water.
3. Cover the vials, and incubate them for 30 min at room temperature or as specified by the reagent manufacturer. Incubation may be performed on a rotator (approximately 20 rpm) to facilitate distribution of the reagent.
4. Remove the reagent from each vial, and rinse the monolayer once with distilled water.
5. Allow the monolayers to air dry completely. Using slightly curved fine-tipped forceps, remove each coverslip and place it cell side down on a drop of buffered-glycerol mounting medium.
6. Using a fluorescence microscope, examine the entire monolayer at $\times 200$, and confirm questionable inclusions at $\times 400$.
7. If coverslips cannot be examined right after staining, store in the dark at 4°C for up to 24 h.

E. Blind subpassage

1. Remove the medium from the vial to be subpassaged, and add 1 ml of CTM and three or four sterile glass beads. Alternatively, if the monolayer appears fragile or is lifting off the coverslip, add the glass beads directly to the vial and proceed to step V.E.2.
2. Recap the vial, and vortex it for 2 min.
3. Inoculate one or two monolayers with 0.1 ml of the harvested material by proceeding with inoculation as described above.

   ◩ **NOTE:** As an alternative to mechanically disrupting cells to perform a blind pass, cultures of *C. pneumoniae* have been recentrifuged on days 3, 4, and 5 and stained on day 7 of the culture protocol. Recentrifugation of the original culture has been reported to increase the number of IFU up to 5,000-fold (9).
4. The remaining material may be transferred to a cryotube and stored at −70°C or lower.

VI. RESULTS

A. Control cultures

1. The positive control must show strongly fluorescent (brilliant apple-green) characteristic intracytoplasmic inclusions (Fig. 10.6–2). Inclusions often occupy >50% of the cellular cytoplasm; smaller ones may be observed in younger cultures or if secondary infection is taking place.
2. The negative control should have a satisfactory monolayer and show minimal to no nonspecific fluorescence.

B. Specimen cultures

1. A positive culture is one showing one or more characteristic fluorescent intracytoplasmic inclusions.
2. A negative culture is one that has minimal or no nonspecific staining and no chlamydial inclusions.
3. An unsatisfactory culture is one showing strong nonspecific fluorescence that interferes with reading or one that does not contain an adequate monolayer (≥75% of that observed in the negative control) as a result of contamination or toxicity. Subpassage, or obtain a replacement sample. Although an attempt may be made to treat a contaminated specimen with an antimicrobial concentrate and then reinoculate it, it is better to obtain a new sample.

VII. PROCEDURE NOTES

A. Tissue and semen samples may be toxic to the cell culture monolayers. Inoculate these samples undiluted and after dilution (1:10 and 1:100) in HBSS. With semen samples, high-speed centrifugation may be used to reduce toxicity (*see* procedure 10.4).

B. Avoid chilling during centrifugation, since EBs attach to but do not efficiently enter the host cell at cool temperatures. Temperatures above 37°C may result in loss of chlamydial viability.

C. Some workers have reported an isolation rate of ≥10% when primary cultures are subpassaged (12). However, the percent increase detected by a blind subpassage should be determined by each laboratory, and the extra cost and time required to complete a blind subpassage should be taken into account.

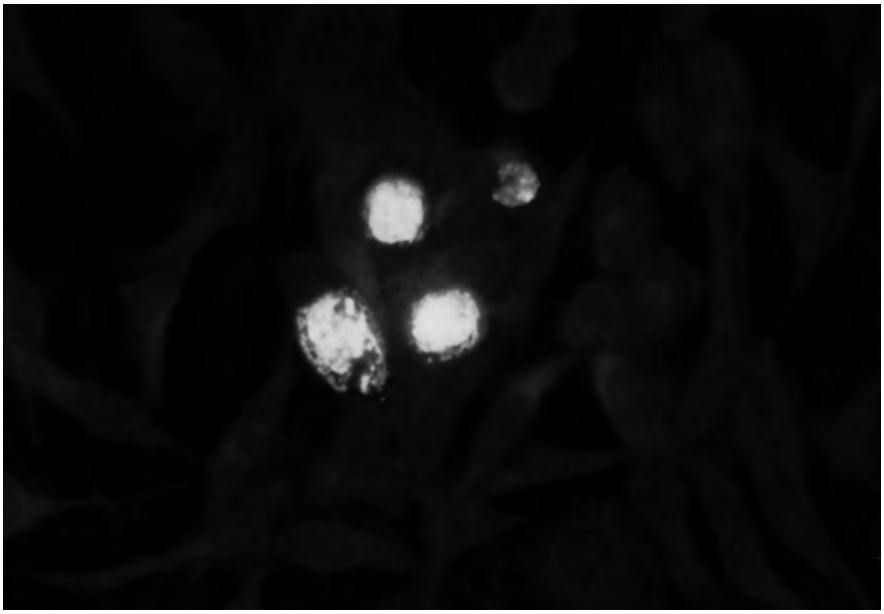

Figure 10.6–2 Typical chlamydial inclusions in a culture stained at 48 h with a monoclonal antibody to *C. trachomatis.*

VII. PROCEDURE NOTES
(continued)

D. Although there are reports of 96-well microtiter plates used for chlamydial cultures, this method has been shown to be less sensitive than culture systems using shell vials (11). A high-volume laboratory, however, might prefer to use microtiter plates for convenience. Seeding of microtiter plates must be done in-house, and centrifuge carriers designed for microtiter plates must be used for the inoculation step. When using microtiter plates, it is essential to incubate the plates in 5% CO_2.

E. While a monoclonal antibody recognizing the major outer membrane protein would be adequate for genital cultures, respiratory cultures should be stained with a lipopolysaccharide monoclonal antibody, since *C. psittaci* and *C. pneumoniae* can be recovered from these sites (Appendix 10.6–4).

F. Alternative staining methods for the detection of chlamydial inclusions are available (2, 3, 11). However, these methods are less sensitive than immunostaining with fluorescein-or enzyme-conjugated antibodies (Appendix 10.6–4).

1. Iodine

Methanol-fixed monolayers are stained for 5 to 10 min with 0.5 to 1 ml of either Lugol's iodine or 5% iodine in 10% potassium iodide. The monolayers are rinsed with PBS and examined by light microscopy for reddish-brown glycogen-containing cytoplasmic inclusions. *C. psittaci* and *C. pneumoniae* inclusions do not stain with iodine.

2. Giemsa

Various versions of the Giemsa stain have been used to visualize chlamydial inclusions, but this method is not routinely used for culture confirmation.

G. A standard chlamydial culture is one in which culture confirmation involves the visual identification of characteristic inclusions; methods that utilize solubilized cell culture monolayers (e.g., solid-phase EIA) may result in false-positive results as a result of cross-reactivity with organisms present in clinical samples (4).

H. Although DEAE-dextran is not routinely included in the isolation medium in the diagnostic laboratory, some workers have reported increased isolation rates when monolayers were treated with DEAE-dextran prior to inoculation (6). Decant medium from the monolayers, add 1 ml of DEAE-dextran in HBSS (30 μg/ml), and incubate the monolayers at room temperature for 30 min. Remove the DEAE-dextran solution prior to inoculation, or, alternatively, remove the solution along with the inoculum after centrifugation. The use of DEAE does not eliminate the need for cycloheximide in the isolation medium.

I. The procedure described for the isolation of *C. trachomatis* can be applied to the isolation of *C. psittaci* and *C. pneumoniae.* The human cell line HeLa 229 has been used for the culture of these two species. In addition, the HL and HEp2 cell lines have been reported to be more sensitive than HeLa 229 and McCoy cells for the primary isolation of *C. pneumoniae* (5, 10).

J. All specimen and culture manipulations should be carried out in a class II biological safety cabinet with appropriate safety precautions. Laboratory infections with *C. psittaci* have been reported, and isolation attempts should be done only by laboratories meeting the biohazard safety requirements.

REFERENCES

1. **Aarnaes, S. L., E. M. Peterson, and L. M. de la Maza.** 1984. The effect of media and temperature on the storage of *Chlamydia trachomatis*. *Am. J. Clin. Pathol.* **81:**237–239.
2. **Barnes, R. C.** 1989. Laboratory diagnosis of human chlamydial infections. *Clin. Microbiol. Rev.* **2:**119–136.
3. **Bird, B. R., and F. T. Forrester.** 1981. *Laboratory Diagnosis of Chlamydial Infections*, p. 55–62. Centers for Disease Control, Atlanta, Ga.
4. **Centers for Disease Control.** 1990. False-positive results with the use of chlamydia tests in the evaluation of suspected sexual abuse. *Morb. Mortal. Wkly. Rep.* **39:**932–935.
5. **Cles, L. D., and W. E. Stamm.** 1990. Use of HL cells for improved isolation and passage of *Chlamydia pneumoniae*. *J. Clin. Microbiol.* **28:**938–940.
6. **Kuo, C.-C., S. P. Wang, B. B. Wentworth, and J. T. Grayston.** 1972. Primary isolation of TRIC organisms in HeLa 229 cells treated with DEAE-dextran. *J. Infect. Dis.* **125:**665–668.
7. **Lees, M. I., D. M. Newnan, H. Plackette, P. W. Traynor, J. R. Forsyth, and S. M. Garland.** 1990. A comparison of cytobrush and cotton swab sampling for the detection of *Chlamydia trachomatis* by cell culture. *Genitourin. Med.* **66:**267–269.
8. **Mahony, J. B., and M. A. Chernesky.** 1985. Effect of swab type and storage temperature on the isolation of *Chlamydia trachomatis* from clinical specimens. *J. Clin. Microbiol.* **22:**865–867.
9. **Pruckler, J. M., N. Masse, V. A. Stevens, L. Gang, Y. Yang, E. R. Zell, S. F. Dowell, and B. S. Fields.** 1999. Optimizing the culture of *Chlamydia pneumoniae* by using multiple centrifugations. *J. Clin. Microbiol.* **37:**3399–3401.
10. **Roblin, P. M., W. Dumornay,** and **M. R. Hammerschlag.** 1992. Use of HEp-2 cells for improved isolation and passage of *Chlamydia pneumoniae*. *J. Clin. Microbiol.* **30:**1968–1971.
11. **Schachter, J.** 1985. Immunodiagnosis of sexually transmitted disease. *Yale J. Biol. Med.* **58:**443–452.
12. **Smith, T. F.** 1982. Role of the diagnostic virology laboratory in clinical microbiology: tests for *Chlamydia trachomatis* and enteric toxins in cell culture, p. 82–119. *In* L. M. de la Maza and E. M. Peterson (ed.), *Medical Virology*. Elsevier Biomedical Press, New York, N.Y.

SUPPLEMENTAL READING

Mahony, J. B., B. K. Coombes, and M. A. Chemesky. 2003. *Chlamydia* and *Chlamydophila*, p. 991–1004. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, D.C.

APPENDIX 10.6–1

Preparation of Chlamydial Control Stock

A. Control material may be obtained from a reference laboratory (e.g., ATCC, Manassas, Va.) or prepared with a clinical isolate.

B. Inoculate vials following the procedure described in item V.B above. Follow instructions provided by the supplier for inoculum dilutions and volumes to use. For example, duplicate vials may be inoculated with 5, 50, and 100 μl of the material.

C. Determine the volume that produces inclusions in 80 to 100% of the cells in 48 h, and subpassage 20 μl of this material into each of 10 vials following the procedure described in item V.E above.

D. After 48 h (72 h for *C. pneumoniae*), stain one or two vials to confirm 80 to 100% infection of the monolayer, and harvest the remaining vials as described for subpassage (*see* item V.E above).

E. Pool the contents of the vials, and aliquot the pool in small volumes (e.g., 0.02 ml).

F. Freeze aliquots at −70°C.

G. Thaw a frozen aliquot, and prepare four or five serial 10-fold dilutions in CTM or HBSS.

H. Inoculate two or three McCoy cell vials (HL or HEp-2 for *C. pneumoniae*) with each dilution (*see* item V.B above).

I. Fix and stain the vials after incubation for 48 h (72 h for *C. pneumoniae*) (*see* items V.C and D above).

J. Count the inclusions at each dilution, and determine the dilution necessary to obtain 50 to 100 inclusions per coverslip monolayer. This dilution contains 50 to 100 IFU/0.2 ml.

APPENDIX 10.6–2

Procedure for Pretesting Swabs

A. For each lot of swabs to be tested, prepare four tubes, each containing 1 to 2 ml of CTM containing chlamydial control stock at 250 to 500 IFU/ml of CTM.
B. Place one swab into each of two tubes. Use the other two tubes as no-swab controls.
C. Incubate the tubes to reflect the conditions of specimen transport as defined by the laboratory. For example, incubate the tubes for 2 h at room temperature and then place them at 4°C for 24 h.
D. Inoculate two cell culture monolayers with each of the test and control samples at each time point (e.g., 2 and 24 h for the example given above) by using the procedure described in item V.B above.
E. Harvest (*see* item V.C above) and stain (*see* item V.D above) cultures in the usual manner.
F. Count the inclusions in each monolayer. Do not use lots producing extensive monolayer toxicity and/or a ≥20% reduction in inclusion counts (compared with the no-swab controls).

APPENDIX 10.6–3

Cell Lines

A variety of cell lines have been reported to support the growth of chlamydiae. However, the most widely used cell lines for culturing *C. trachomatis* are McCoy and HeLa 229. The HL and HEp-2 cell lines are preferred for the primary isolation of *C. pneumoniae* (1). Cell monolayers are prepared on coverslips in shell vials or microtiter plates. Centrifugation of the specimen onto the monolayer (3, 4) and incorporation of inhibitors of eukaryotic cell protein synthesis into the cell culture medium give higher chlamydial yields. Although many metabolic inhibitors, including 5-iododeoxyuridine, cytochalasin ementine, and irradiation, have been used, cycloheximide is the cell inhibitor most commonly used for chlamydial cultures. Pretreatment of the monolayers with DEAE-dextran has also been reported to enhance recovery when a strain is grown from the trachoma biovar. However, this pretreatment is not commonly used for primary isolation (2).

◩ **NOTE:** Refer to procedure 10.2 for commercial cell culture sources and maintenance.

References

1. **Cles, L. D., and W. E. Stamm.** 1990. Use of HL cells for improved isolation and passage of *Chlamydia pneumoniae. J. Clin. Microbiol.* **28:**938–940.
2. **Kuo, C.-C., S. P. Wang, B. B. Wentworth, and J. T. Grayston.** 1972. Primary isolation of TRIC organisms in HeLa 229 cells treated with DEAE-dextran. *J. Infect. Dis.* **125:**665–668.
3. **Ripa, K. T., and P.-A. Mardh.** 1977. New simplified culture technique for *Chlamydia trachomatis*, p. 323–327. *In* K. K. Holmes and D. Hobson (ed.), *Non-Gonococcal Urethritis and Related Infections.* American Society for Microbiology, Washington, D.C.
4. **Ripa, K. T., and P.-A. Mardh.** 1977. Cultivation of *Chlamydia trachomatis* in cycloheximide-treated McCoy cells. *J. Clin. Microbiol.* **6:**328–331.

APPENDIX 10.6–4

Detection of Inclusions

Chlamydial inclusions, which contain 100 to 1,000 EBs (1), can be detected by a variety of staining methods. Before the advent of monoclonal antibodies, both Giemsa and iodine stains were used to detect the typical intracytoplasmic inclusions in infected cultures. Giemsa stain was used primarily on direct smears, and iodine was used to identify the glycogen-containing inclusions that are produced by *C. trachomatis* but not *C. pneumoniae* or *C. psittaci* (4). However, monoclonal antibodies directed at either the major outer membrane protein of *C. trachomatis* or the lipopolysaccharide of chlamydiae have proven to be more sensitive than either of these staining methods (2, 3). It is important to remember that in general, the monoclonal antibodies to major outer membrane protein react only with *C. trachomatis*, while the monoclonal antibodies to lipopolysaccharide detect all species within this genus.

References

1. **de la Maza, L. M., and E. M. Peterson.** 1981. Scanning electron microscopy of McCoy cells infected with *Chlamydia trachomatis. Exp. Mol. Pathol.* **36:**217–226.
2. **Munday, P. E., A. P. Johnson, B. J. Thomas, and D. Taylor-Robinson.** 1980. A comparison of immunofluorescence and Giemsa for staining *Chlamydia trachomatis* inclusions in cycloheximide-treated McCoy cells. *J. Clin. Pathol.* **33:**177–179.
3. **Schachter, J.** 1985. Immunodiagnosis of sexually transmitted disease. *Yale J. Biol. Med.* **58:**443–452.
4. **Schachter, J., and C. Dawson.** 1978. *Human Chlamydial Infections.* Publishing Sciences Group, Littleton, Mass.

10.7 Direct Detection of Viruses and *Chlamydia* in Clinical Samples

I. MOLECULAR DETECTION ASSAYS

Commercially available assays for diagnostic testing are available for several viral agents and for *Chlamydia trachomatis* (Table 10.7–1) and are discussed in section 12.

Table 10.7–1 Direct specimen testing: viral and chlamydial infections[a]

Method[b]	Specimen type or source[c]	Representative agent(s)
Antigen detection kits and reagents (e.g. IF, EIA, OIA, latex agglutination)	Blood leukocytes	CMV
	Genitourinary tract	*C. trachomatis*
	Lesion	HSVs, varicella-zoster virus
	Ocular	HSV (negative results may require confirmation by culture), *C. trachomatis*
	Respiratory tract	Adenoviruses, influenza virus types A and B, measles virus, parainfluenza virus types 1–4, respiratory syncytial virus, *C. trachomatis. Note:* Pools containing antibodies to several respiratory viruses are available commercially.
	Serum, plasma	HBV, HIV-1
	Stool	Adenoviruses (group), enteric adenoviruses (types 40 and 41), rotaviruses
Nucleic acid assays		
Nonamplified	Genitourinary tract	*C. trachomatis*, HPV
Amplified	Blood leukocytes	CMV DNA, HIV-1 DNA
	Genitourinary tract	*C. trachomatis*
	Serum, plasma	HBV DNA, HCV RNA, HIV-1 RNA, CMV mRNA

[a] Abbreviations: OIA, optic immunoassay; HBV, hepatitis B virus; HIV-1, human immunodeficiency virus type 1; HPV, human papillomavirus.

[b] Refer to kit manufacturer regarding specimens for which the reagent can be used and specific instructions regarding collection, transport, and storage of specimens.

[c] Anti-chlamydial LPS MAbs may cross-react with bacteria present in vaginal, rectal, and oral specimens, and false-positive results have been reported with EIA testing of vaginal specimens from children (2, 5, 6, 11) and of rectal specimens from adults (12) and children (11) and with direct IF staining of rectal smears from adults and children (6, 11).

II. ANTIGEN DETECTION ASSAYS: IMMUNOFLUORESCENCE (IF) PROCEDURE

NOTE: Other antigen detection procedures are discussed elsewhere in this publication.

IF is very widely applied in the diagnostic virology setting and has been shown to be very useful for the detection of viruses in leukocytes (e.g., cytomegalovirus [CMV]) and other cells derived from respiratory, genital, ocular, and cutaneous samples. Although the method requires expertise using a fluorescence microscope and interpretation of staining patterns, it has been shown to be a rapid and convenient method and allows for assessment of each sample for adequacy.

The procedure presented below is useful for developing assays using analyte-specific reagents and also provides generic technical information that is useful when following manufacturers' instructions for diagnostic kits. Kits and reagents are available for detection of numerous agents using either the direct IF (labeled primary antibody) or indirect IF (unlabeled primary antibody followed by labeled secondary antibody) method. Limitations imposed by the very high specificity of monoclonal antibodies (MAbs) and lower binding efficiencies are overcome by using mixtures of MAbs directed against different viral epitopes (10). Mixtures of MAbs (blends, pools) directed against two or more viruses (8, 13) are also available, as are preparations containing antibodies to two different viruses, each labeled with a different fluorochrome.

A. Supplies, reagents, and equipment

1. Sterile individually wrapped pipettes (1, 2, 5, and 10 ml) and safety pipetting device
2. Waste containers
   Discard specimens, cell cultures, and other infectious waste based on local regulations.
3. Acetone-cleaned, Teflon-coated microscope slides with 5- to 8-mm wells and no. 1 coverslips
4. Coplin jars or staining dishes and racks
5. Slide storage boxes and desiccant
6. Scalpels, tongue depressors, or spatulas (for tissue impression smears)
7. PPE
8. Most antibody reagents used for IF assays are MAbs; because of their high specificity and lower binding efficiencies, a mixture of MAbs directed against different viral epitopes is generally more useful than individual MAb preparations. Mixtures of MAbs (blends or pools) directed against two or more viruses are also available for detecting multiple viruses by using a single reagent. Recently, mixed-antibody preparations labeled with different fluorescing reagents have been introduced for the simultaneous identification of common virus pairs (e.g., influenza virus types A and B, herpes simplex virus [HSV], and CMV).
9. Phosphate-buffered saline (PBS) (pH 7.1 to 7.6)
10. Acetone (reagent grade) or fixative recommended by reagent manufacturer (e.g., methanol, ethanol, paraformaldehyde)
11. Buffered-glycerol mounting medium, pH 8.0 for IF
12. Cytofuge and funnels, optional, for cytospin preparations
13. Centrifuge, low speed, with carriers to hold 15-ml centrifuge tubes
14. Rotator (15 to 20 rpm) or rocker
15. Fluorescence microscope equipped with filters suitable for fluorescein isothiocyanate (FITC) or fluorochrome of choice
16. Refrigerator
17. Incubator, 37°C

II. ANTIGEN DETECTION ASSAYS: IMMUNOFLUORESCENCE (IF) PROCEDURE *(continued)*

B. QC

1. Include positive and negative cell spots in each run.
2. Unless predetermined and stated by the manufacturer, determine the optimal working dilution to use for antibodies, and conjugate and assess cross-reactivity with other viruses.

C. Cell spot preparation

1. Aspirates and fluids containing cells (other than blood)
   Follow the procedure described in Fig. 10.7–1. Alternatively, cytospin samples may be prepared by following the cytofuge manufacturer's instructions.
2. Swab specimens or scrapings
   Follow the procedure described in Fig. 10.7–2.
   ◩ **NOTE:** Swab specimens or scrapings may be placed into 1 to 2 ml of viral transport medium or PBS. After vortexing to release cells from the swab, the specimen may be processed in the same manner as aspirates and fluids; cell yield may be low for specimens processed in this manner.
3. Blood
   Kits are commercially available for detection of CMV antigen in the nuclei of peripheral blood PMNs (Fig. 10.7–3).
   a. Collect blood with anticoagulant (e.g., EDTA, heparin) as recommended by the manufacturer. Store blood at room temperature before cell separation; for optimal results, prepare slides within several hours of specimen collection.
   b. The test can be applied quantitatively by applying a known number of leukocytes to the slides; however, it is important to determine whether an adequate number of cells are actually present at the time of reading. The number of cells can be estimated visually with experience; the number can also be estimated by inserting a micrometer disk in the ocular lens of the microscope and counting the cells in several representative fields. The average number of cells per field multiplied by the number of fields per well will give an estimate of the number of cells.
4. Tissues
   a. Prepare impression smears as described in Fig. 10.7–2.
   b. Prepare cell spots from tissue homogenates as described for aspirates in Fig. 10.7–1. Homogenates may be prepared by using a tissue grinder (Fig. 10.4–4) or a Tekmar Stomacher tissue grinder.
   c. Acetone- or formalin-fixed frozen sections 4 to 6 μm thick are suitable for staining.
   d. Paraffin-embedded tissues may be used after deparaffinization.

D. IF staining

Follow the procedure described in Fig. 10.5–8.

E. Observation of viral antigens

Use a 10× objective to focus the slide. Then examine the entire cell spot at 20×. Cell morphology should be clear, with nuclear and cytoplasmic details defined. Use a higher magnification (×40 or ×63) for closer examination of cellular detail and for examining questionable areas.

1. Assess control slides before examining test slides; repeat the assay if the controls do not yield the expected results. Refer to Table 10.7–2 for troubleshooting guidelines.
2. The positive control slide (virus-infected cells with homologous antibody) should have numerous cells with characteristic and intense (3+ to 4+) apple-green (for FITC) fluorescence. The number or percentage of fluorescing cells can also be determined, if desired.
3. The negative control should exhibit no (0) or minimal dim (<1+) fluorescence.

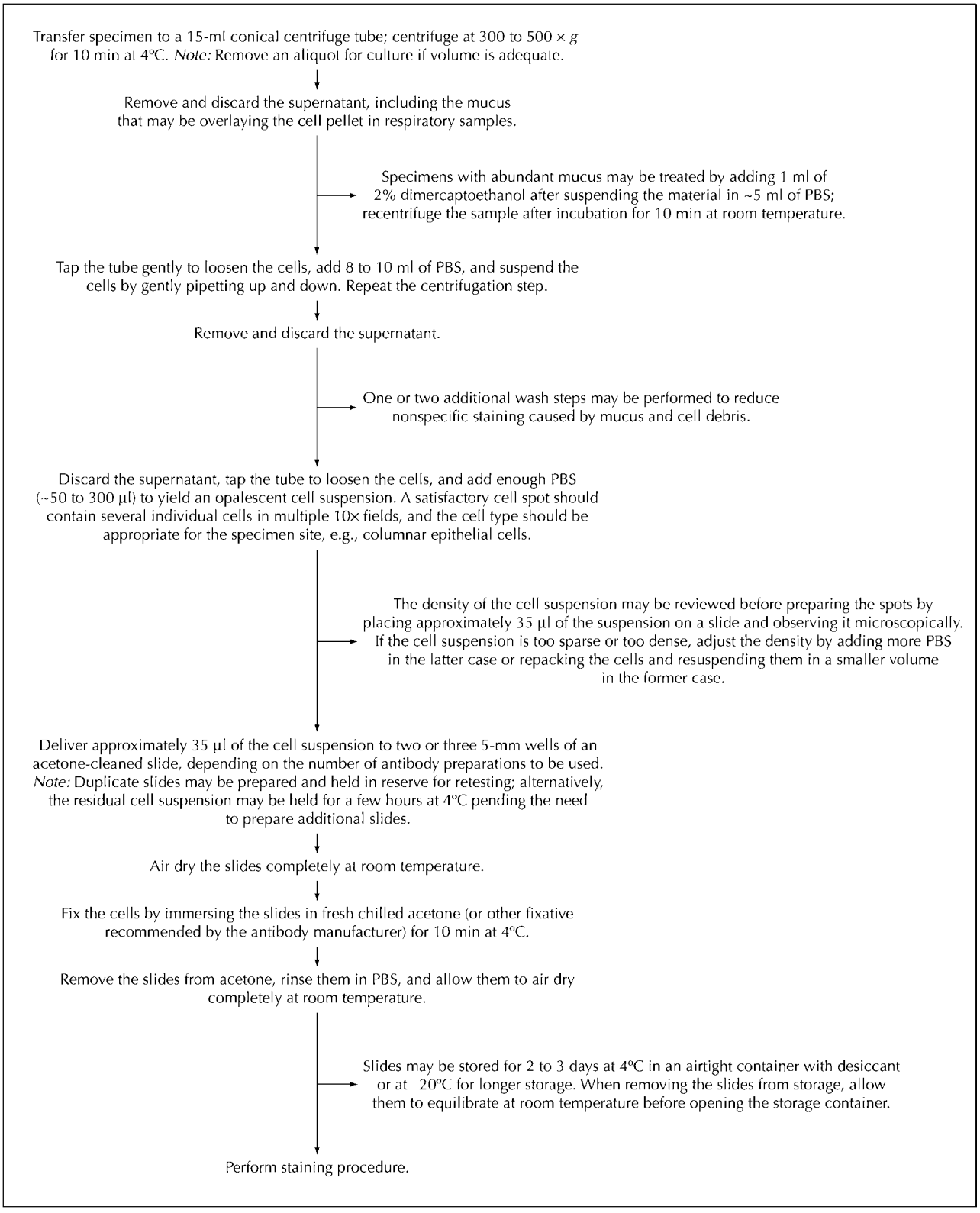
Transfer specimen to a 15-ml conical centrifuge tube; centrifuge at 300 to 500 × g for 10 min at 4°C. Note: Remove an aliquot for culture if volume is adequate.
Remove and discard the supernatant, including the mucus that may be overlaying the cell pellet in respiratory samples.
Specimens with abundant mucus may be treated by adding 1 ml of 2% dimercaptoethanol after suspending the material in ~5 ml of PBS; recentrifuge the sample after incubation for 10 min at room temperature.
Tap the tube gently to loosen the cells, add 8 to 10 ml of PBS, and suspend the cells by gently pipetting up and down. Repeat the centrifugation step.
Remove and discard the supernatant.
One or two additional wash steps may be performed to reduce nonspecific staining caused by mucus and cell debris.
Discard the supernatant, tap the tube to loosen the cells, and add enough PBS (~50 to 300 µl) to yield an opalescent cell suspension. A satisfactory cell spot should contain several individual cells in multiple 10× fields, and the cell type should be appropriate for the specimen site, e.g., columnar epithelial cells.
The density of the cell suspension may be reviewed before preparing the spots by placing approximately 35 µl of the suspension on a slide and observing it microscopically. If the cell suspension is too sparse or too dense, adjust the density by adding more PBS in the latter case or repacking the cells and resuspending them in a smaller volume in the former case.
Deliver approximately 35 µl of the cell suspension to two or three 5-mm wells of an acetone-cleaned slide, depending on the number of antibody preparations to be used. Note: Duplicate slides may be prepared and held in reserve for retesting; alternatively, the residual cell suspension may be held for a few hours at 4°C pending the need to prepare additional slides.
Air dry the slides completely at room temperature.
Fix the cells by immersing the slides in fresh chilled acetone (or other fixative recommended by the antibody manufacturer) for 10 min at 4°C.
Remove the slides from acetone, rinse them in PBS, and allow them to air dry completely at room temperature.
Slides may be stored for 2 to 3 days at 4°C in an airtight container with desiccant or at –20°C for longer storage. When removing the slides from storage, allow them to equilibrate at room temperature before opening the storage container.
Perform staining procedure.

Figure 10.7–1 Preparation of aspirates and fluids for IF.

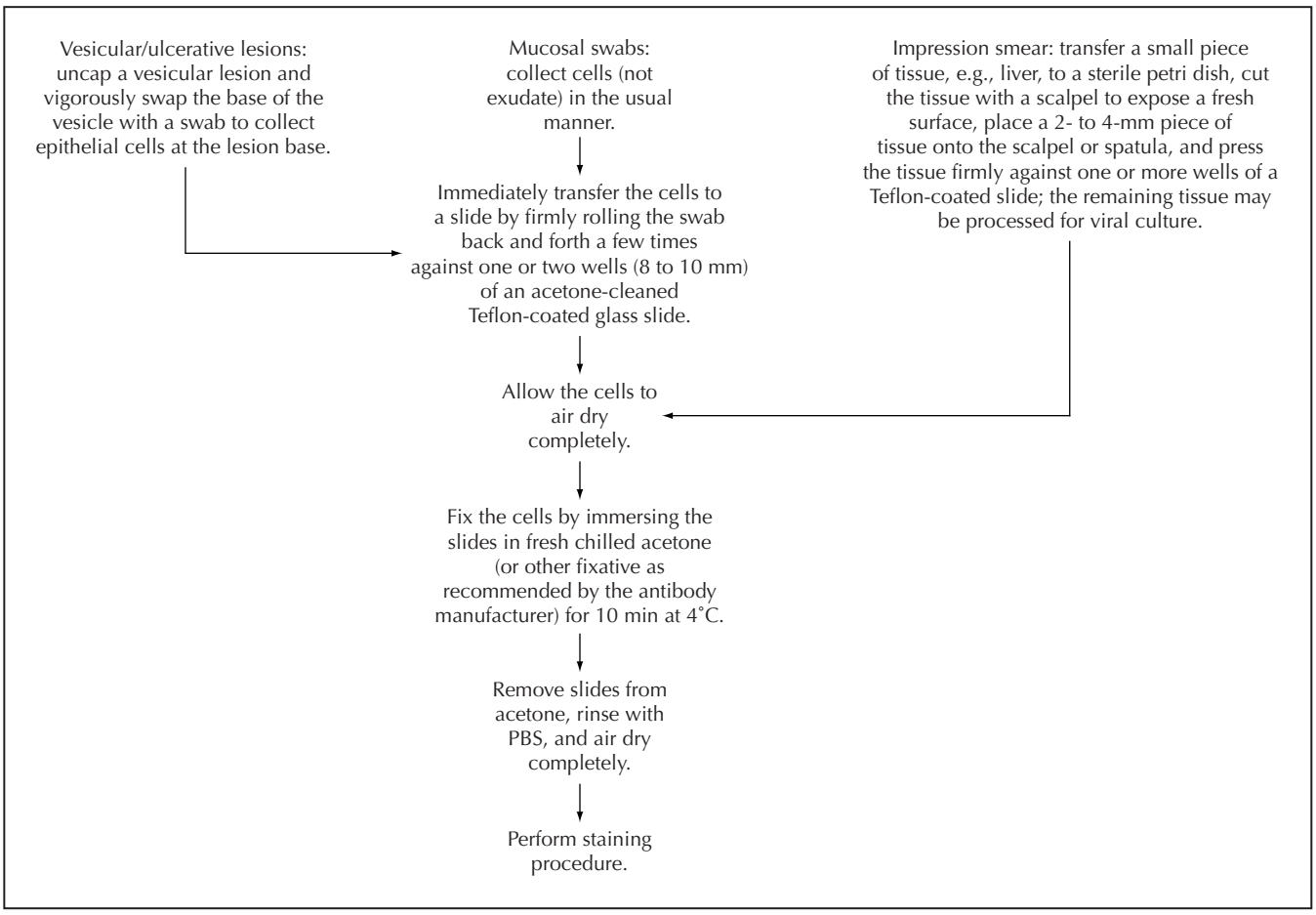
Vesicular/ulcerative lesions: uncap a vesicular lesion and vigorously swap the base of the vesicle with a swab to collect epithelial cells at the lesion base.
Mucosal swabs: collect cells (not exudate) in the usual manner.
Impression smear: transfer a small piece of tissue, e.g., liver, to a sterile petri dish, cut the tissue with a scalpel to expose a fresh surface, place a 2- to 4-mm piece of tissue onto the scalpel or spatula, and press the tissue firmly against one or more wells of a Teflon-coated slide; the remaining tissue may be processed for viral culture.
Immediately transfer the cells to a slide by firmly rolling the swab back and forth a few times against one or two wells (8 to 10 mm) of an acetone-cleaned Teflon-coated glass slide.
Allow the cells to air dry completely.
Fix the cells by immersing the slides in fresh chilled acetone (or other fixative as recommended by the antibody manufacturer) for 10 min at 4°C.
Remove slides from acetone, rinse with PBS, and air dry completely.
Perform staining procedure.

Figure 10.7–2 Preparation of swabs, scrapings, and impression smears for IF.

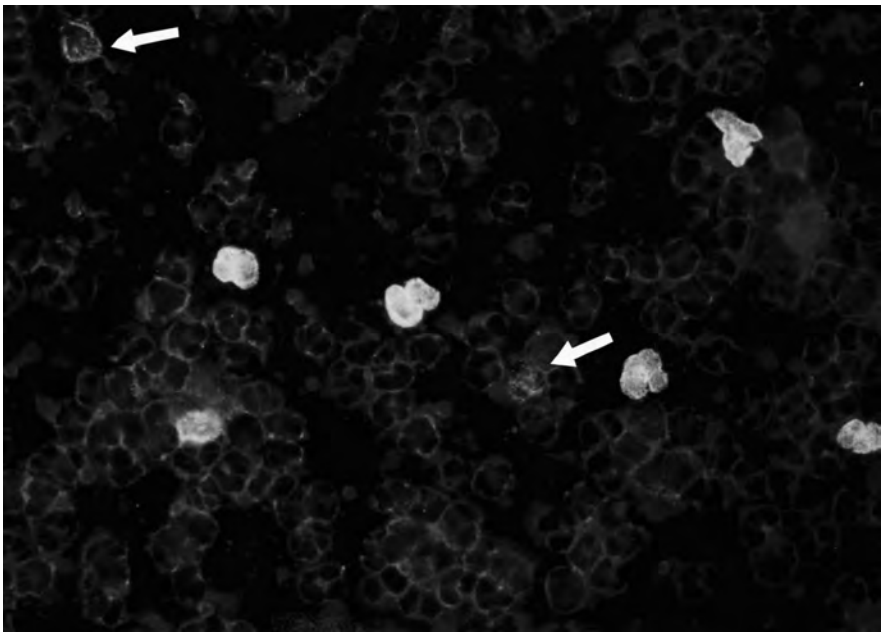

Figure 10.7–3 Leukocyte preparation with several cells showing intense CMV pp65 staining and a few cells (arrows) showing weak speckled nuclear staining (×400). Provided by Chemicon International.

Table 10.7–2 Troubleshooting IF problems

Problem	Causes	Solution
Weak or no specific fluorescence, including positive controls	Wrong (too weak) concn of immunoreagent used	Retitrate reagent.
	Deterioration of reagent	Retitrate and/or replace. Store properly, and aliquot if necessary.
	Counterstain too strong	Review concn and counterstaining time.
	Rapid fading of fluorescence	pH of mounting medium must be greater than that of wash buffer; use mounting medium that contains photobleaching inhibitor.
	Wrong filters or inadequate light source	Review use and maintenance of equipment; replace light bulb.
	Cannot achieve sharp focus because of dirty or improperly focused optics	Review procedures for maintenance and use of optics.
	Nonspecific glare or haze masking specific fluorescence	Allow cell spots to air dry completely before mounting; use fresh acetone.
	Antigens destroyed by fixative	Use alternative fixative.
Weak or no fluorescence with test slides but positive controls acceptable	Controls do not adequately reflect actual test conditions	Review procedure. Determine whether same or different reagents or methods were used for test and control slides.
	Improper slide cleaning, preparation, or fixation	Review procedures.
Nonspecific fluorescence and/or false positives with test slides and negative controls	Wrong concn (too strong) of immunoreagent used	Retitrate reagent.
	Cross-reactive immunoreagent	Identify non-cross-reactive concn of reagent, or obtain a better product.
	Binding of antibody via Fc receptors	Use conjugated $F(ab')_2$ fragments.
	Trapping of immunoreagent on heavy or raised cell preparations	Avoid heavy cell preparations, which obscure cellular morphology and complicate reading. Increase number of washes during staining.
	Inappropriate immersion oil	Use immersion oil designated for fluorescence microscopy.
	Inadequate removal of fixative	Air dry cell spots completely after fixing. Rinse slides with PBS and/or water just prior to staining.
	Water in acetone	Replace acetone.
	Slides dried during staining	Use properly sealed moist chamber during incubation. Apply adequate reagent volumes to cell spots.
	Mounting medium not appropriate for IF, or pH too low	Obtain proper reagents. pH of mounting medium must be greater than that of wash buffer.
	Mounting medium applied to wet cells	Allow cell spots to air dry completely before mounting.
	Wrong filters or equipment	Review use and maintenance of equipment.

Table 10.7–2 *(continued)*

Problem	Causes	Solution
	Reading error	Must be able to differentiate specific patterns of fluorescence from nonspecific staining.
	Precipitated material in immunoreagent	Microcentrifuge reagent for 1 min, or filter through a 0.45-μm-pore-size filter.
Nonspecific fluorescence and/or false positives with test slides but negative controls acceptable	Controls do not adequately reflect test conditions	Review procedure. Determine whether same or different reagents or methods were used for test and control slides.
	Reading error	More reading experience needed, since controls may be easier to read and interpret than test slides.
	Reagent cross-contamination	Take care to confine reagents to wells.
	Inoculum debris or microorganisms on test slide	Increase number of washes.

II. ANTIGEN DETECTION ASSAYS: IMMUNOFLUORESCENCE (IF) PROCEDURE *(continued)*

4. A positive specimen is one in which one or more intact cells exhibit a characteristic staining pattern with a 2+ or greater staining intensity.

   ◩ **NOTE:** Slides with weak staining and/or a single positive cell might be considered equivocal.
5. A negative specimen is one that contains an adequate number of cells typical of the sampling site and in which all cells exhibit minimal (<1) or no (0) fluorescence.
6. An unsatisfactory specimen is one in which strong nonspecific staining interferes with reading, or the slide contains too few intact cells typical of the site. Clinical samples should contain several cells in several 10× fields. Prepare new cell spots from the residual sample, and repeat the assay or request a new sample.

   ◩ **NOTE:** The specific pattern of fluorescence depends on several variables, including the viral protein(s) being targeted and the type of antibody preparation (e.g., single MAb, mixed MAbs, or polyclonal antiserum). The expected pattern with a particular antibody preparation should be provided by the manufacturer. Positive controls, provided that the cells represent those in the test slides, are the best tool for determining characteristic staining. Refer to Fig. 10.5–9 in procedure 10.5 for examples of IF staining.

F. Observation for *C. trachomatis* elementary bodies (EBs) (staining for other chlamydial species may be performed in the same manner)

◩ **NOTE:** Fix slides for *Chlamydia pneumoniae* by using acetone, not methanol.

Extracellular EBs are approximately 300 nm in diameter and appear as bright apple-green punctate structures; EBs show a smooth edge when stained with anti-major outer membrane protein (anti-MOMP) antibodies. Chlamydial reticulate bodies or intermediate forms of the organism may stain with a peripheral halo and are larger than EBs; do not include these structures in the EB counts.

1. Examine control slides and determine whether they are acceptable before observing specimen slides. Examine slides at 40×; use 63× or 100× to facilitate confirmation of suspected EBs (glycerol or oil objectives will facilitate reading).

II. ANTIGEN DETECTION ASSAYS: IMMUNOFLUORESCENCE (IF) PROCEDURE *(continued)*

2. A positive specimen is one that contains a quantity of EBs equal to or greater than the established cutoff number (e.g., 1, 2, or 5).
3. A negative specimen is one that contains an adequate number of columnar epithelial cells and no EBs.

   ◩ **NOTE:** Report as positive for *C. trachomatis* if an anti-MOMP MAb is used; report as positive for *Chlamydia* spp. if an antilipopolysaccharide (anti-LPS) antibody is used.
4. An equivocal sample is one that contains fewer EBs than the established positive cutoff number.
5. An unsatisfactory sample is one in which EBs were not observed but which exhibits strong nonspecific staining that interferes with reading or that contains an insufficient number of cells. Specimen slides should contain two to three columnar epithelial cells per high dry field.

III. ELECTRON MICROSCOPY (EM)

EM (1, 3, 4, 9) can be used to visualize viral particles in specimens and can be very useful for studying virus-cell relationships and for attempting to assign a viral origin to a disease of unknown etiology. However, EM is generally less sensitive than other methods and is of limited availability. Negative staining, usually with phosphotungstic acid, can be used to visualize viral particles in specimen fluids and extracts containing at least 10^5 to 10^6 particles per ml; thin sectioning is useful for detecting viruses in tissues. Enhancement methods such as immunoelectron microscopy and/or concentration by ultracentrifugation increase the sensitivity of this method.

EM is useful in investigating suspected outbreaks of viral gastroenteritis caused by viruses for which other methods are not readily available (e.g., Norwalk agent, astroviruses, and caliciviruses). Collect stool within 2 to 3 days of onset of symptoms. Store and ship at 4°C; do not freeze. Contact the local or state public health laboratory or the Enteric Diseases Branch, CDC, Atlanta, GA 30333.

IV. CYTOHISTOPATHOLOGY

Examination of exfoliated cells, biopsy samples, and autopsy tissues may reveal nonspecific changes suggestive of viral infections, and several viruses are associated with the development of characteristic inclusion bodies (7) (Table 10.7–3). Cytohistopathology is generally less sensitive than other viral and chlamydial detection methods. However, this method is useful for detecting agents for which other diagnostic assays are not available (e.g., molluscum contagiosum) and to demonstrate organ or tissue involvement. For example, CMV inclusions in alveolar epithelial cells may be a better indicator of pulmonary CMV disease than positive culture or PCR results on bronchoalveolar lavage samples.

Table 10.7–3 Inclusion morphology

Agent[a]	Inclusion
CMV (Fig. 10.7–4A)	Enlarged (cytomegalic) cells with a large single basophilic intranuclear inclusion that nearly fills the nucleus and is surrounded by a clear halo (owl's eye appearance); associated with small Papanicolaou-positive cytoplasmic inclusions
C. trachomatis (Fig. 10.7–4B)	Giemsa- or Papanicolaou-stained smears may reveal typical intracytoplasmic inclusions. Sensitive method for examination of ocular specimens, for which the sensitivity has been estimated to approach 95% compared with culture, but insensitive for urogenital samples
HSV, VZV	Large eosinophilic Cowdry type A intranuclear inclusion with a halo of nuclear chromatin (mature form); associated with multinuclearity with indented (molded) nuclei. The Tzanck smear (Fig. 10.7–4C) is a classic method involving Giemsa staining of epithelial cells scraped from the lesion base.
HPV	Squamous cells appear swollen and contain an enlarged nucleus and perinuclear halo with poor cytoplasmic keratinization resulting in irregular staining (koilocytic change).
Measles virus	Large multinucleated giant cells with eosinophilic nuclear and cytoplasmic inclusions; high sensitivity for giant-cell pneumonia in immunocompromised patients (Warthin-Finkelday inclusions)
Molluscum contagiosum (Fig. 10.7–4D)	Large dense intracytoplasmic eosinophilic inclusions (molluscum bodies) occupying the entire squamous cell and resulting in peripheral displacement of a flattened nucleus
Rabies virus	Seller's stain reveals large eosinophilic cytoplasmic inclusions (Negri body), often with blue-staining granules or inner bodies arranged in concentric layers; several inclusion bodies, usually of variable size, may be present in one neuron.

[a] Abbreviations: VZV, varicella-zoster virus; HPV, human papillomavirus.

REFERENCES

1. **Biel, S. S., and H. R. Gelderblom.** 1999. Diagnostic electron microscopy is still a timely and rewarding method. *J. Clin. Virol.* **13:**105–119.
2. **Centers for Disease Control.** 1991. False-positive results with the use of chlamydia tests in the evaluation of suspected sexual abuse. *Morb. Mortal. Wkly. Rep.* **39:**932–935.
3. **Doane, F. W., and N. Anderson.** 1987. *Electron Microscopy in Diagnostic Virology: Practical Guide and Atlas.* Cambridge University Press, New York, N.Y.
4. **Fong, C. K. Y.** 1994. Electron microscopy and immunoelectron microscopy, p. 99–107. *In* G. D. Hsiung, C. K. Y. Fong, and M. L. Landry (ed.), *Hsiung's Diagnostic Virology,* 4th ed. Yale University Press, New Haven, Conn.
5. **Goudswaard, F., L. Sabbe, and C. van Belzen.** 1989. Interference by gram-negative bacteria in the enzyme immunoassay for detecting *Chlamydia trachomatis. J. Infect. Dis.* **18:** 94–96.
6. **Hammerschlag, M. R., P. J. Rettig, and M. E. Shields.** 1988. False positive results with the use of chlamydia antigen detection tests in the evaluation of suspected sexual abuse in children. *Pediatr. Infect. Dis. J.* **7:**11–14.
7. **Hsiung, G. D.** 1994. Histocytochemical staining, p. 83–90. *In* G. D. Hsiung, C. K. Y. Fong, and M. L. Landry (ed.), *Hsiung's Diagnostic Virology,* 4th ed. Yale University Press, New Haven, Conn.
8. **Matthey, S., D. Nicholson, S. Ruhs, B. Aiden, M. Knock, K. Schultz, and A. Schmuecker.** 1992. Rapid detection of respiratory viruses by shell vial culture and direct staining by using pooled and individual monoclonal antibodies. *J. Clin. Microbiol.* **30:**540–544.
9. **Miller, S. E.** 1986. Detection and identification of viruses by electron microscopy. *J. Electron. Microsc. Technol.* **4:**265–301.

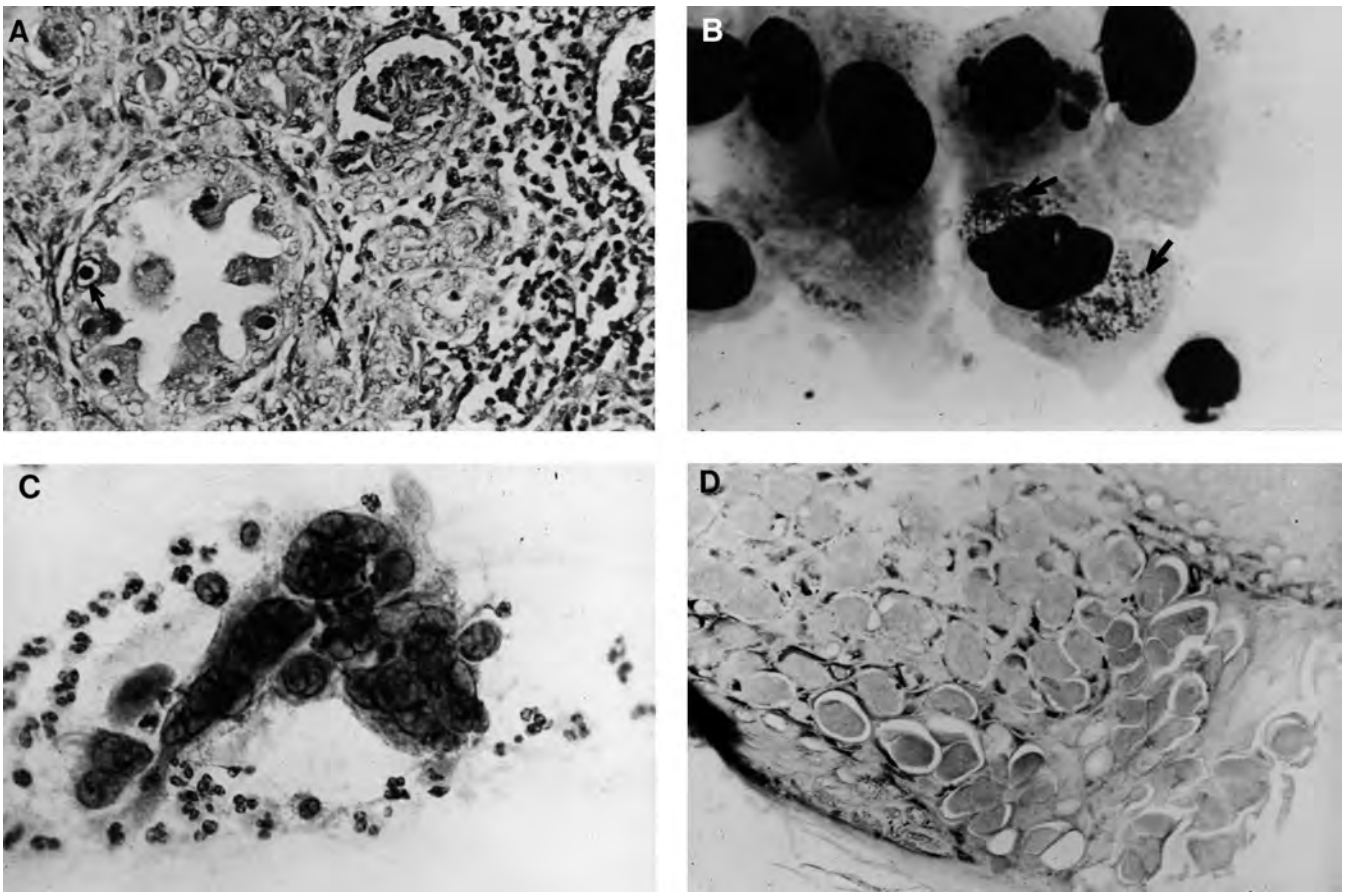

Figure 10.7–4 (A) Kidney tubule with characteristic "owl eye" inclusion of CMV (arrow). Courtesy of W. L. Drew. (B) Giemsa stain of conjunctival cells showing cytoplasmic inclusion (arrow) of *C. trachomatis.* Courtesy of W. L. Drew. (C) Cytologic examination (Tzanck smear) of scrapings from the base of an ulcerative HSV lesion showing multinucleated giant cells. Courtesy of W. L. Drew. (D) Large eosinophilic inclusions fill the cytoplasms of several infected basal cells (molluscum bodies). Courtesy of W. L. Drew.

REFERENCES *(continued)*

10. **Payne, W. J., Jr., D. L. Marshall, R. K. Shockley, and W. J. Martin.** 1988. Clinical laboratory applications of monoclonal antibodies. *Clin. Microbiol. Rev.* **1:**313–329.
11. **Poder, K., N. Sanchez, P. M. Robin, M. McHugh, and M. R. Hammerschlag.** 1989. Lack of specificity of Chlamydiazyme for detection of vaginal chlamydial infection in prepubertal girls. *Pediatr. Infect. Dis. J.* **8:**358–360.
12. **Pratt, B. C., I. A. Tait, and W. L. Anyaegbunam.** 1989. Rectal carriage of *Chlamydia trachomatis* in women. *J. Clin. Pathol.* **42:**1309–1310.
13. **Stout, C., M. D. Murphy, S. Lawrence, and S. Julian.** 1989. Evaluation of a monoclonal antibody pool for rapid diagnosis of respiratory viral infections. *J. Clin. Microbiol.* **27:**448–452.

SECTION 11 Immunology

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11.1.1 Introduction

Advances in the clinical immunology laboratory have continued at a rapid pace since the previous edition of CMPH. The goal of this section is to highlight procedures for major assays that are called upon in the clinical microbiology and immunology laboratories and to assist those laboratory directors to conduct the highest quality of laboratory testing. In this section it is impossible to cover all aspects of immunology laboratory testing, as was noted in the previous edition. We therefore again provide Table 11.1.2 from *Essential Procedures for Clinical Microbiology* (2), which provides the reader with generic descriptions of assays used in the clinical immunology laboratory. Table 11.1.3 is also provided again as a summary of those assays that are emerging from research laboratories; these may find their way into the clinical immunology laboratory with time.

Each procedure in this immunology section has been reviewed by the original author and updated when appropriate. For 11.2.1, Serologic Diagnosis of Group A Streptococcal Infections, revisions have been made to the introduction and to summary table 11.2.1–1. A few edits have also been made to the principle and analytical considerations sections for the anti-streptolysin procedure (11.2.2). Similar updates have been made to the principle, materials, and results and conclusion for the procedure on the anti-DNase B test (11.2.3). Procedure 11.3, Detection of *Legionella* Antigen by Direct Immunofluorescence, and the procedure on Urinary Antigen Detection for *Legionella* spp. (11.4) have been reviewed and updated. Table 11.5.1 in the introduction of the Laboratory Diagnosis of Syphilis procedure (11.5) has been modified to include a number of new vendors. A number of updates and modifications have been made to procedure 11.6, Detection of *Borrelia burgdorferi* Antibodies. Specific attention is paid to the V.2 western immunoblots and V.2.1, which deals with the scoring of the Western blot banding patterns. The Serodiagnosis of Rickettsial Infections material (procedure 11.7) has been revised in a number of areas with specific attention drawn to Table 11.7.2.2, in which sensitivities and specificities of testing formats are summarized. Procedure 11.8, Immunoassay Detection of Shiga Toxin-Producing *Escherichia coli*, has been revised and now includes two new sections on the use of immunochromatographic assays and immunoassay for detection of Shiga toxin. A number of revisions have also been made to Table 1, noting new vendors of EIA kits. For the flow cytometry and ICS procedure, 11.15, appendix 11.15–1 was revised to make corrections in the Brefeldin A concentrations that were noted to be incorrect in the earlier edition of CMPH. This current edition of CMPH now includes a new procedure (11.19), gamma-interferon enzyme-linked immunospot assay. Having been first described about 13 years ago, this assay has found its way into many immunology laboratories as one of the most standardized assays to measure antigen-specific T cells.

As in the first edition of CMPH, the reader is again referred to the *Manual of Molecular and Clinical Laboratory Immunology* for additional information related to laboratory immunology procedures and interpretation of results obtained from these procedures (1).

REFERENCES

1. **Detrick, B., R. G. Hamilton, and J. D. Folds (ed.).** 2006. *Manual of Molecular and Clinical Laboratory Immunology*, 7th ed. ASM Press, Washington, DC.
2. **Janda, W. M.** 1998. Immunology, p. 561–577. *In* H. D. Isenberg (ed.), *Essential Procedures for Clinical Microbiology*. ASM Press, Washington, DC.

11.1.2 Immunologic Assays Used in the Serologic Diagnosis of Infectious Diseases

The following table is a summary of various tests that can be used in clinical microbiology laboratories for the serologic diagnosis of infectious diseases.

Table 11.1.2–1 Generic description of immunologic assays used in the serologic diagnosis of infectious diseases[a,b]

Test	Basic principle	Specimen type evaluated	Basic materials	Basic QC
Latex agglutination for antigen detection	Latex spheres coated with specific immunoglobulins agglutinate in the presence of antigen.	CSF, urine, or serum	1. Latex beads coated with specific antibodies 2. Positive and negative antigen controls 3. Mixing sticks 4. Reaction slides 5. Heat block or boiling water bath 6. Centrifuge	Positive and negative controls should be performed with each batch of patient specimens.
Latex agglutination for antibody detection	Latex spheres coated with specific antigens agglutinate in the presence of serum containing the corresponding specific antibody.	Serum or plasma	1. Latex beads coated with specific antibodies 2. Mixing sticks 3. Glass agglutination slides 4. Appropriate positive and negative controls	Inclusion of positive and negative controls each time test is performed
Staphylococcal coagglutination	Staphylococcal cells are sensitized with antibody against specific antigens by the ability of staphylococcal protein A to bind immunoglobulin to the bacterial cell surface by the Fc regions of the antibody molecules.	Suspension of the organism to be identified prepared in PBS, buffered saline, etc., depending on the kit (usually used for bacterial identification)	1. Staphylococcal cells sensitized with specific antibody 2. Nonsensitized staphylococcal cells (negative control reagent) 3. Reaction slides or cards 4. Mixing sticks	Inclusion of positive and negative controls each time the test is performed

Basic procedure	Expected results	Basic limitations	Generic examples
Mix specimen with reagent on a glass slide and inspect for specific agglutination.	1. Positive control must yield a strong agglutination reaction within 10 min. 2. Negative control must show no agglutination. 3. Specimens showing agglutination are positive. 4. Specimens showing no agglutination are negative.	1. Tests should not be used to replace culture (CSF antigen detection). 2. Urine and serum specimens require additional processing to prevent uninterpretable results. 3. Low antigen levels may result in false-negative tests.	Several commercial kits for detection of bacterial antigens (group B streptococcus, *Haemophilus influenzae* type b, *Streptococcus pneumoniae*, and *Neisseria meningitidis* serogroups A, C, Y, and W135) in CSF; also kits for detection of *Cryptococcus neoformans* capsular polysaccharide in CSF
1. Mix serum specimen on a glass slide. 2. Rock or rotate slide for specified period. 3. Inspect for agglutination.	1. Positive control must produce appropriate specific agglutination reaction. 2. Negative control must not show agglutination. 3. Patient specimens showing agglutination contain specific antibody. 4. Negative specimens show no agglutination.	1. Low levels of antibody may not be reliably detected. 2. Some kits may not be able to produce results that correlate with traditional antibody titers.	Latex agglutination tests for detection of rubella virus and CMV antibodies
1. Standardized, properly processed bacterial cell suspension (1 drop) is reacted with antibody-sensitized staphylococcal cells (1 drop) and cells that are not sensitized in circles or walls of a reaction slide. 2. Mix and rock or rotate the reaction slide for specified period. 3. Inspect for specific agglutination.	1. Positive control suspension must produce at least 2+ agglutination with the sensitized cells and no agglutination with the control cells. 2. Negative control suspension must show no agglutination with either the sensitized or the control staphylococcal cells. 3. Agglutination reactions of 2+ or greater with the sensitized cells and no agglutination with the nonsensitized cells on patient isolates are positive.	1. Isolates that show no agglutination must be identified by other methods. 2. Not all strains of an organism may be identified by a particular test. For example, not all strains of *Neisseria gonorrhoeae* are identified by commercially available coagglutination tests due to lack of appropriate monoclonal antibodies on the surfaces of the sensitized staphylococcal cells.	Coagglutination tests for the identification of *N. gonorrhoeae* (e.g., Phadebact OMNI, Gonogen, Meritec)

(continued)

Table 11.1.2–1 Generic description of immunologic assays used in the serologic diagnosis of infectious diseases[a,b] *(continued)*

Test	Basic principle	Specimen type evaluated	Basic materials	Basic QC
EIA for antibody detection	Antigen is bound to a solid phase (bead or microtiter well). Antibody present in the specimen binds to the antigen on the solid phase. This complex is then reacted with a second antibody that is enzyme labeled and also directed against the antigen (the conjugate is often an anti-human immunoglobulin raised in goats). Subsequent exposure of this complex to the enzyme substrate results in the generation of a colored end product that is detected spectrophotometrically.	Serum or plasma	1. Microtiter walls/beads coated with antigen 2. PBS with Tween 80 for serum dilution 3. Detection antibody-enzyme conjugate 4. Chromogenic enzyme substrate 5. Spectrophotometer 6. Incubator 7. Pipettes	Inclusion of sera known to be positive and negative for antibody being detected; diluent buffer control
EIA for antigen detection	In this case, antibody (e.g., capture antibody) instead of antigen is bound to a solid phase. Antigen present in a specimen binds to the capture antibody on the solid phase. Following wash steps, this complex is reacted with a second enzyme-labeled antibody that is directed against another epitope of the captured antigen. After washing, enzyme substrate is added, resulting in the generation of a colored end product that is detected visually or spectrophotometrically.	Serum, plasma, other body fluids (e.g., stool for detection of *Giardia lamblia* antigen, respiratory tract secretions for detection of RSV, etc.)	1. Antibody-sensitized microtiter wells, beads, or membrane filters 2. Second enzyme-conjugated antibody directed against other epitopes of the antigen 3. Positive and negative controls that contain antigen or lack the antigen, respectively 4. Some antigen detection EIAs contain calibrators and standards to allow semiquantitation of antigen levels present in certain specimens. 5. Spectrophotometric detection of positive results is used with some kits, although many rely on visual reading for detection of positive and negative tests.	Known positive and negative control samples

Basic procedure	Expected results	Basic limitations	Generic examples
1. Diluted serum specimen is incubated in antigen-sensitized wall or with antigen-sensitized bead. 2. Incubation and wash stage 3. Addition of enzyme-labeled conjugate directed against antibody (e.g., anti-human IgG raised in goats and linked to HRP) 4. Incubation and wash steps 5. Addition of enzyme substrate and generation of signal 6. Addition of stop solution 7. Generated color assessed spectrophotometrically	Positive results are determined by comparison with negative control; generally absorbance values 2- to 3-fold greater than the mean absorbance of a group of negative controls are considered positive. Some kits may include low- and high-titer positive controls, calibrations, etc.	1. Sera obtained during the acute phase of infection may contain only antibodies of the IgM class and will not be identified in IgG-specific assays; the converse is also true. 2. Contaminated, icteric, lipemic, heat-inactivated, or hemolyzed sera may produce erroneous results. 3. Departure from specific procedure may affect test results.	EIA kits for detection of antibodies are available for a wide variety of agents, particularly certain bacteria (e.g., *Borrelia burgdorferi*) and viruses (HIV-1, CMV, rubella, HSV, measles, mumps, HIV-1/2)
1. The specimen is incubated in a microtiter well or tube containing the capture antibody. 2. Incubate for specified period. 3. Wash steps 4. A second enzyme-labeled antibody that is also directed against epitopes of the antigen is added. 5. Incubate for specified period. 6. Wash steps 7. Enzyme substrate is added and allowed to incubate for a specified period. 8. Addition of stop solution 9. Color reaction is read visually or with a spectrophotometer. 10. In semiquantitative assay, standard curves are prepared from calibrators run in the same assay.	1. Positive results are determined visually by the presence of a colored endpoint. This may also be done with a spectrophotometer. 2. Semiquantitative results may be determined by standard curves with cutoffs determined by negative controls and regression analysis of standards.	1. Tests must be used exactly as described in package inserts. 2. Low levels of antigen may not be detected. 3. Antigens already present in serum specimens as antigen-antibody complexes will not be detected unless immune complexes are first dissociated.	Immunoassays for detection of group A streptococci directly in throat swabs; immunoassays for detection of RSV and influenza A virus in respiratory tract specimens; assays for detection of antigen in stool of patients with *G. lamblia* and *Cryptosporidium parvum;* assays for detection of HIV p24 antigen in serum

(continued)

Table 11.1.2–1 Generic description of immunologic assays used in the serologic diagnosis of infectious diseases[a,b] *(continued)*

Test	Basic principle	Specimen type evaluated	Basic materials	Basic QC
Direct fluorescent-antibody test for antigen detection	Specific antibody conjugated to a fluorescent tag (e.g., FITC) is reacted directly with a specimen on a glass slide or with a smear of an organism prepared from a culture plate. After incubation and washing, the slide is examined under a fluorescence microscope and examined for specific immunofluorescence.	Specimens may include smears made directly from clinical specimens (e.g., endocervical smears for detection of *Chlamydia trachomatis*) or from cultures, where this procedure may be used for identification (e.g., identification of *Legionella* spp. from cultures grown on BCYE medium, identification of respiratory viruses growing in shell vials, etc.)	1. Appropriate clinical specimens 2. Teflon-coated slides used for immunofluorescence (i.e., with specimen wells) 3. Specific antibodies conjugated directly to a fluorophore (e.g., FITC) 4. Humidor for slide incubation 5. Pipettes	Positive and negative controls performed along with clinical specimens
IFA test for antigen detection	A specimen fixed on a slide is overlaid with an excess of unlabeled immune serum directed against the antigen and incubated. After a wash step, the specimen-antibody complex is reacted with a fluorochrome-labeled antibody directed against the species of the first unlabeled antibody (e.g., goat anti-human immunoglobulin conjugated to FITC). After a wash step, the specimen is examined under a fluorescence microscope fitted with the appropriate barrier filters for detection of specific immunofluorescence.	IFA tests can be used for detection of antigens in various clinical specimens (e.g., IFA test for *Pneumocystis carinii* in bronchoalveolar lavage specimens).	1. Appropriate clinical specimens 2. Unlabeled specific antibodies directed against the antigen of interest 3. Fluorochrome-conjugated antibodies directed against the unlabeled antibodies (e.g., FITC-labeled anti-goat antibodies raised in mice) 4. Teflon-coated immunofluorescence slides with multiple wells 5. Humidor for incubation 6. Pipettes	Positive and negative control slides prepared from previously tested patient specimens are processed through the procedure along with unknown patient specimens.

Basic procedure	Expected results	Basic limitations	Generic examples
1. Specimen is applied to a well on a Teflon-coated immunofluorescence slide and fixed. 2. Specimen is overlaid with labeled conjugate and incubated in humidor for 15- to 30-min period. 3. Slide is washed to remove unbound conjugate. 4. Air dry specimen and apply mounting fluid and coverslip. 5. Examine under a microscope outfitted with a UV light source and appropriate barrier filters for assay under consideration.	Positive control and positive patient specimens show specific apple-green immunofluorescence (FITC); negative control and negative patient samples show no fluorescence.	Usually used only for antigen detection; while more rapid and less nonspecific, direct fluorescence procedures may be less sensitive and produce duller fluorescence than indirect immunofluorescence antigen detection methods.	Direct fluorescent-antibody test for *C. trachomatis*
1. Appropriate clinical specimen and positive and negative control material are placed in walls of Teflon-coated immunofluorescence slide. 2. Slides are dried and fixed. 3. The wells are overlaid with unlabeled antibodies directed against the antigen of interest. 4. Incubate slides for 15–30 min in humidor. 5. Wash and blot slide dry. 6. Overlay wells with fluorochrome-conjugated antibody. 7. Incubate in humidor for 15–30 min. 8. Wash and blot slide dry. 9. Add mounting medium and place a coverslip over wells. 10. Read wells for specific immunofluorescence under a fluorescence microscope with appropriate filter system for fluorochrome detection.	1. Positive control material reveals presence of specific immunofluorescence of the antigen. 2. No immunofluorescence should be observed with negative control material. 3. Positive and negative patient specimens are determined by comparison of unknowns with the immunofluorescence observed in the positive control.	1. Positive and negative controls of human origin should be tested to verify reagent performance. 2. Care must be taken to minimize nonspecific immunofluorescence caused by "trapping" of the fluorochrome (e.g., treatment of sputa with mucolytic agents when looking for *P. carinii* by IFA test). 3. Test may not detect antigens present in low concentrations or in inappropriately collected specimens.	*P. carinii* IFA test.

(continued)

Table 11.1.2–1 Generic description of immunologic assays used in the serologic diagnosis of infectious diseases[a,b] *(continued)*

Test	Basic principle	Specimen type evaluated	Basic materials	Basic QC
IFA test for antibody detection	This test is performed as described for the indirect assay; however, the first unlabeled antibody used in the test is that of the patient. Patient serum is serially diluted and placed on a series of immunofluorescence slide wells containing the same antigen. After an incubation and wash step, fluorochrome-labeled goat anti-human antibody is applied to each well. After another incubation-wash step, the slide is observed with a fluorescence microscope. The reciprocal of the highest dilution of the patient's serum that shows specific immunofluorescence is called the titer. Titers may be determined for both IgG and IgM. In the latter determination, the fluorochrome-labeled conjugate is directed against human IgM.	An antibody titer can be determined with a single serum. To document recent infection, an acute-phase specimen (collected early in the illness) and a convalescent-phase specimen (collected 2–3 weeks later) are needed to demonstrate an increase in titer of antibody against the antigen being tested.	1. Teflon-coated immunofluorescence slides, each well of which contains the antigen of interest (e.g., cells infected with CMV and expressing CMV-specific antigens) 2. Patient serum (acute and convalescent) 3. Low- and high-titer positive and negative control sera 4. Fluorochrome-labeled anti-human immunoglobulin raised in another animal species (e.g., FITC-labeled anti-human immunoglobulins raised in goats) 5. Humidor for incubations 6. Pipettes	1. Negative, low-titer positive, and high-titer positive control sera are included with each test run performed on patient specimens. 2. Negative control serum is usually tested at titers of 4 and 16, while the low- and high-titer positive controls are run at 4 dilutions, usually 1 twofold dilution below and 2 twofold dilutions above the expected titer (e.g., if the low positive control has an expected titer of 64, it should be tested at titers of 32, 64, 128, and 256). 3. A conjugate control using PBS is also included.

Basic procedure	Expected results	Basic limitations	Generic examples
1. Prepare twofold serial dilutions of the control sera and patients' sera. Depending on individual lab procedures, patient serum may be screened at 1 dilution (e.g., 1:16) or at 2 dilutions (e.g., 1:16 and 1:64). 2. Place 0.10 ml of the diluted control and patient sera on appropriately labeled wells of the immunofluorescence slide. 3. Incubate slide in humidor for 30 min at 37°C. 4. Rinse off sera and gently blot slide dry. 5. Place a drop of fluorochrome-tagged, appropriately diluted goat anti-human immunoglobulin on each of the slide wells. 6. Incubate in humidor for 30 min at 37°C. 7. Rinse slides, and blot dry. 8. Place small drops of buffered glycerol on each well and place a coverslip on the slide. 9. Read for specific immunofluorescence under the high-dry objective of the fluorescence microscope.	1. No immunofluorescence should be observed with either dilution of the negative control or with the PBS conjugate control. 2. Low- and high-titer control sera should agree with the predetermined titers, or neither titer should be $> \pm 1$ dilution off the expected titer. 3. If the controls are correct, the antibody titer is defined as the reciprocal of the highest dilution giving characteristic immunofluorescence.	Rheumatoid factor present in patient sera may cause false-positive results in tests for both IgG and IgM.	IFA tests are available for the detection of antibodies against several viral agents (e.g., CMV, HSV, HIV-1) and toxoplasma.

(continued)

Table 11.1.2–1 Generic description of immunologic assays used in the serologic diagnosis of infectious diseases[a,b] *(continued)*

Test	Basic principle	Specimen type evaluated	Basic materials	Basic QC
Immunoblot procedure	In this modified EIA, a mixture of different antigens (e.g., a lysate prepared from a virus growing in culture) is separated into its component proteins by molecular weight with electrophoresis. The separated components are then electrophoretically transblotted onto a sheet of nitrocellulose paper. The sheet is cut into strips that are used as the solid phase for reaction with patient sera. Subsequently, the strip is incubated with enzyme-labeled anti-human immunoglobulin. After wash steps, enzyme substrate is added. The presence of antibody in patient serum that is reactive with antigens separated on the nitrocellulose strip is indicated by the appearance of a colored band at the location of that particular antigen on the strip.	Serum or plasma	1. Nitrocellulose strips containing electrophoresed antigen (these may be prepared in-house or purchased from a vendor if available) 2. Plastic container with troughs for holding and incubating nitrocellulose blot strips 3. Pipettes 4. Positive and negative control sera 5. HRP-labeled goat anti-human antibodies (alternatively, biotinylated goat anti-human conjugate and avidin-HRP can be used in place of the HRP-labeled goat anti-human immunoglobulin) 6. HRP substrate	Negative and low- and high-titer controls should be performed with each test.

Basic procedure	Expected results	Basic limitations	Generic examples
1. The nitrocellulose strip with the electrophoresed antigens is placed in an incubation trough. 2. A dilution of the serum to be tested is placed in the trough along with the antigen strip. 3. After incubation and wash steps, the strip is incubated with goat anti-human IgG labeled with an enzyme. Alternatively, goat anti-human immunoglobulin conjugated to biotin may be used as the conjugate. 4. After incubation and wash steps, the enzyme substrate is added to the trough. The appearance of colored bands at the locations of the various antigens indicates the presence of antibody to that antigen in the original serum specimen. If a biotinylated conjugate is used, then avidin-enzyme is added and, after incubation and wash steps, the enzyme substrate is added for color development.	The appearance of colored bands on the strip indicates that antibody against that particular antigen was present in the original serum specimen.	1. Test must be performed exactly as described in the package insert. 2. Western blotting is as sensitive as and more specific than standard colorimetric EIA.	HIV-1 Western immunoblot supplemental test for detection of specific anti-HIV-1 antibodies

(continued)

Table 11.1.2–1 Generic description of immunologic assays used in the serologic diagnosis of infectious diseases[a,b] *(continued)*

Test	Basic principle	Specimen type evaluated	Basic materials	Basic QC
CF	Terminal components of the complement cascade (C7, C8, C9) damage cell membranes in the presence of specific antibody, which fixes complement to the cell surface. In the CF test, erythrocytes are used as the target cells and complement-induced "leakiness" can be detected colorimetrically or visually as an increase in free (rather than cell-bound) hemoglobin. In the presence of specific antibodies to an infectious agent, any complement added to the test system becomes bound, leaving no residual complement for reaction with antibodies to the "indicator" target erythrocytes. Therefore, the presence of specific antibody is indicated by the absence of hemolysis	Serum	1. Sheep erythrocyte suspension 2. Hemolysin (commercially available)	Known antibody-positive and antibody-negative controls; serum control to detect anticomplementary activity; antigen controls without serum to detect anticomplementary activity; tissue control (the cells or tissue in which the antigen was prepared); buffer control; back titration of commercial complement to ensure use of 5 CH_{50} units in each well; cell control to demonstrate absence of hemolysis; reference hemolysis standards

Basic procedure	Expected results	Basic limitations	Generic examples
Sensitization of erythrocytes 1. Serial dilutions of hemolysin incubated with 2.8% solution of sheep RBCs in buffer 2. As hemolysin concentration increases, degree of hemolysis increases, to achieve a maximal "plateau." Quantitation of complement 1. Various amounts of a 1:400 dilution of cold, reconstituted guinea pig complement are incubated with sensitized RBCs. 2. Volume of complement producing 50% lysis of RBCs (CH_{50} unit) is calculated from graphed results of the hemolysis. CF test 1. Block titration of serial dilutions of both antigen and antibody is performed in presence of 5 CH_{50} units of complement at 4°C for 16 h. 2. Patient sera are heated at 56°C for 30 min to inactivate endogenous complement. 3. RBCs that have been sensitized with the optimal dilution of hemolysin are added and the mixture is incubated at 37°C for 30 min. 4. Plates are centrifuged to sediment unlysed erythrocytes.	Highest dilution of antibody providing 3+ to 4+ fixation of complement (<30% hemolysis) is the endpoint.	All reagents must be free of anticomplementary activity, the correct quantity of complement must be present, and controls must produce expected reactions.	The CF test is considered the reference method for detection of antibodies against a wide variety of viral agents and *Mycoplasma pneumoniae.* The CF test has been largely supplanted by EIA methodology.

(continued)

Table 11.1.2–1 Generic description of immunologic assays used in the serologic diagnosis of infectious diseases[a,b] *(continued)*

Test	Basic principle	Specimen type evaluated	Basic materials	Basic QC
Hemagglutination inhibition	Some viruses produce surface proteins that agglutinate erythrocytes from various species. The presence of antibodies to such viruses in patient serum can be determined by specific inhibition of that hemagglutination.	Serum or plasma	1. Erythrocytes from appropriate species (usually chicken) collected in Alsever's solution or heparin 2. Diluent of appropriate pH 3. Solutions to remove nonspecific agglutinins from serum 4. Standardized viral antigen	Known positive and negative sera; back titration of antigen to ensure that the correct concentration of hemagglutinating units was tested

Basic procedure	Expected results	Basic limitations	Generic examples
Determination of hemagglutinating titer 1. Diluted suspension of RBCs is incubated at 4°C or at room temperature with serial dilutions of viral antigen until RBCs in tubes lacking virus settle as a button. 2. Highest antigen dilution that produces partial or complete agglutination equals 1 HAU. Treatment of sera Treat serum by physical means (kaolin), enzymes (neuraminidase), etc., to remove nonspecific agglutinins. Inhibition test Dilutions of antigen containing 4 HAU are mixed with erythrocytes and twofold dilutions of pretreated patient sera and incubated as for the determination of HAU titer.	End point is the last well in which partial or complete inhibition of viral agglutination of RBCs occurs.	Use of test is limited to detection of antibodies against those viruses that possess surface hemagglutinins (e.g., rubella).	

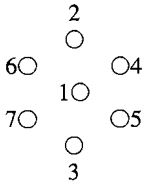
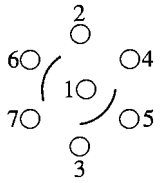
(continued)

Table 11.1.2–1 Generic description of immunologic assays used in the serologic diagnosis of infectious diseases[a,b] *(continued)*

Test	Basic principle	Specimen type evaluated	Basic materials	Basic QC
Immunodiffusion test	Double immunodiffusion is used to detect antigen or antibody. Known and unknown reactants are placed in adjoining wells of an agarose matrix and are allowed to passively diffuse towards one another for 12–24 h. A precipitin line forms where optimal levels of antigen and antibody are present. Double diffusion is usually used for the detection of antibodies. CIE is similar to double diffusion, except that the migration of one or both reactants is directed by an electrical field. (Cells to the right describe materials and procedures for fungal immunodiffusion testing.)	Patient serum or plasma for detection of antibody. CIE has been used with other body fluids (e.g., CSF) primarily for the detection of bacterial antigens.	Materials listed are those needed for fungal immunodiffusion. 1. *Blastomyces dermatitidis* immunodiffusion antigen 2. *B. dermatitidis* immunodiffusion rabbit antisera 3. *Coccidioides immitis* immunodiffusion antigen 4. *C. immitis* immunodiffusion rabbit antisera 5. *Histoplasma capsulatum* immunodiffusion antigen 6. *H. capsulatum* immunodiffusion rabbit antisera 7. 1% Purified agar 8. Immunodiffusion matrix pattern containing a single central well and 6 peripheral wells located equidistant from the central well and from adjacent wells 9. Reading box containing an incandescent light source with a flat, black background	For fungal immunodiffusion, control serum containing the antibodies being sought is required to discern lines of identity that may form between the antigen, the known antisera, and the patient's specimen. In addition to homologous testing, each test serum and antigen should also be tested for immunodiffusion reactivity with heterologous sera and antigens.

[a] Reproduced from **W. M. Janda.** 1998. Immunology, p. 561–577. *In* H. D. Isenberg (ed.), *Essential Procedures for Clinical Microbiology.* ASM Press, Washington, DC.

[b] Abbreviations: CMV, cytomegalovirus; PBS, phosphate-buffered saline; HIV-1, human immunodeficiency virus type 1; HSV, herpes simplex virus; IgM, immunoglobulin M; IgG, immunoglobulin G; RSV, respiratory syncytial virus; BCYE, buffered charcoal-yeast extract; IFA, indirect fluorescent antibody; HRP, horseradish peroxidase; CF, complement fixation; CH_{50}, 50% hemolytic complement; HAU, hemagglutinating unit(s); CIE, countercurrent immunoelectrophoresis; FITC, fluorescein isothiocyanate.

Basic procedure	Expected results	Basic limitations	Generic examples
1. Each immunodiffusion grid can be used to detect antibodies against a given antigen for four patients. 2. Using *B. dermatitidis* immunodiffusion testing, as an example, *B. dermatitidis* antigen is placed in the central well (1), and reference antisera are placed in wells 2 and 3. 3. Patients' sera to be tested for *B. dermatitidis* antibodies are placed in wells 4, 5, 6, and 7.  4. Similar plates are set up for *C. immitis* and *H. capsulatum* antigens and reference antisera. The patient sera are again placed in the four wells as stated in step 3 above. 5. Incubate plates at room temperature and read for lines of precipitation after 24 and 48 h of incubation.	The location and identity of precipitin lines should be noted. Reactions of identity (i.e., precipitin lines formed by the test reagents which are continuous with precipitin lines formed by reference reagents) indicate that the patient serum specimen contains antibodies directed against the antigen in the central well.  	Low levels of antibodies may not be detected by this method.	Fungal immunodiffusion for detection of antibodies to the systemic molds and *Aspergillus* spp.

11.1.3 Emerging Immunological Assays

Table 11.1.3–1 Emerging immunological assays using flow cytometry[a]

Test	Basic principle	Specimen type
Sub-G_0-G_1	Apoptosis can be characterized as cells with subdiploid DNA content. These cells can be measured utilizing PI staining and DNA software.	Whole blood, heparinized, is preferred. Isolate PBMCs.
TUNEL	The TUNEL method allows rapid phenotypic identification of individual apoptotic cells using flow cytometry. Cleavage of the genomic DNA during apoptosis is detected by labeling the free 3′ OH termini with fluorescein-labeled nuleotides. The reaction is catalyzed by terminal deoxynucleotidyl-transferase in a template-independent fashion. The fluoresceinated DNA is then detected on a single-cell level by flow cytometry. Cell phenotype, activation, or maturation state is established by cell surface labeling with MAb prior to the TUNEL reaction.	Blood should be drawn in ACD. A total of 20 ml is used for this assay.
Annexin V	Apoptosis can be characterized by changes in cell membrane structure. During apoposis, the cell membrane's phospholipid asymmetry changes: PS is exposed on the outer membrane while membrane integrity is maintained. Annexin V specifically binds PS, while PI or 7-AAD is a DNA binding fluorochrome. When a cell population is exposed to both reagents, apoptotic cells will stain positive for annexin V and negative for PI or 7-AAD, necrotic cells will stain positive for both, and live cells will stain negative for both.	
Caspase 3	Caspase 3 has been implicated as a key protease that is activated during the early stages of apoptosis. The caspases are synthesized as inactive proenzymes that are processed in cells undergoing apoptosis by self-proteolysis and/or cleavage by another protease. The processed forms consist of a large heterodimer of 17–21 kDa and a small one at 12 kDa, subunits which derive from the 32-kDa proenzyme. These subunits form an active enzyme. Active caspase 3 proteolytically cleaves and activates the other caspases, as well as relevant targets in the cytoplasm and nucleus. Caspases trigger a cascade of proteolytic cleavage events within the apoptotic cell, which include numerous cytoplasm and nuclear proteins as well as other caspase family members.	
Chemokines	Chemokines are a new subset of cytokines, which participate in the activation of leukocytes and play a critical role in controlling their movement during inflammation, infectious diseases, tumor growth, and hematopoietic progenitor cell proliferation. Chemokines are 8 to 10 kDa proteins sharing 20–70% homology in amino acids. According to the relative positions of their cysteine residues, chemokines can be subdivided into 4 families, including CXC, CC, C, and CX3C. Chemokines exert their function through a family of 7 G protein-coupled transmembrane receptors.	
DCs	DCs are specialized for antigen uptake, processing and presentation to the T cells. DCs are distinguished from the antigen-presenting cells, such as B cells and monocytes, by their ability to induce a primary immune response, such as activation of immunologically naive T cells. DCs are present in low frequencies in peripheral lymphoid tissue and blood and in nonlymphoid organs. The two distinct peripheral dendritic subsets can be detected using 3- or 4-color flow cytometry, $CD123^+$ and $CD11c^+$ HLA-DR lineage (CD3, -14, -16, -19, -20, and -56) dim or negative.	Whole blood collected in either EDTA, ACD, or sodium heparin, or PBMCs. Use blood within 24 h of draw.

Basic materials	Basic QC	Basic procedure	Flow analysis	Basic limitations
1. PBMCs adjusted to 10^6/ml. 2. PI in H_2O for fresh cells 3. For fixed cells, use RNase (incubate for 1–2 h at 37°C); then add PI in PBS. 4. DNA analysis software	Set flow compensation.			
1. In situ cell death detection kit 2. PBS 3. FACS permeabilizing solution 4. CD4 PE, CD8 cytochrome, CD8 FITC, CD8 PE 5. DNase 6. Culture medium	Set flow compensation.	1. Prepare and adjust PBMCs at 10^6/ml in culture medium. 2. Culture PBMCs for 24 h at 37°C. 3. Add 100 µl of cells to tubes with MAb; incubate for 30 min at 4°C in the dark. 4. Wash cells, fix with 2% paraformaldehyde, and incubate for 30 min or overnight. 5. Follow manufacturer instructions for TUNEL method. 6. Do flow analysis.	1. Apoptotic-cell number is reported as a percentage of total CD4 and CD8 cells. 2. Look at dot plot: FSC vs SSC, FL2 vs SSC, and FL1 vs SSC. Also look at FL1 (TUNEL) histogram of CD4 (FL2) and CD8 (FL3) cells.	DNA cleavage can be absent or incomplete in some forms of apoptotic cell death. Sterical hindrance such as extracellular matrix components can prevent access of TdT to DNA strand breaks.
1. 10× Binding buffer (BD catalog no. 66121A) 2. PI 3. Annexin V-FITC (BD catalog no. 65874×) 4. 1× PBS				
1. For a 3-color assay: lineage cocktail 1 (lineage 1) FITC, which contains CD3, -14, -16, -19, -20, and -56. 2. CD123 PE 3. HLA-DR PerCp 4. CD11c PE 5. Isotypes 6. Lyse	Set flow compensation.	Basic immunophenotyping: 1. 100 µl of whole blood 2. Add MAb. 3. Incubate for 15 min. 4. Lyse for 10 min. 5. Wash twice. 6. Fix and analyze.	1. Gate 1 = R1 = all cells minus debris and dead cells 2. Gate 2 = R2 = all cells from gate 1 and further selecting cells that are dim and negative for lineage [(CD3, -14, -16, -19, -20, and -56) FITC and HLA-DR PerCp] 3. Gate 3 = R3 = gate 1 + gate 2. Using gate 3, create an HLA-DR/CD11c plot and an HLA-DR/CD123 plot. 4. Draw regions to define basophiles as [HLA DR^-/$CD123^+$] and DCs as [HLA-DR^+/$CD123^+$] and [HLA-DR^+/$CD11c^+$]. 5. DCs are defined as lineage negative/HLA-DR^+/$CD123^+$/$CD11c^+$.	DCs occur with low frequency in peripheral blood. Need to acquire at least 50,000 events.

(continued)

Table 11.1.3–1 Emerging immunological assays using flow cytometry[a] *(continued)*

Test	Basic principle	Specimen type
Tetramer assay	This assay is a peptide epitope-specific assay for antigen-specific T cells. Normally this is done to detect virus-specific (influenza virus, HIV, CMV, EBV, etc.) $CD8^+$ T cells, but it can be done with any antigen. Sample of blood needs to be prescreened for HLA-A2; it must be HLA-$A2^+$ in order for the assay to work using the tetramer. HLA-A2 forms a tetramer in solution when bound to a microglobulin and an epitope peptide. Once the class I chain-peptide-microglobulin is ready, it is mixed with fluoresceinated streptavidin, thus forming a tetramer of peptide-loaded MHC class I molecules. The dominant epitope of the antigenic peptide is bound to the appropriate class of HLA. Since this mimics how macrophages, B cells, or DCs present antigens, it can identify antigen-specific cells. The binding affinity of the complex to the T cell is near that of Ab-Ag complexes and is suitable for flow cytometry.	Whole blood, heparinized, is preferred over PBMCs. Whole blood has a lower background, and the tetramer-positive population has a higher MFI in whole blood. PBMC staining is adequate, and fresh and frozen PBMCs give similar results. Staining at room temperature is best.

[a] Abbreviations: PI, propidium iodide; TUNEL, terminal deoxynucleotidyltransferase-mediated duTP-biotin nick end labeling; MAb, monoclonal antibody; PS, phosphotidylserine; 7-AAD, 7-aminoactinomycin D; PBS, phosphate-buffered saline; PBMCs, peripheral blood mononuclear cells; FACS, fluorescence-activated cell sorter; PE, phycoerythrin; FITC, fluorescein isothiocyanate; ACD, acid citrate dextrose; FSC, forward scatter; SSC, side scatter; TdT, terminal deoxynucleotidyl transferase; DCs, dendritic cells; HIV, human immunodeficiency virus; CMV, cytomegalovirus; EBV, Epstein-Barr virus; MHC, major histocompatibility complex; MFI, mean fluorescence intensity.

Basic materials	Basic QC	Basic procedure	Flow analysis	Basic limitations
1. Tetramers labeled with PE (FL2) less background or antigen-presenting cells (FL4) 2. FACS Lysing Solution 3. CD8-, CD3-labeled MAb 4. 1% Fixative 5. Flow cytometer 4-color acquisition/analysis	Set flow compensation.	1. Use MAb to identify a tetramer-specific population included in the CD3 CD8 population. 2. Gate on $CD3^+$ lymphocytes and plot tetramer vs CD8. All tetramer-positive cells will be $CD8^+$.	Due to the infrequency of antigen-specific cells, a minimum of 150,000 lymphocyte events in a flow cytometer must be collected to detect an antigen-specific population of less than 0.1% or higher of T cells. To detect a lower frequency, one will need to stain more cells and collect more events.	Procedure is MHC class I restricted like HLA-$A2^+$

11.2.1 Introduction

A number of extracellular antigenic products have been identified in cultures of group A streptococci, primarily enzymatic proteins. These include streptolysin O, streptokinase, hyaluronidase, deoxyribonucleases (DNases A, B, C, and D), and adenine dinucleotide glycohydrolase.

Infections by group A streptococci are unique because they can be followed by the serious nonpurulent complications acute rheumatic fever and glomerulonephritis. Rheumatic fever is associated with infection by certain M serotypes (M3, M5, M6, M18, and others), while glomerulonephritis follows infection by nephritogenic serotypes (M1, M2, M4, M6, M12, M49, and M60). Rheumatic fever follows untreated tonsillitis-pharyngitis. Glomerulonephritis occurs in as many as 10 to 15% of individuals involved in outbreaks of impetigo. These complications occur after a period of latency following the infection, during which the patient is asymptomatic. The latency period for acute glomerulonephritis is approximately 10 days, and that for acute rheumatic fever is about 20 days.

Serologic tests have been developed to provide evidence for an antecedent group A streptococcal infection, utilizing assays for serum antibodies against one or more of the antigenic streptococcal extracellular products or cellular components (Table 11.2.1–1). The use of these assays for the diagnosis of acute group A streptococcal infection is rarely, if ever, indicated. Occasionally, they may be used to confirm that an acute illness is related to group A streptococcal infection, such as in a patient with suspected scarlet fever. For these patients, acute- and convalescent-phase sera obtained 2 to 4 weeks apart can be assayed in tandem so as to demonstrate a rise in titer.

TESTS FOR ANTIBODIES TO EXTRACELLULAR PRODUCTS OF GROUP A STREPTOCOCCI

The anti-streptolysin O (ASO) test was devised by Todd (1) to measure serum neutralizing antibody against streptolysin O. Hemolysin activity is measured by the capacity of the enzyme to lyse erythrocyte membranes, and this activity is neutralized by serum antibody. The ASO test was followed by the development of other neutralizing-antibody tests for enzymes produced by group A streptococci. The ASO test remains the best standardized, most reproducible, and most universal. The use of other tests, such as the antihyaluronidase and antistreptokinase tests, has declined in part because of difficulties in reproducibility and the limited availability of some reagents. In contrast to these tests, the anti-DNase B test has gained usage. DNase B is one of four deoxyribonuclease isozymes (A, B, C, and D) produced by group A streptococci, and because the B isozyme is produced predominantly by group A streptococci, the anti-DNase B test is highly specific for recent group A streptococcal infection. The test is highly reproducible, and reagents are also available commercially.

Table 11.2.1–1 Tests of antibodies to extracellular products of group A streptococci

Type of test	Name	Supplier	Comment(s)
ASO	Inverness Wampole Colorcard ASO	Wampole, Cranbury, NJ	Rapid, semiquantitative
	Remel Sera Test ASO Latex Test Kit	Remel, Lenexa, KS	Rapid, qualitative
	Immage ASO	Beckman Coulter, Fullerton, CA	Nephelometric assay
Anti-DNase B	Streptonase-B	Wampole, Cranbury, NJ	Standard assay
	Immage Anti-DNase B	Beckman Coulter, Fullerton, CA	Nephelometric assay

TESTS FOR ANTIBODIES TO EXTRACELLULAR PRODUCTS OF GROUP A STREPTOCOCCI *(continued)*

Although the ASO test is quite reliable, the ASO response is not universal: elevated serum ASO titers are found in only about 85% of individuals with acute rheumatic fever. While the same holds true for other streptococcal antibody tests, a significant proportion of individuals with normal antibody titers for one test have elevated antibody titers for another test. Thus, the number of false negatives can be reduced by performing two or more tests of antibodies to different group A antigens. In addition, skin infection, in contrast to throat infection, is associated with a poor ASO response; thus, the ASO is far less reliable than the anti-DNase B test in cases of postimpetigo nephritis.

The Streptozyme test is an agglutination test that detects antibodies against several streptococcal antigens. Five products (streptolysin O, DNase B, streptokinase, hyaluronidase, and adenine dinucleotide glycohydrolase) are used to coat sensitized erythrocytes. Antibody against these antigens is tested for by hemagglutination of the coated erythrocytes on a slide. Discrepancies between the Streptozyme test and the ASO and anti-DNase B tests on the same serum have been reported in several studies. Use of the Streptozyme test is discouraged.

Serum antibody responses to the extracellular products of group A streptococci peak 2 to 3 weeks after the acute infection. Because the nonpurulent complications present 2 to 3 weeks after the acute infection, a single serum sample obtained at presentation of a patient with acute nephritis or acute rheumatic fever should reveal elevated ASO and/or anti-DNase B titers in most patients. An upper limit of normal titer must be established for these tests for proper interpretation for various ages (school-age childhood values differ from preschool or adult values).

We recommend that a laboratory establishing procedures for providing evidence of group A streptococcal infection limit itself to two tests for which reagents are readily available and which can be depended on to yield reproducible results. Because interpretation of a single titer depends on the upper limit of normal, it is advisable to survey a healthy population to establish this value for populations of different age groups within a geographic region. In addition, serum controls of known low and high titers should be included in each assay. The ASO and anti-DNase B tests are the most commonly utilized assays. Details of the performance and interpretation of these tests are given in procedures 11.2.2 and 11.2.3.

REFERENCE

1. **Todd, E. W.** 1932. Antigenic streptococcal hemolysis. *J. Exp. Med.* **55:**267–280.

SUPPLEMENTAL READING

Ayoub, E. M. 1982. Streptococcal antibody tests in rheumatic fever. *Clin. Immunol. Newsl.* **3:**107–111.

Ferrieri, P. 1986. Immune responses to streptococcal infections, p. 336–346. *In* N. R. Rose, H. Friedman, and J. L. Fahey (ed.), *Manual of Clinical Laboratory Immunology*, 3rd ed. American Society for Microbiology, Washington, DC.

11.2.2 Anti-Streptolysin O

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Many individuals respond to respiratory infection by group A (and to a lesser degree to group C or G) streptococci with antibody to the hemolysin streptolysin O (anti-streptolysin O [ASO]) that is elaborated by the bacteria. The presence of ASO activity is indicated by the ability of diluted serum to neutralize the hemolytic activity of the streptolysin O reagent. There is a newer nephelometric method for assaying ASO antibodies in human sera (Immage; Beckman Coulter).

II. SPECIMEN COLLECTION

Observe standard precautions.

Peripheral blood should be collected using aseptic techniques. Blood is stored preferably at 4°C prior to serum separation. Storage for over 72 h may lead to hemolysis, which can result in misinterpretation of the ASO test, as can grossly hemolyzed serum of other causes. The blood sample should be kept refrigerated, on ice, or at room temperature, but it should not be frozen. Shipped specimens should have serum separated prior to shipment.

The serum is clarified by centrifugation of erythrocytes at 500 × *g* for 5 min, and the serum is transferred to a sterile tube or vial for storage. Sera that will be assayed within a week can be stored at 4°C. If longer storage is necessary, the serum should be stored at −20 or −70°C. Repeated freezing and thawing should be avoided.

Protective gloves are used during the handling of the blood samples and subsequently during the dilution of the sera. Proper precautions for the disposal of blood-contaminated material should be observed.

III. MATERIALS

A. Equipment

1. Disposable 96-well, round-bottom microtiter plates (USA Scientific Plastics, Ocala, FL)
2. Micropipette, 100- to 1,000-μl capacity (P1000 Rainin pipette, Rainin Instruments, Woburn, MA)
3. Micropipette, 1- to 100-μl capacity (P100 Rainin pipette)
4. Multichannel pipette, 12 row, 25- to 100-μl variable capacity (Finn)
5. Plastic disposable tips for micropipettes
6. Disposable test tubes (13 by 100 mm), sterilized
7. Microtiter plate reading platform with mirror (Dynatech Laboratories Inc., Alexandria, VA)
8. Centrifuge with rotor and carriers for microtiter plates
9. Conical graduated centrifuge tubes

B. Reagents

1. Streptolysin O reagent, 10-ml vial (Difco Laboratories, Detroit, MI; Difco Laboratories, Grand Island, NY; Fisher Scientific, Orangebury, NY)
2. Citrated fresh type O human erythrocytes (rabbit or sheep RBC, can be substituted)

III. MATERIALS *(continued)*

3. Two internal control sera of known antibody titer, one with high titer and one provided with the streptolysin reagent by the manufacturer
4. ASO buffer concentrate (no. 2679-79; Fisher Scientific)

ANALYTICAL CONSIDERATIONS

IV. PROCEDURE

Observe standard precautions.

The ASO assay can be performed by a macrodilution or a microdilution assay. The microdilution assay has the advantage of requiring small amounts of serum and reagents. Accurate micropipetting instruments allow for high test reproducibility. The microdilution procedure described by Klein et al. (1) was adapted in our laboratory to use a 0.1-log serum dilution scheme. This scheme has the advantage of determining relatively small increments in antibody titers.

The major precautions for proper test performance include (i) the use of clean instruments and utensils, (ii) the use of fresh streptolysin O reagent with active sulfhydryl-reducing compounds to prevent oxidation and inactivation of the enzyme, (iii) the use of freshly collected (within 1 week) human type O or rabbit or sheep erythrocytes, and (iv) the inclusion of proper controls.

V. ERYTHROCYTE SUSPENSION (2.5%)

Observe standard precautions.

Citrate-treated human type O erythrocytes are collected in 3.8% sodium citrate dihydrate in a ratio of 1.0 volume of blood to 1.2 volumes of citrate. Commercially citrate-treated blood bank type O erythrocytes may be used. Pipette 3 to 4 ml of citrate-treated blood into a 12- to 15-ml conical centrifuge tube. Add 2 to 3 volumes of physiological saline (0.9%) to 1 volume of blood. Mix gently by inverting the tube. Centrifuge at 400 $\times$ *g* for 5 min. Remove the supernatant, and wash the cells twice in physiological saline. After the second wash, suspend the cells in physiological saline and centrifuge for 10 min. The supernatant should be clear. Note the volume of packed erythrocytes, and make a 2.5% suspension by adding 3.9 ml of phosphate-buffered saline (7.4 g of NaCl, 3.17 g of KH_2PO_4, and 1.8 g of Na_2HPO_4 per liter of distilled water; adjust to pH 6.5 to 6.7 with NaOH) to each 0.1 ml of packed erythrocytes. The cell suspension can be stored at 4°C but should be discarded at the first sign of hemolysis.

VI. STREPTOLYSIN O REAGENT

The streptolysin O reagent is reconstituted by adding 10 ml of sterile distilled water until the lyophilized preparation is dissolved. This should be done immediately prior to use. Store at 2 to 8°C.

VII. SERUM DILUTIONS

Observe standard precautions.

A. Stock dilution

Make a 1:10 serum dilution by adding 50 μl of serum to 450 μl of Bacto streptolysin O buffer or phosphate-buffered saline described above. Cover this dilution and inactivate it at 56°C for 30 min.

B. Serum dilution in the microtiter plate

Each serum is assigned three vertical columns on the microtiter plate; thus, four sera can be assayed in each plate. The top row of wells is used for the initial serum dilutions. We start with initial dilutions of 1:25, 1:30, and 1:40 in the three top wells, but this can be altered to higher or lower starting dilutions. The scheme outlined here is a 0.1-log dilution scheme. Top wells are not used in the assay but only to initiate the dilution process. The bottom row of wells is used for controls that lack serum or lack streptolysin O reagent.

VII. SERUM DILUTIONS *(continued)*

Make the initial dilutions as follows. To each of the three top wells, add 30 µl of the stock 1:10 serum dilution. Then add 45 µl of diluent phosphate-buffered saline to the first of the three wells to obtain a 1:25 dilution, 60 µl of diluent to the second well to obtain a 1:30 dilution, and 90 µl of diluent to the third well to obtain a 1:40 dilution. To the remaining wells on the plate, add 50 µl of dilution buffer. Then make twofold serial dilutions with the multichannel pipette by transferring vertically 50 µl from the preceding horizontal row of wells to the following row, mixing gently with the multichannel pipette (three or four strokes) while avoiding the production of air bubbles, and discarding the 50 µl removed from the next to last row of wells. Then add 25 µl of the freshly reconstituted streptolysin O reagent to all wells except those in the top row. Incubate the plate for 30 min at 37°C, followed by addition of 25 µl of the erythrocyte suspension to all the wells. Reincubate plates at 37°C, for 45 min, with careful shaking (by gently tapping the plate) at 15-min intervals. Centrifuge the plates on the carriers at 400 × *g* for 5 min. Place the plate on the plate reader, using a fluorescent lamp or daylight as a source of light. Cover the top of the plate with clear white paper while reading to avoid light distortion.

POSTANALYTICAL CONSIDERATIONS

VIII. READING AND INTERPRETATION OF TEST

With the plate in the position described above, examine the wells for hemolysis. The ASO titer of a serum is equal to the inverse of the highest serum dilution that inhibits the action of streptolysin, or the serum dilution in the last well that shows no hemolysis. The titer may be expressed in Todd units if the streptolysin reagent used was adjusted against the Todd standard serum, as the Bacto reagent is.

A test is considered uninterpretable if the streptolysin O control well (containing streptolysin O reagent, RBCs, and buffer) does not show total hemolysis or if the erythrocyte control well (without streptolysin O reagent) shows hemolysis. In addition, if the reference serum standards yield titers that differ by more than 1 dilution from the designated titer for that serum, the test should be repeated.

To determine a rise in antibody between acute- and convalescent-phase sera, both sera should be assayed in tandem on the same plate. A difference of at least 0.2 log unit (two wells if the 0.1-log unit serum dilution scheme is used) between the titers of the two sera is considered to be a significant difference. When one serum is assayed, an elevated titer should clearly exceed the upper limit of the normal range as described above. In our laboratory, the upper limit of normal titer is 240 for adults and at least 340 for children. An elevated ASO titer or other streptococcal antibody titer is not diagnostic of acute rheumatic fever but provides evidence for an antecedent group A streptococcal infection in the modified Jones criteria (2). Performance of several streptococcal antibody tests may increase the frequency of finding an elevated antibody titer indicative of a preceding streptococcal infection.

In addition, falsely high titers may be obtained with sera that are contaminated by certain bacterial organisms during shipment or storage or from and in patients with liver disease in whom high serum lipoprotein concentrations may mimic antibody activity by inhibiting the lytic action of the streptolysin on the erythrocyte membrane. Preassay precipitation of these lipoproteins with dextran sulfate will remove this nonspecific inhibitory activity (3).

IX. NEPHELOMETRIC ASSAY

The nephelometric assay measures the rate of increase of light scatter due to particles in suspension as a result of immune complexes formed during the reaction between streptolysin O antigen and antibody to streptolysin O in human serum. Plasma can also be used. The antigen is attached to polystyrene particles. Light scatter is read by a nephelometer.

REFERENCES

1. **Klein, G. C., E. C. Hall, C. N. Baker, and B. V. Addison.** 1970. Antistreptolysin O test: comparison of micro and macro techniques. *Am. J. Clin. Pathol.* **53:**159–162.
2. **Special Writing Group of the Committee on Rheumatic Fever, Endocarditis, and Kawasaki Disease of the American Heart Association.** 1992. Guidelines for the diagnosis of rheumatic fever: Jones criteria, 1992 update. *JAMA* **268:**2069–2073.
3. **Winblad, S.** 1966. Studies on non-specific antistreptolysin O titre. 1. The influence of serum beta-lipoproteins on the non-specific antistreptolysin O titre. *Acta Pathol. Microbiol. Scand.* **66:**93–104.

11.2.3 Anti-DNase B Test

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The anti-DNase test evaluates the ability of diluted serum to neutralize the effect of DNase B to digest a DNA substrate complexed to a colored dye and thus to block the loss of color expressed by the digested substrate. Two microtechniques are available for the performance of the test; one is described by Nelson et al. (1), and the other is available as a commercial kit from Wampole. The latter method is described here. There is also a nephelometric assay for quantitative determination of antibodies to DNase B (Immage; Beckman Coulter).

II. SPECIMEN COLLECTION

Specimen collection is as described above for the anti-streptolysin O (ASO) test.

III. MATERIALS

A. Reagents

Anti-DNase B test kit (Streptonase-B; Wampole, Cranbury, NJ)

1. DNase B enzyme reagent (stable for 3 months at 2 to 8°C after opening)
2. Calf thymus DNA with color indicator (DNase substrate). Use within 5 days of preparation.
3. Buffer concentrate (imidazole, calcium chloride, magnesium sulfate, and gelatin, containing 0.5% sodium azide) (stable at 2 to 8°C)
4. Anti-DNase B positive control serum, titer of 1:240 (stable at 2 to 8°C). Some laboratories also utilize a higher-titer positive external control human serum.

B. Supplies

1. Negative control serum (human serum previously found to be negative for anti-DNase B antibodies) (stable at 2 to 8°C)
2. Disposable round-bottom 96-well microtitier plates
3. Serological pipettes, 1.0, 0.05, and 0.025 ml
4. Test tubes (10 by 75 mm) and racks
5. Incubator, 37 ± 2.0°C
6. Distilled water

ANALYTICAL CONSIDERATIONS

IV. PROCEDURE

Observe standard precautions.

A. Preparation of reagents

1. Buffer

   Bring buffer concentrate to room temperature. If buffer is lyophilized, mix with appropriate amount of distilled water (usually 100 ml). Store at 2 to 8°C.

IV. PROCEDURE *(continued)*

2. DNase B enzyme reagent
Prepare a stock solution of DNase B by reconstituting with the correct amount of buffer (usually 2 ml) according to the manufacturer's directions. Stock DNase B is stable for 3 months at 4°C. On the day of the test, prepare working DNase B reagent by adding 0.1 ml of the stock to 6.9 ml of buffer. Store at 2 to 8°C.

3. Calf thymus DNA substrate with color indicator
If lyophilized, add appropriate amount of distilled water (usually 12 ml) to the contents of the vial *at least* 3 h before use. Allow substrate to dissolve at room temperature, swirling the vial every 30 min. The dissolved substrate must be used within 5 days. The substrate can be stored at 4°C but must be at room temperature before use.

4. Positive control serum
Use the positive control supplied with the kit or a past positive specimen with a known titer. Make an initial 1:10 dilution by adding 0.5 ml of buffer. The positive control supplied with the kit is already at a 1:10 dilution. Final dilution is 1:240.

B. Serum dilution and assay method

Make a 1:10 dilution of the patient serum by adding 50 µl of serum to 450 µl of buffer. Inactivate serum by placing the Parafilm-covered tube in a 65°C water bath for 30 min. Serum dilutions for titration are performed as described above for the ASO test, except that after the initial dilutions are made, 25 µl of diluent is added to the remaining wells. Using a 25-µl multichannel pipette, make two-fold serum dilutions, leaving a 25-µl volume in each well.

Add 25 µl of the appropriate dilution of DNase B enzyme reagent to each well. Incubate the plates at 37°C for 30 min. Remove the plates from the incubator, and add 50 µl of DNA substrate to each well. Cover the plate carefully with clear sealing tape, using the plate-sealing device. Incubate the plates at 37°C for 15 min, gently mix, and reincubate for 4 h at 37°C. Remove the plates from the incubator, and read the endpoint of each serum titration. As with the ASO test, the two internal control sera are included in the assay. In addition, appropriate enzyme and substrate controls should be included.

V. READING AND INTERPRETATION

DNase activity on the DNA substrate with color indicator is reflected by digestion of the DNA and depolymerization of the colored complex. This results in a pink or pinkish-violet color in the solubilized substrate. Thus, the endpoint of antibody neutralization is reflected in a decrease or loss of the blue or blue-violet color in the well. The antibody titer is the reciprocal of the highest dilution of serum showing a bluish color. As with the ASO test, divergence of the control serum titers by more than 1 dilution requires repetition of the assay. The negative control should read $<$1:50. The serum control and substrate control wells should show no enzyme activity (blue), and the enzyme control well must show enzyme activity (pink), for valid results.

POSTANALYTICAL CONSIDERATIONS

VI. RESULTS AND CONCLUSIONS

Stability of the reagents provides a decided advantage in reproducibility of the anti-DNase B test over that of the ASO test. The DNase B enzyme can be stored for 3 months at 4°C without any appreciable loss of activity. In practice, we divide the concentrated stock solution into aliquots and store them at -70°C. An aliquot is thawed and diluted in the working buffer to 10 times the working dilution and stored at 4°C for use over a period of about 3 months. Other advantages are that

VI. RESULTS AND CONCLUSIONS *(continued)*

the calf thymus DNA is available commercially and the substrate (the DNA-color conjugate) is stable when stored at 4°C. This eliminates the need for a fresh source of erythrocytes and the requirement for processing the cells before each ASO test.

To ensure the reproducibility and specificity of the assay, certain precautions need to be considered. Group A streptococci as well as other streptococcal serogroups (B, C, and G) produce DNase isozymes (DNases A, C, and D). Although the isozymes produced by other streptococci are antigenically distinct from the group A streptococcal nucleases, assessment of the purity of the DNase B preparation by the availability of a specific antiserum with known titers for DNase B is important in the standardization and performance of the test. The use of a pure DNAse B preparation ensures specificity of the assay for group A streptococcal infections.

In contrast to ASO titers, anti-DNase B titers are elevated following either streptococcal pharyngitis or pyoderma.

REFERENCE

1. **Nelson, J., E. M. Ayoub, and L. W. Wannamaker.** 1968. Streptococcal antideoxyribonuclease B: microtechnique determination. *J. Lab. Clin. Med.* **11:**868–873.

11.3 Detection of *Legionella* Antigen by Direct Immunofluorescence

Legionnaires' disease is a type of bacterial pneumonia caused by *Legionella* spp. (2, 14). It is estimated that about 1 to 4% of adults with pneumonia requiring hospitalization have Legionnaires' disease and that about 20,000 to 100,000 adults with community-acquired pneumonia have this disease in the United States each year. About 5 to 20% of patients with Legionnaires' disease die of the disease, with major dependence on promptness of specific antibiotic therapy and underlying health of the patient. Legionnaires' disease occurs worldwide, in both epidemic and sporadic form, with sporadic cases being far more common. The disease is more prevalent in some geographic regions than others, for unclear reasons. The primary host risk factors for the disease include immunosuppression, cigarette smoking, and travel. *Legionella pneumophila* is the cause of more than 90% of cases of community-acquired Legionnaires' disease, with *L. pneumophila* serogroup 1 being by far the most common causative agent of the disease. Immunosuppressed patients, and those with nosocomial pneumonia, may have infections caused by other *L. pneumophila* serogroups and other *Legionella* spp., in particular *L. micdadei, L. longbeachae, L. bozemanae,* and *L. dumoffii.* Distribution of the common serogroups and species causing infection may be quite different in various geographic regions. *Legionella* bacteria are commonly found in the aqueous environment, including tap water and sometimes even distilled water. The preponderance of *L. pneumophila* as the cause of Legionnaires' disease has resulted in the development of reagents optimized for detection of this species; detection of other *Legionella* species can be problematic, for reasons of both test sensitivity and specificity. Rarely, organs other than the lungs and pleural space may be infected by the bacterium, causing such diseases as prosthetic heart valve endocarditis, assorted soft tissue abscesses, and systemic infection.

The *Legionella* direct immunofluorescence antibody (DFA) test can be used for two purposes in the clinical laboratory: (i) detection of the bacteria in clinical specimens and (ii) identification and serotyping of bacterial isolates suspected to be *Legionella* spp. The first use has fallen out of favor because of its requirement for expertise in interpretation and performance, its low sensitivity, and under certain circumstances its low specificity (13). The DFA test is commonly used by many laboratories for rapid and relatively accurate confirmation of bacterial identity.

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

A thin layer of sputum, tissue homogenate, or bacterial suspension is heat fixed to a slide, which is then stained with anti-*Legionella* antibody conjugated with fluorescein isothiocyanate (FITC). The slide is then washed to eliminate unbound antibody and examined microscopically using UV light illumination. The FITC fluoresces under UV light, which causes the *Legionella* bacteria to appear a bright green color. The presence in clinical specimens of fluorescent bacteria of the proper size and shape is diagnostic of Legionnaires' disease, providing that the test has been performed correctly. A positive test of a bacterial suspension confirms the identity of the bacterium as a *Legionella* sp., again assuming proper performance of the assay and selection of the correct bacteria to test.

II. SPECIMEN TYPES, COLLECTION, AND TRANSPORT

Observe standard precautions.

The DFA test has been used to detect the bacterium in a variety of tissues (Table 11.3–1). Lower respiratory tract secretions, including expectorated sputum, are the most frequently tested specimen types. The test can be quite valuable for detecting *Legionella* bacteria in premortem or postmortem lung tissue. The bacteria are very stable in refrigerated or frozen sputum and tissues, and the major determinants of specimen handling are other requirements for the specimen, such as bacterial cul-

Table 11.3–1 Suitable specimen types for *L. pneumophila* DFA testing

Sputum	Vascular grafts
Endotracheal suction	Kidney
Bronchoalveolar lavage fluid	Liver
Transbronchial biopsy specimen	Spleen
Lung biopsy specimen	Soft tissue abscesses, including muscle
Pleural biopsy specimen or pleural fluid	Brain
Bronchoscopic aspirates	Heart valve
Bronchial brushings	Pericardium

II. SPECIMEN TYPES, COLLECTION, AND TRANSPORT *(continued)*

ture. If the specimen will be tested within 1 to 5 days, then store the specimen in a tightly sealed sterile container at 3 to 5°C. Longer-term storage is best done at −70°C. Since *Legionella* bacteria, and cross-reacting bacteria, are ubiquitous in water, avoid contamination of the specimen with non-filter-sterilized water. Test lots of empty specimen containers for intrinsic contamination before buying them, as on rare occasion, false-positive results may be obtained because of water contamination of the containers before they are sterilized. Preservation of tissues in 10% neutral Formalin obscures the antigen detected by the Bio-Rad monoclonal antibody to *L. pneumophila*, making freezing at -70°C a preferred alternative for long-term storage. However, polyclonal antibodies to the individual *Legionella* serogroups still react with Formalin-fixed materials, making possible examination of Formalin-fixed specimens.

Identification of bacteria by DFA testing requires that the bacteria first be suspended in Formalin. The Formalin concentration used depends on the type of antibody used in the DFA test; polyclonal antibodies work correctly with bacteria that have been suspended in 1 to 10% neutral Formalin, whereas the commercial monoclonal antibody works best with 1% Formalin, as higher Formalin concentrations may cause false-negative reactions with prolonged fixation.

III. MATERIALS

All commercial antibody preparations are available with the antibody conjugated to FITC and with few exceptions are rabbit polyclonal antibodies (Table 11.3–2). The polyclonal antibodies are generally, but not always, serogroup specific and may be available either as polyvalent pools or as monovalent reagents. A monoclonal antibody to all *L. pneumophila* serogroups is also available. Many commercially available antibodies have not been extensively evaluated in terms of their analytical or clinical specificity and sensitivity. The Bio-Rad monoclonal antibody reagent has had the most extensive clinical evaluation (3). Only FDA-cleared reagents, and preferably the Bio-Rad monoclonal product, should be used to detect *L. pneumophila* in clinical specimens.

Evaluations of the non-FDA-cleared reagents have not been published. However, in many cases they are the only reagents available for a broad range of species, and they are exceptionally useful in serogroup and species identification of culture isolates. Since these reagents have not had extensive evaluations, unusual results obtained using them must be confirmed by a reference laboratory.

Microscope slides made specifically for immunofluorescent microscopy give the best results. The slides are ringed or masked by a hydrophobic material, which prevents the antibody from running off the slide or onto an adjacent well. The slides must be cleaned in alcohol and manufactured in such a way that dead *Legionella* bacteria are not present on the slides. One manufacturer is Scientific Device Laboratory (http://www.scientificdevice.com).

Table 11.3–2 Commercial DFA reagents

Manufacturer	Antibody type	Notes	FDA cleared?
Bio-Rad, http://www.bio-rad.com	Monoclonal	Reacts with all *L. pneumophila* serogroups. Formerly known as Genetic Systems or Sanofi Pasteur monoclonal antibody.	Yes
M-Tech, http://www.4m-tech.com	Polyclonal, monovalent, and polyvalent	Very wide range of antibodies available	No
Trinity Biotech, http://www.trinitybiotech.com	Polyclonal, polyvalent only	Four polyvalent reagents are available: *L. pneumophila* serogroups 1–6, *L. pneumophila* serogroups 1–14, and two reagents reacting with non-*L. pneumophila* spp. Formerly known as MarDx reagents and, before that, BioDx.	Only the *L. pneumophila* serogroup 1–6 reagent is FDA cleared
SciMedx, http://www.scimedx.com	Polyclonal, polyvalent, and monovalent	*L. pneumophila* serogroup 1–6 monovalents and pooled; a pooled reagent and monovalent reagents to non-*L. pneumophila* spp. Formerly known as BioDx reagents.	Yes
Wampole Laboratories, http://www.wampolelabs.com	Polyclonal, monovalent, and polyvalent	Individual antibodies to *L. pneumophila* serogroups 1–6 and *L. micdadei*, as well as a single pooled antibody. Formerly known as Zeus reagents.	Yes
Remel, http://www.remelinc.com	Polyclonal, polyvalent	Pooled antibody preparation to a number of species	Yes

ANALYTICAL CONSIDERATIONS

IV. QUALITY ASSURANCE

Include QC information on reagent container and in QC records.

Each time the DFA test is performed, positive and negative controls should be performed. A good negative control is a Formalin suspension of *Escherichia coli* ATCC 25922. This preparation is stable for years, if not decades. A positive control is either one recommended by the manufacturer, or the type strain of the *L. pneumophila* serogroup, or the *Legionella* spp., detected by the antibody; type strain information and the bacteria themselves can be obtained from the ATCC (http://w.atcc.org). Many of the commercially available kits provide a set of QC strains. If at all possible, known positive and negative clinical specimens should be used as an additional QC, especially when training new personnel. Sputum or lung homogenates can be suspended in Formalin and a small amount can be used for QC purposes.

In addition to verifying the proper performance of the antibody being used, specimen containers should be tested by examination of known negative sputum specimens placed in the containers. Several different lots of new specimen containers should be tested before wide-scale purchase of the containers. Similarly, although much less of a problem, plasticware used to prepare bacterial isolate suspensions should be similarly tested.

IV. QUALITY ASSURANCE *(continued)*

At least 75% of all DFA-positive clinical specimens should be culture positive for the same serogroup or species. In the case of *L. pneumophila* serogroup 1 DFA-positive specimens, the *L. pneumophila* serogroup 1 urinary antigen test should be positive at least 90% of the time. Lower culture or urine antigen positivity rates may indicate a problem with false-positive DFA tests, which are usually due to operator inexperience or cross-contamination of the specimen during processing.

V. PROCEDURE

Observe standard precautions.

Instructions for use of the FDA-cleared products are available from the manufacturer, but in other cases, few or no instructions are provided. All of the rabbit polyclonal antibodies use a protocol developed by Cherry and McKinney for the detection of *L. pneumophila* serogroup 1 in clinical specimens and from culture, and the procedure used by any of the manufacturers can be used for any of the polyclonal antibodies made by other manufacturers (reference 1 and references therein). The Bio-Rad monoclonal antibody uses a slightly different protocol, which must be followed exactly to obtain accurate results. Since the CDC protocol may not be readily available, a modified abbreviated version of it follows.

A. Sample application onto a slide

1. For clinical specimens such as expectorated sputum or lower respiratory tract specimens, a very thin layer of the sample is smeared onto a Teflon-ringed or masked slide. The best way to do this is to use a wooden applicator stick to squeegee the sample onto the slide. Fresh lung specimens are best prepared by making a thin impression smear directly onto the slide, and Formalin-fixed lung specimens are best prepared by scraping the lung with a scalpel and then applying the sample to the slide in a very thin layer. It is very important to make the material on the slide one cell layer thick, as thick smears cannot be reliably read using this procedure. Only one clinical specimen should be placed on a slide. All slides must be individually processed to avoid slide-to-slide cross-contamination. The slide is air dried and then heat fixed at 50 to 60°C for 2 to 10 min on a heating block. Methanol fixation has not been studied and is not recommended as a substitute for heat fixation. Formalin (10% neutral) is added to the slide and the sample is fixed on the slide for 10 min in a moisture chamber at room temperature; this can be omitted for previously Formalin-fixed specimens.
2. Bacterial colonies to be tested must first be suspended in 10% neutral Formalin to approximate the density of a no. 1 MacFarland barium sulfate turbidity standard ($\approx 3 \times 10^8$ cells/ml). Then the suspension is pipetted onto the slide and immediately sucked back into the pipette. Emulsifying bacteria directly onto the slide can cause false-negative results due to a prozone phenomenon. Formalin fixation is not crucial for antigen exposure, so if only a single very small bacterial colony is available for testing, then the suspension can be made in sterile water to facilitate subculture and simultaneous DFA testing; the slide is heat fixed on a heating block to kill the bacteria. Procedures involving the manipulation of live bacterial suspensions must be performed under appropriate biosafety conditions.

B. Slide staining

1. The slides are washed with filter-sterilized distilled water and air dried. It is important to use filter-sterilized water to avoid false-positive results.
2. Antibody is added to the slide, which is incubated at room temperature in individual moisture chambers for 15 to 30 min.

V. PROCEDURE *(continued)*

C. Removal of excess antibody

1. Wash the slide with filter-sterilized phosphate-buffered saline (PBS), pH 7.4, using a syringe. Perform an additional 5-min soak in PBS, which need not be filter sterilized.
2. Rinse the slides in distilled water, and then air dry.

D. Mounting of coverslips

Alkaline buffered glycerol (Bio-Rad) is applied to the slide, and a coverslip is mounted. Antifade mounting fluid (Molecular Probes, Eugene, OR) can be used but is usually not necessary.

E. Slide examination

1. The slide is examined microscopically using a microscope equipped with the appropriate filters and illumination for FITC microscopy. Optimal total magnification ranges from $\times 200$ to $\times 1{,}000$. The lower magnification can be used when screening culture isolates and by very experienced technicians when screening clinical samples. Confirmation of positive samples should be done at $\times 500$ to $\times 1{,}000$.
2. Clinical specimens containing more than one brightly staining coccobacillus are considered positive. Some authorities require up to 25 fluorescent bacterial cells to be present to designate a sample as positive, and others require 5. However, both of these criteria are too conservative and will result in an unacceptably high false-negativity rate. Only those with months or years of experience in reading these sorts of slides can reliably use ≥ 2 cells/specimen as a cutoff, whereas those with less experience are advised to use the higher cutoff. Positive and negative controls must perform acceptably.
3. Organism suspensions are considered positive when large numbers of brightly fluorescent bacteria are seen. Rare fluorescent bacteria are considered negative.

POSTANALYTICAL CONSIDERATIONS

VI. INTERPRETATION

Properly performed by experienced laboratorians, the DFA test for detecting *L. pneumophila* in clinical specimens is highly specific but only moderately sensitive (3, 6, 9, 12, 16, 17). With training and experience, the sensitivity of the DFA test for *L. pneumophila* in clinical specimens ranges from 50 to 75%, and the specificity ranges from 94 to 99%. The best performance of the DFA test is with open-lung biopsy specimens or necropsy specimens; providing that the biopsied specimens are from areas with acute pneumonia, the sensitivity and specificity of the DFA test are more than 95 and 99%, respectively, if the antibody being used reacts with the *Legionella* species and serogroup causing the infection.

Use of the test by inexperienced laboratorians may result in a very insensitive and nonspecific test. Several pseudoepidemics of Legionnaires' disease have been reported when nonfiltered Formalin, PBS, or water was used in the test or when there was cross-contamination between clinical specimens and positive controls (8, 10). A number of fluorescent artifacts and cross-reactive bacteria may be present in clinical specimens, which the expert can discriminate from *L. pneumophila* by morphological appearance. The cross-reactive bacteria are generally gram-positive coccal bacteria, such as staphylococci and streptococci; gram-positive bacilli, such as *Bacillus* spp. and lactobacillus-like bacteria; and *Candida*-like yeasts. In addition, there may be cross-reactions with gram-negative bacilli, including *Pseudomonas aeruginosa, Pseudomonas fluorescens,* and *Bacteroides fragilis;* a number of these cross-reactive bacteria are available from the ATCC, for those interested in developing new reagents (ATCC strain numbers 49266 to 49269, 43935 to 43937, and 49270 and 49271). Using polyclonal antibodies, even experts may not be able to

VI. INTERPRETATION
(continued)

reliably discriminate between cross-reactive *B. fragilis,* or *P. aeruginosa,* and *L. pneumophila* bacteria. The Bio-Rad monoclonal antibody does not cross-react with other gram-negative bacteria, although it may stain some *Bacillus* spores, or some gram-positive cocci (7, 15).

More than 60 different *Legionella* serogroups have been reported, many with little or no cross-reactivity with one another. This means that the DFA test of clinical specimens will be negative if the antibody being used does not react with the *Legionella* bacterium in the clinical specimen. Fortunately, more than 90% of all cases of community-acquired Legionnaires' disease are caused by *L. pneumophila,* most by *L. pneumophila* serogroup 1. However, the sensitivity and specificity of the DFA test for the detection of other serogroups and species in clinical specimens have not been established. One polyvalent polyclonal reagent cross-reacted with a number of non-*Legionella* bacteria and stained non-*Legionella* bacteria in clinical specimens, mirroring the problems observed with some monovalent polyclonal reagents (4). Use of multiple polyvalent polyclonal pools to screen clinical specimens leads to a high false-positivity rate (P. Edelstein, unpublished data). Unfortunately, no genus-specific monoclonal antibody is commercially available. Because of the problems with cross-reactions observed with polyvalent pools, it is inadvisable to use them for testing clinical specimens.

One key for proper interpretation of DFA testing of clinical specimens is that *L. pneumophila* is a small coccobacillus in clinical specimens, not the long filamentous rod sometimes found in positive control reagents. This is in contrast to the appearance of the bacterium when isolated from culture, in which case it varies from a short to a very long filamentous bacillus but is almost never coccobacillary.

Identification of *Legionella* bacteria isolated from culture with DFA reagents can be very useful, but it does have some pitfalls. False-negative results can be obtained if the bacterial suspension put on the slide is too dense (4) or if the serogroup of the bacterium does not match that of the antibody being used. False-positive tests can be obtained if the antibody is allowed to dry on the slide during incubation, if nonfluorescent bacteria are misinterpreted as staining with the antibody, or if the organism cross-reacts with the antibody being used. It is important to view isolated bacterial cells to be certain that they are brightly fluorescent, as sometimes bacterial clumps will be fluorescent because of trapped antibody. Bacteria stained with polyclonal antibodies stain very brightly around their periphery but not at all in the central portion, creating a doughnut-like appearance. In contrast, the Bio-Rad monoclonal antibody stains *L. pneumophila* bacteria uniformly but less intensely than do the polyclonal antibodies. Since there can be considerable cross-reactivity between some serogroups and species, the DFA test may assign a *Legionella* bacterium to the wrong species or serogroup. Identification of *L. pneumophila* serogroup 1 with polyclonal antibodies, and of *L. pneumophila* with the Bio-Rad monoclonal antibody, is quite reliable. However, some *L. pneumophila* serogroup 1 strains may fail to react with the polyclonal antibody made to the Philadelphia strain but will react with antibody made to the Bellingham or Knoxville strain. Rarely, a non-*Legionella* bacterium will stain with the DFA reagent; however, with the exception of *Francisella tularensis,* none of these bacteria have a nutritional requirement for L-cysteine (5, 7, 11). In the case of *F. tularensis* and several other cross-reacting bacteria, review of the colony morphology and Gram stain of the bacterium by an experienced microbiologist quickly discriminates between the cross-reacting and *Legionella* bacteria. Molecular methods are sometimes the only means by which some *Legionella* species may be differentiated and identified.

REFERENCES

1. **Cherry, W. B., B. Pittman, P. P. Harris, G. A. Hebert, B. M. Thomason, L. Thacker, and R. E. Weaver.** 1978. Detection of Legionnaires disease bacteria by direct immunofluorescent staining. *J. Clin. Microbiol.* **8:**329–338.
2. **Edelstein, P. H.** 1993. Legionnaires' disease. *Clin. Infect. Dis.* **16:**741–749.
3. **Edelstein, P. H., K. B. Beer, J. C. Sturge, A. J. Watson, and L. C. Goldstein.** 1985. Clinical utility of a monoclonal direct fluorescent reagent specific for *Legionella pneumophila:* comparative study with other reagents. *J. Clin. Microbiol.* **22:**419–421.
4. **Edelstein, P. H., and M. A. C. Edelstein.** 1989. Evaluation of the Merifluor-Legionella immunofluorescent reagent for identifying and detecting 21 *Legionella* species. *J. Clin. Microbiol.* **27:**2455–2458.
5. **Edelstein, P. H., R. M. McKinney, R. D. Meyer, M. A. C. Edelstein, C. J. Krause, and S. M. Finegold.** 1980. Immunologic diagnosis of Legionnaires' disease: cross-reactions with anaerobic and microaerophilic organisms and infections caused by them. *J. Infect. Dis.* **141:**652–655.
6. **Edelstein, P. H., R. D. Meyer, and S. M. Finegold.** 1980. Laboratory diagnosis of Legionnaires' disease. *Am. Rev. Respir. Dis.* **121:**317–327.
7. **Flournoy, D. J., K. A. Belobraydic, S. L. Silberg, C. H. Lawrence, and P. J. Guthrie.** 1988. False positive Legionella pneumophila direct immunofluorescent monoclonal antibody test caused by Bacillus cereus spores. *Diagn. Microbiol. Infect. Dis.* **9:**123–125.
8. **Johnson, D. A., K. F. Wagner, J. Blanks, and J. Slater.** 1985. False-positive direct fluorescent antibody testing for Legionella. *JAMA* **253:**40–41. (Letter.)
9. **Ramirez, J. A., and J. T. Summersgill.** 1994. Rapid tests for the diagnosis of Legionella infections. *J. Ky. Med. Assoc.* **92:**62–65.
10. **Ristagno, R. L., and L. D. Saravolatz.** 1985. A pseudoepidemic of Legionella infections. *Chest* **88:**466–467.
11. **Roy, T. M., D. Fleming, and W. H. Anderson.** 1989. Tularemic pneumonia mimicking Legionnaires' disease with false-positive direct fluorescent antibody stains for Legionella. *South. Med. J.* **82:**1429–1431.
12. **Saravolatz, L. D., G. Russell, and D. Cvitkovich.** 1981. Direct immunofluorescence in the diagnosis of Legionnaires' disease. *Chest* **79:**566–570.
13. **She, R. C., E. Billetdeaux, A. R. Phansalkar, and C. A. Petti.** 2007. Limited applicability of direct fluorescent-antibody testing for *Bordetella* sp. and *Legionella* sp. specimens for the clinical microbiology laboratory. *J. Clin. Microbiol.* **45:**2212–2214.
14. **Stout, J. E., and V. L. Yu.** 1997. Legionellosis. *N. Engl. J. Med.* **337:**682–687.
15. **Tenover, F. C., P. H. Edelstein, L. C. Goldstein, J. C. Sturge, and J. J. Plorde.** 1986. Comparison of cross-staining reactions by *Pseudomonas* spp. and fluorescein-labeled polyclonal and monoclonal antibodies directed against *Legionella pneumophila. J. Clin. Microbiol.* **23:**647–649.
16. **Winn, W. C., Jr., W. B. Cherry, R. O. Frank, C. A. Casey, and C. V. Broome.** 1980. Direct immunofluorescent detection of *Legionella pneumophila* in respiratory specimens. *J. Clin. Microbiol.* **11:**59–64.
17. **Zuravleff, J. J., V. L. Yu, J. W. Shonnard, B. K. Davis, and J. D. Rihs.** 1983. Diagnosis of Legionnaires' disease. An update of laboratory methods with new emphasis on isolation by culture. *JAMA* **250:**1981–1985.

APPENDIX 11.3–1

Buffered Glycerin Mounting Medium

I. MATERIALS

A. 250-ml beaker or flask

B. Magnetic stir bar

II. REAGENTS

glycerin, neutral	10 ml
Na_2CO_3	5.3 g
$NaHCO_3$	4.2 g
distilled water	200 ml

III. PREPARATION

A. Add the carbonate and bicarbonate powders to separate flasks, each containing 100 ml of water. Stir to dissolve. This will make 0.5 M solutions of each salt.

B. Mix 4.4 ml of 0.5 M Na_2CO_3 with 100 ml of 0.5 M $NaHCO_3$. The final pH should be 9.0; if it is not, adjust by addition of appropriate salt.

C. Add 1 part buffer, pH 9.0, with 9 parts glycerin.

IV. STORAGE

Store in a small-volume tightly stoppered plastic container at room temperature. Discard if cloudy or if the pH is <9. The shelf life is weeks to months, depending on the amount of CO_2 gas that is absorbed into the buffer.

11.4 Urinary Antigen Detection for *Legionella* spp.

Patients with Legionnaires' disease excrete soluble serogroup-specific *Legionella* antigen into their urine. Urinary antigen tests to detect Legionnaires' disease were developed by several groups soon after the 1976 Philadelphia, PA, outbreak of Legionnaires' disease (3, 37) and then further refined by Kohler et al. and other groups (5, 16, 27–30, 34–36). The antigen being detected has never been extensively purified, but it is known that it is resistant to boiling, is trypsin resistant, has a molecular mass of approximately 10 kDa, and is most likely a lipopolysaccharide (29, 39).

The first urinary antigen tests developed were designed to detect *Legionella pneumophila* serogroup 1 infections, and they were initially thought to be capable of detecting only this serogroup. It is now known that the *L. pneumophila* serogroup 1 antigenuria test will detect an unknown fraction of infections caused by other *L. pneumophila* serogroups and possibly some other species (2, 13). Regardless, the analytical sensitivity of all commercially available test kits is 100- to 1,000-fold greater for the detection of *L. pneumophila* serogroup 1 antigen than for other *L. pneumophila* antigens (2, 11, 23). The vast majority of positive tests will be for patients with *L. pneumophila* serogroup 1 infections, and most specimens from patients with infections caused by other *L. pneumophila* serogroups and other *Legionella* species will be negative. *L. pneumophila* serogroup 1 causes more than 90% of cases of community-acquired Legionnaires' disease and is almost without exception the cause of epidemic Legionnaires' disease.

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Two different methods are used in these tests: EIA antigen capture and immunochromatographic assay. In the EIA method, microtiter plates containing antibody to *L. pneumophila* serogroup 1 (or other *Legionella* species and serogroups, depending on the kit) are provided by the manufacturer. Urine is added to the microwells and allowed to incubate for a short period, and the urine is then removed by washing with buffer. If *L. pneumophila* serogroup 1 antigen is present in the urine, it binds to the antibody in the well. The antigen-antibody complex bound to the well is detected by the addition of the same antibody as used to capture the antigen. This detection antibody is supplied already conjugated to an enzyme, such as peroxidase or alkaline phosphatase. The well is again washed to removed unbound detection antibody, and an enzyme substrate and chromogen are added. If the antigen is present in the specimen, the detection antibody bound to it will cleave the enzyme substrate, which, in turn, changes the color of the chromogen. A strong acid is added to stop the reaction, and a significant color change is detected either visually (for one kit) or by using a microplate spectrophotometer (for other kits). The test is interpreted based on the intensity of the color change.

In the immunochromatographic assay, capture rabbit antibody to *L. pneumophila* serogroup 1 antigen is bound in a line to a nitrocellulose filter. On a second line on the filter is bound goat anti-rabbit antibody. Dried encapsulated detection rabbit antibody, conjugated to colored particles, is also applied to an inert fibrous support. Urine is applied to a spot on the fibrous support, and then a citrate-phosphate buffer is applied to the same fibrous support. The urinary antigen and the rehydrated detection antibody migrate to the filter areas containing the capture antibody and anti-rabbit antibody by capillary action. The detection antibody binds to the antigen while both are being eluted onto the filter membrane. The detection antibody-antigen complex binds to the capture antibody in an easily visible colored line, as well as to the anti-rabbit antibody, producing a second line. If the urine contains no antigen, then the detection antibody is eluted past the capture antibody, binding only to the control anti-rabbit antibody, which produces only a single line.

II. SPECIMEN COLLECTION AND TRANSPORT

Observe standard precautions.

Only a few milliliters of freshly collected urine is required for the assay. The urine should be collected in a clean screw-top container and does not require refrigeration during short (hours) transport periods. For longer transport, refrigeration of the specimen at 3 to 5°C may be beneficial, although this has never been studied. Addition of boric acid (1% final concentration) preserves the antigen for longer than a week when stored at room temperature and for up to many years when stored at 3 to 5°C. The use of evacuated tubes containing preservative that are designed for preserving urine for bacterial culture has not been studied for the transport of urine used for the antigen test. Freezing the specimen should be avoided if possible, as freeze-thawing can very rarely cause false-positive results. Long-term (months to years) storage of urine at −70°C has been reported to rarely cause false-negative results (7, 33).

III. MATERIALS

Four manufacturers sell kits for urinary antigen detection, with four kits cleared for marketing by the FDA (Table 11.4–1). The kit manufacturers provide needed controls. Some kits require making buffer solutions from supplied stock solutions, wash bottles, or plate washers. All of the EIA kits, except the Bartels kit, require the use of a microplate spectrophotometer.

ANALYTICAL CONSIDERATIONS

IV. QUALITY ASSURANCE

Include QC information on reagent container and in QC records.

Proper performance of the external negative and positive kit controls gives assurance of proper test performance. The EIA kits must include positive and negative controls with every run. Since the immunochromatographic card assays contain internal controls, per the American College of Physicians standards, external QC testing is required only with receipt of each new kit lot before the lot is put into service. QC failure has never been observed with the Binax NOW kit after many years of testing in my laboratory.

An external QC scheme exists for the urinary antigen test in some countries, including the United States (Medical Laboratory Evaluation, http://www.acponline.org/running_practice/mle/index.html) and European countries (http://www.ewgli.org/eqa.htm). These should be subscribed to if available. If they are not available in a particular country or region, trading blinded specimens with other regional laboratories can help to detect testing problems not detected with the kit control reagents.

Table 11.4–1 Urinary antigen kits

Company	Test kit name	Notes	FDA cleared?
Trinity Biotech, http://www.trinitybiotech.com	Bartels Prima EIA	Formerly known as Itracel/Bartels	Yes
	Trinity Uni-Gold Legionella urinary antigen	Immunochromatographic assay	No
Binax, http://www.binax.com	NOW Legionella	Immunochromatographic assay	Yes
	Binax Legionella urinary antigen EIA	EIA, also sold by Wampole	Yes
Biotest, http://www.biotest.de	Biotest Legionella Urin Antigen EIA	Not available in the United States	No
SAS, http://www.sascientific.com	SAS Legionella	Immunochromatographic assay	Yes

IV. QUALITY ASSURANCE *(continued)*

Since urine antigen testing may be more sensitive than other means of laboratory diagnosis of Legionnaires' disease, it may be difficult to use the results of other laboratory tests for Legionnaires' disease to determine the proper performance of the urine test, especially for a single case of the disease. However, the majority of patients with a positive urine test should have a positive sputum culture or seroconvert to *L. pneumophila* serogroup 1 positivity. Every effort should be made to confirm the laboratory diagnosis of the disease, if at all possible. Also, it should be determined if the patients have an illness compatible with Legionnaires' disease.

V. PROCEDURE

Observe standard precautions.

Use of the test kits is straightforward and should be familiar to any laboratorian experienced with EIAs. As with all immunoassays, particular attention must be paid to equilibration of reagents to room temperature before use, to use of proper incubation temperatures, to adequate plate washing, and to use of a calibrated spectrophotometer. The immunochromatographic card kits are very simple to use, requiring relatively little training or prior experience.

Three modifications of the sample preparation procedure for all of the test kits can enhance test sensitivity and specificity. Test sensitivity can be increased by up to 30% by concentrating urine prior to testing (12, 14). This is done by concentrating the urine 25-fold using an Amicon Minicon B15 or Ultra-4 concentrator (6). Concentrating the urine does not appear to affect test specificity. The other modification is to boil urine for 10 min to inactivate rheumatoid-like factors that can, rarely, cause false-positive tests (about 1 to 5% of positive tests). In addition, clarification of urine by brief low-speed centrifugation can also reduce the frequency of false-positive tests. Whether and when to perform these specimen modifications are dependent somewhat on the test format being used. All urine samples giving a positive result in the assay should be retested after clarification and boiling. Users of an EIA format assay will probably find it most cost-effective and expeditious to boil and clarify all urine specimens prior to testing, as repeating the assay requires several hours and can be quite costly.

Immunochromatographic card assay users will probably find it easiest to boil and clarify only the urine samples giving a positive result, since the vast majority of urine specimens tested will give negative results and because repeating a test is quick and relatively inexpensive. Since concentrating urine samples is time-consuming and expensive, some concentrate only those giving an equivocal result in an EIA (e.g., a ratio between 2 and 3 in the Binax EIA). No one has offered guidelines for when to concentrate urine specimens giving a negative result in the Binax NOW assay. In investigation of an outbreak of Legionnaires' disease, it may be wise to concentrate all urine samples tested.

POSTANALYTICAL CONSIDERATIONS

VI. INTERPRETATION

The urine antigen tests are very specific and sensitive for the detection of *L. pneumophila* serogroup 1. The levels of performance of all kits have been roughly equivalent in most published studies (1, 2, 7, 10–13, 15, 18–21, 23, 35). One retrospective comparison of the Bartels and Binax EIAs showed that the clinical and analytical sensitivity of the Bartels kit was significantly greater than its comparator if nonconcentrated samples were tested and that the tests were roughly equivalent when testing concentrated urine samples (11). An analysis of 178 frozen urine samples collected during the Murcia, Spain, Legionnaires' disease epidemic showed that the Bartels and Biotest assays were 1.8 to 1.9 times more sensitive than the Binax NOW assay when unconcentrated urine samples were tested and roughly equivalent when the urine samples were concentrated (19). The SAS Legionella

VI. INTERPRETATION
(continued)

test was 83% sensitive when incubated for 10 min, versus 91% for the Binax NOW assay, but prolonging incubation led to sensitivities of 97 and 94% for the SAS and Binax tests, respectively (10). Freezing reduced overall sensitivity by 11% in this study and could account for the differences observed between the assays if the antigen concentration in the fresh urine samples was above the limit of detection for all kits.

Test sensitivity is primarily dependent on the serologic identity of the infecting bacterium, nature of exposure, and severity of illness. The urine tests are most sensitive, around 90%, for detection of one monoclonal antibody type of *L. pneumophila* serogroup 1, interchangeably called "Pontiac," "MAB2," or "MAB3/1," and only 50 to 80% sensitive for the detection of other *L. pneumophila* serogroup 1 monoclonal antibody types (24). The MAB2 monoclonal antibody type is responsible for more than 90% of cases of Legionnaires' disease due to *L. pneumophila* serogroup 1 community-acquired pneumonia but a substantially lower fraction of Legionnaires' disease in nosocomial infections and infections in immunocompromised patients (22). Urine test sensitivity is only about 40% for patients with *L. pneumophila* serogroup 1 MAB2-negative infections. Patients with community-acquired or travel-related Legionnaires' disease are much more likely to have a positive urine test than are those who are severely immunosuppressed or with nosocomial disease. This is because the latter groups have higher rates of infections caused by *Legionella* spp. other than *L. pneumophila,* and when the infecting species is *L. pneumophila,* it is less likely to be serogroup 1 MAB2 positive (22). This means that the negative predictive value of the urine test can be quite low for immunocompromised patients or those with nosocomial Legionnaires' disease, and practically that a negative test result by itself does not exclude the disease. Finally, increased disease severity has a dramatic effect on test sensitivity, with the most severely ill patients most likely to have a positive test result. The positivity rate is about 80 to 99% for urine specimens taken from patients with *L. pneumophila* serogroup 1 MAB2 community-acquired pneumonia severe enough to require hospitalization, and close to 100% for those with respiratory failure. In contrast, those with less severe illness have positive urine antigen tests 10 to 50% of the time, with mildly ill ambulatory patients having the lowest test positivity rate (22, 38). Ninety-five to 100% of patients with culture-proven *L. pneumophila* serogroup 1 pneumonia, who are most likely to have more severe disease, have positive urine tests (1). The test has had its greatest utility in the investigation of outbreaks of Legionnaires' disease, where the sensitivity is about 65%, providing rapid diagnoses in far more patients than does culture diagnosis (9, 17, 26, 38). Since about 35% of patients with epidemic Legionnaires' disease have negative urine tests, it is advisable that epidemic-associated cases be treated with a drug active for Legionnaires' disease regardless of the urine test result, even though such patients generally have milder disease and suffer less from a delay in specific therapy (31).

The specificity of the Binax EIA is extraordinarily high, somewhere in the range of 99.9%, if urine boiling and clarification are performed for initially positive samples (P. Edelstein, unpublished data). The specificities of the other test kits appear to be similar (11, 13, 21). One study reported that the specificity of the Binax NOW assay was only 97% but that this could be enhanced by reincubation of the test cards for 60 min, after which very faint lines that were false positive became negative (23). My experience with the NOW assay is that its specificity is $\geq$99%, using the specimen clarification and boiling protocol detailed above. The specificity of the Bartels kit may be slightly lower when the test wells are read visually rather than with a spectrophotometer, leading the authors of one study to recommend that visual reading not be used for this test (21). The very high specificity of these assays means that a patient with a compatible clinical illness has a 75 to 90% chance of truly having Legionnaires' disease.

VI. INTERPRETATION
(continued)

False-negative tests can occur for several different reasons. Patients with severe renal failure may not concentrate the antigen in the urine, and for those patients with oliguric renal failure, simply obtaining an adequate urine specimen can be difficult. Overly dilute urine may mask the presence of a positive test, especially after a fluid load or administration of diuretics. Concentrating urine or testing another urine specimen may alleviate this problem. Patients with Legionnaires' disease caused by a *Legionella* bacterium other than *L. pneumophila* serogroup 1 may have a negative test with any of the kits. It is claimed, but unproven, that the Biotest kit is more sensitive in this circumstance. In fact, the analytical and clinical sensitivities of the Biotest and Binax kits appear to be very similar for non-*L. pneumophila* serogroup 1 *Legionella* antigens (2, 13, 25). Some patients with very early pneumonia may have a negative urine assay, a cause for repeating negative tests for patients with pneumonia present for less than 2 days (4, 28, 29). Finally, improper test performance or inherent limitations of the assay may also cause false-negative tests.

False-positive tests are usually the result of the presence of rheumatoid-like factors in the urine, which can be eliminated by boiling the urine. Freeze-thawing of urine has also been reported to be rarely responsible for false-positive tests (1). A unique case of a false-positive test was reported for a patient with anti-rabbit antibodies, after immunotherapy for kidney transplant rejection with rabbit antibody (8). Although not determined by the authors of the report, boiling of the urine should have eliminated this false-positive reaction. Inherent errors in device performance may also be very rarely responsible for some false-positive tests. Improper test performance or interpretation of results has been reported to be the cause of a pseudo-outbreak of Legionnaires' disease (32). Patients with very severe pneumonia may excrete detectable urinary antigen for months after recovery from Legionnaires' disease (28). Such patients usually have multilobar pneumonia, accompanied by respiratory failure, and have been hospitalized in intensive care units because of the severity of their illness. Such prolonged urinary antigen excretion is not seen in patients with mild pneumonia (Edelstein, unpublished). The concentration of urinary antigen excreted in such severe cases of Legionnaires' disease diminishes fairly quickly, so if testing both acute- and convalescent-phase urine samples using an EIA method, it is easy to distinguish the two. However, for a patient with two episodes of pneumonia spaced weeks to months apart, it may be difficult to determine if a positive urinary antigen test is due to the first, second, or both episodes of pneumonia, especially if the first episode was severe and no semiquantitative antigen assay was performed with the first pneumonia episode.

REFERENCES

1. **Aguero-Rosenfeld, M. E., and P. H. Edelstein.** 1988. Retrospective evaluation of the Du Pont radioimmunoassay kit for detection of *Legionella pneumophila* serogroup 1 antigenuria in humans. *J. Clin. Microbiol.* **26:**1775–1778.
2. **Benson, R. F., P. W. Tang, and B. S. Fields.** 2000. Evaluation of the Binax and Biotest urinary antigen kits for detection of Legionnaires' disease due to multiple serogroups and species of *Legionella. J. Clin. Microbiol.* **38:**2763–2765.
3. **Berdal, B. P., C. E. Farshy, and J. C. Feeley.** 1979. Detection of *Legionella pneumophila* antigen in urine by enzyme-linked immunospecific assay. *J. Clin. Microbiol.* **9:**575–578.
4. **Bernander, S., B. Gastrin, S. Lofgren, and A. M. Olinder-Nielsen.** 1994. Legionella urinary antigen in early disease. *Scand. J. Infect. Dis.* **26:**777–778.
5. **Bibb, W. F., P. M. Arnow, L. Thacker, and R. M. McKinney.** 1984. Detection of soluble *Legionella pneumophila* antigens in serum and urine specimens by enzyme-linked immunosorbent assay with monoclonal and polyclonal antibodies. *J. Clin. Microbiol.* **20:**478–482.
6. **Blanco, S., C. Prat, M. A. Pallares, L. Matas, and J. Dominguez.** 2004. Centrifugal ultrafiltration method for rapid concentration of *Legionella pneumophila* urinary antigen. *J. Clin. Microbiol.* **42:**4410.
7. **Chang, F. Y., J. E. Stout, and V. L. Yu.** 1996. Assessment of enzyme immunoassay versus radioimmunoassay for detection of *Legionella pneumophila* serogroup 1 antigen in frozen urine specimens. *J. Clin. Microbiol.* **34:**2628–2629.

REFERENCES *(continued)*

8. **DeForges, L., P. Legrand, J. Tankovic, C. Brun-Buisson, P. Lang, and C. J. Soussy.** 1999. Case of false-positive results of the urinary antigen test for *Legionella pneumophila*. *Clin. Infect. Dis.* **29:**953–954.
9. **De Schrijver, K., K. Dirven, K. Van Bouwel, L. Mortelmans, P. Van Rossom, T. De Beukelaar, C. Vael, M. Fajo, O. Ronveaux, M. F. Peeters, A. Van der Zee, A. Bergmans, M. Ieven, and H. Goossens.** 2003. An outbreak of Legionnaire's disease among visitors to a fair in Belgium in 1999. *Public Health* **117:**117–124.
10. **Diederen, B. M., and M. F. Peeters.** 2007. Evaluation of the SAS Legionella test, a new immunochromatographic assay for the detection of *Legionella pneumophila* serogroup 1 antigen in urine. *Clin. Microbiol. Infect.* **13:**86–88.
11. **Domínguez, J., N. Gali, S. Blanco, P. Pedroso, C. Prat, L. Matas, and V. Ausina.** 2001. Assessment of a new test to detect Legionella urinary antigen for the diagnosis of Legionnaires' disease. *Diagn. Microbiol. Infect. Dis.* **41:**199–203.
12. **Domínguez, J. A., N. Galí, L. Matas, P. Pedroso, A. Hernandez, E. Padilla, and V. Ausina.** 1999. Evaluation of a rapid immunochromatographic assay for the detection of Legionella antigen in urine samples. *Eur. J. Clin. Microbiol. Infect. Dis.* **18:**896–898.
13. **Domínguez, J. A., N. Galí, P. Pedroso, A. Fargas, E. Padilla, J. M. Manterola, and L. Matas.** 1998. Comparison of the Binax *Legionella* urinary antigen enzyme immunoassay (EIA) with the Biotest *Legionella* urine antigen EIA for detection of *Legionella* antigen in both concentrated and nonconcentrated urine samples. *J. Clin. Microbiol.* **36:**2718–2722.
14. **Domínguez, J. A., J. M. Manterola, R. Blavia, N. Sopena, F. J. Belda, E. Padilla, M. Gimenez, M. Sabria, J. Morera, and V. Ausina.** 1996. Detection of *Legionella pneumophila* serogroup 1 antigen in nonconcentrated urine and urine concentrated by selective ultrafiltration. *J. Clin. Microbiol.* **34:**2334–2336.
15. **Domínguez, J. A., L. Matas, J. M. Manterola, R. Blavia, N. Sopena, F. J. Belda, E. Padilla, M. Gimenez, M. Sabria, J. Morera, and V. Ausina.** 1997. Comparison of radioimmunoassay and enzyme immunoassay kits for detection of *Legionella pneumophila* serogroup 1 antigen in both concentrated and nonconcentrated urine samples. *J. Clin. Microbiol.* **35:**1627–1629.
16. **Fehrenbach, F. J., I. Horbach, B. Ruf, D. Shurmann, and H. D. Pohle.** 1986. Rapid detection of Legionella antigen in tissues and body fluids. *Isr. J. Med. Sci.* **22:**706–710.
17. **Fiore, A. E., J. P. Nuorti, O. S. Levine, A. Marx, A. C. Weltman, S. Yeager, R. F. Benson, J. Pruckler, P. H. Edelstein, P. Greer, S. R. Zaki, B. S. Fields, and J. C. Butler.** 1998. Epidemic Legionnaires' disease two decades later: old sources, new diagnostic methods. *Clin. Infect. Dis.* **26:**426–433.
18. **Franzin, L., and D. Cabodi.** 2000. Comparative evaluation of two commercially available antigen enzyme immunoassays (EIA) for the detection of Legionella pneumophila urinary antigen in frozen non-concentrated urine samples. *New Microbiol.* **23:**383–389.
19. **Guerrero, C., C. M. Toldos, G. Yagüe, C. Ramírez, T. Rodríguez, and M. Segovia.** 2004. Comparison of diagnostic sensitivities of three assays (Bartels enzyme immunoassay [EIA], Biotest EIA, and Binax NOW immunochromatographic test) for detection of *Legionella pneumophila* serogroup 1 antigen in urine. *J. Clin. Microbiol.* **42:**467–468.
20. **Hackman, B. A., J. F. Plouffe, R. F. Benson, B. S. Fields, and R. F. Breiman.** 1996. Comparison of Binax *Legionella* urinary antigen EIA kit with Binax Ria urinary antigen kit for detection of *Legionella pneumophila* serogroup 1 antigen. *J. Clin. Microbiol.* **34:**1579–1580.
21. **Harrison, T. G., and N. Doshi.** 2001. Evaluation of the Bartels *Legionella* urinary antigen enzyme immunoassay. *Eur. J. Clin. Microbiol. Infect. Dis.* **20:**738–740.
22. **Helbig, J. H., S. A. Uldum, S. Bernander, P. C. Luck, G. Wewalka, B. Abraham, V. Gaia, and T. G. Harrison.** 2003. Clinical utility of urinary antigen detection for diagnosis of community-acquired, travel-associated, and nosocomial Legionnaires' disease. *J. Clin. Microbiol.* **41:**838–840.
23. **Helbig, J. H., S. A. Uldum, P. C. Luck, and T. G. Harrison.** 2001. Detection of Legionella pneumophila antigen in urine samples by the Binax NOW immunochromatographic assay and comparison with both Binax Legionella Urinary Enzyme Immunoassay (EIA) and Biotest Legionella Urine Antigen EIA. *J. Med. Microbiol.* **50:**509–516.
24. **Helbig, J. H., S. A. Uldum, P. C. Lück, and T. G. Harrison.** 2003. Detection of *Legionella pneumophila* antigen in urine samples: recognition of serogroups and monoclonal serogroups, p. 204–206. *In* R. Marre, Y. Abu Kwaik, C. Bartlett, N. P. Cianciotto, B. S. Fields, M. Frosch, J. Hacker, and P. C. Lück (ed.), Legionella. ASM Press, Washington, DC.
25. **Horn, J.** 2001. Binax and Biotest *Legionella* urinary antigen kits. *J. Clin. Microbiol.* **39:**1682. (Letter.)
26. **Jernigan, D. B., J. Hofmann, M. S. Cetron, C. A. Genese, J. P. Nuorti, B. S. Fields, R. F. Benson, R. J. Carter, P. H. Edelstein, I. C. Guerrero, S. M. Paul, H. B. Lipman, and R. Breiman.** 1996. Outbreak of Legionnaires' disease among cruise ship passengers exposed to a contaminated whirlpool spa. *Lancet* **347:**494–499.
27. **Kohler, R., and L. J. Wheat.** 1982. Rapid diagnosis of pneumonia due to Legionella pneumophila serogroup 1. *J. Infect. Dis.* **146:**444.

REFERENCES *(continued)*

28. **Kohler, R. B., W. C. Winn, Jr., and L. J. Wheat.** 1984. Onset and duration of urinary antigen excretion in Legionnaires' disease. *J. Clin. Microbiol.* **20:**605–607.
29. **Kohler, R. B., S. E. Zimmerman, E. Wilson, S. D. Allen, P. H. Edelstein, L. J. Wheat, and A. White.** 1981. Rapid radioimmunoassay diagnosis of Legionnaires' disease: detection and partial characterization of urinary antigen. *Ann. Intern. Med.* **94:**601–605.
30. **Lebrun, L., C. Tram, F. Lapierre, L. Grangeot-Keros, and J. Pillot.** 1983. Detection of Legionella pneumophila antigen by ELISA in urine or [sic] experimentally infected guinea pigs. *Ann. Microbiol. (Paris)* **134A:**155–161.
31. **Lettinga, K. D., A. Verbon, G. J. Weverling, J. F. Schellekens, J. W. den Boer, E. P. Yzerman, J. Prins, W. G. Boersma, R. J. van Ketel, J. M. Prins, and P. Speelman.** 2002. Legionnaires' disease at a Dutch flower show: prognostic factors and impact of therapy. *Emerg. Infect. Dis.* **8:**1448–1454.
32. **Regan, C. M., Q. Syed, K. Mutton, and B. Wiratunga.** 2000. A pseudo community outbreak of Legionnaires' disease on Merseyside: implications for investigation of suspected clusters. *J. Epidemiol. Community Health* **54:**766–769.
33. **Rigby, E. W., J. F. Plouffe, B. A. Hackman, D. S. Hill, R. F. Benson, and R. F. Breiman.** 1997. Stability of Legionella urinary antigens over time. *Diagn. Microbiol. Infect. Dis.* **28:**1–3.
34. **Sathapatayavongs, B., R. B. Kohler, L. J. Wheat, A. White, and W. C. Winn, Jr.** 1983. Rapid diagnosis of Legionnaires' disease by latex agglutination. *Am. Rev. Respir. Dis.* **127:**559–562.
35. **Sathapatayavongs, B., R. B. Kohler, L. J. Wheat, A. White, W. C. Winn, Jr., J. C. Girod, and P. H. Edelstein.** 1982. Rapid diagnosis of Legionnaires' disease by urinary antigen detection. Comparison of ELISA and radioimmunoassay. *Am. J. Med.* **72:**576–582.
36. **Tang, P. W., D. de Savigny, and S. Toma.** 1982. Detection of *Legionella* antigenuria by reverse passive agglutination. *J. Clin. Microbiol.* **15:**998–1000.
37. **Tilton, R. C.** 1979. Legionnaires' disease antigen detected by enzyme-linked immunosorbent assay. *Ann. Intern. Med.* **90:**697–698.
38. **Wever, P. C., E. P. Yzerman, E. J. Kuijper, P. Speelman, and J. Dankert.** 2000. Rapid diagnosis of Legionnaires' disease using an immunochromatographic assay for *Legionella pneumophila* serogroup 1 antigen in urine during an outbreak in The Netherlands. *J. Clin. Microbiol.* **38:**2738–2739.
39. **Williams, A., and M. S. Lever.** 1995. Characterisation of Legionella pneumophila antigen in urine of guinea pigs and humans with Legionnaires' disease. *J. Infect.* **30:**13–16.

11.5.1 Introduction

Syphilis is a sexually transmitted disease that is caused by the organism *Treponema pallidum* subsp. *pallidum.* The disease goes through several stages if untreated (2, 15). The primary chancre occurs at the site of inoculation approximately 3 to 4 weeks (range, 10 to 90 days) after the initial exposure. Treponemes may be visualized in lesion exudates using either dark-field microscopy or direct fluorescent antibody for *T. pallidum* (DFA-TP). About 7 to 10 days after the chancre appears, antibodies to *T. pallidum* are detectable using the routine serologic tests for syphilis. The chancre spontaneously heals after 1 to 4 weeks. The symptoms of secondary syphilis appear about 6 weeks later (range, 2 weeks to 6 months). All serologic tests are generally reactive during secondary syphilis. The most common symptoms are a generalized or localized maculopapular rash that occurs on the palms of the hands and soles of the feet (palmar plantar rash) or on the trunk of the body, mucosal membrane lesions, generalized lymphadenopathy, and condylomata lata. These symptoms will resolve without treatment. The patient then enters a period of latency when there are no symptoms. In about 20 to 25% of individuals, secondary symptoms may reoccur during the early part of this latent period. In early latency (<1 year) the results for the serologic tests for syphilis are reactive. As patients progress into late latency, the nontreponemal tests may become nonreactive, but the treponemal tests will remain reactive. About 65% of persons with untreated syphilis will remain in this stage for life (15). In the remaining 35%, late manifestations of syphilis will occur.

Late syphilis can be divided into benign syphilis, cardiovascular syphilis, and neurosyphilis. Benign syphilis and cardiovascular syphilis are rarely seen since most people receive adequate antimicrobial therapy for some other reason, such as an upper respiratory infection. Neurosyphilis, which is the most common manifestation of late syphilis, can occur during any stage of syphilis, including as a late manifestation of the disease, and is characterized by meningitis, peripheral neuropathy, meningovascular brain lesions, and/or psychiatric illness. Neurosyphilis may be asymptomatic, with the only indication being elevated levels of WBCs (>5 WBCs per ml) and protein (>50 mg per dl) in the CSF. The Veneral Disease Research Laboratory (VDRL) CSF test may or may not be reactive for persons with asymptomatic neurosyphilis, but the fluorescent treponemal antibody absorption (FTA-ABS) test with CSF should be nonreactive for those who do not have syphilis (5). Persons coinfected with *T. pallidum* and human immunodeficiency virus (HIV) should be evaluated for neurosyphilis.

Syphilis serologies can be complicated by a positive HIV status. For some patients who are coinfected, syphilis tests may be nonreactive (2), while for others the nontreponemal titers may be higher (12). For patients with HIV infection only, nontreponemal tests may be falsely reactive (6), perhaps due to polyclonal activation of B lymphocytes. This may also be the cause of higher nontreponemal test titers in HIV- and syphilis-coinfected individuals.

Syphilis is one of the few diseases that can be transmitted from a mother to her unborn child. Rates of transmission to the fetus are dependent on the stage of syphilis in the mother and when in the pregnancy the mother became infected with *T. pallidum* (14). Syphilis contracted in utero may result in fetal death, low birth weight, premature delivery, or various symptoms at birth, or it may be asymptomatic. Diagnosis is usually a presumptive one based on the mother's serology and lack of history of adequate treatment. If immunoglobulin M (IgM) antibodies against *T. pallidum* can be detected in the neonate's serum, a stronger case can be made for a diagnosis of congenital syphilis since IgM antibodies do not cross the placenta as IgG antibodies do. Treponemes are frequently demonstrable using Steiner stain, DFA-TP, or immunohistochemical staining of the placenta or umbilical cord of neonates with congenital syphilis. The best prevention for congenital syphilis is identification and treatment of pregnant women who have syphilis.

In the United States, several states (Alabama, Alaska, California, Connecticut, Georgia, New Jersey, Oklahoma, Pennsylvania, West Virginia) and the District of Columbia still require premarital testing for syphilis (4). Screening for syphilis is carried out in sexually transmitted disease clinics, detention centers, and outreach programs. All blood donations are screened for syphilis. Traditionally, the serologic diagnosis of syphilis has been made using a nontreponemal test to screen for the disease and a treponemal test to confirm the results of the nontreponemal test. The rationale for this algorithm is that confirmation of original test results should be done using the more specific test. The most commonly used nontreponemal tests are the rapid plasma reagin (RPR) test or the VDRL test to screen and the *T. pallidum* particle agglutination test (TP-PA) or FTA-ABS to confirm. Outside the United States, screening is frequently done using a treponemal test such as the *T. pallidum* hemagglutination assay followed by a nontreponemal test such as the RPR or VDRL test to distinguish active syphilis from latent or late syphilis. This algorithm is being used more in the

Table 11.5.1–1 List of suppliers for syphilis diagnostic tests

Supplier	Product(s)
Ampcor Technologies, Inc., Denville, NJ http://www.ampcordx.com	RPR card test kits and control sera
Arlington Scientific Inc. (ASI), Springville, UT http://www.ArlingtonScientific.com	RPR card test reagents
Avanti Polar Lipids, Inc., Alabaster, AL http://www.avantilipids.com	VDRL test kits
Baxter Healthcare Corp., Miami, FL http://www.baxterhealthcare.com	RPR card test reagents, FITC-labeled rabbit or human anti-*T. pallidum* globulin
Becton Dickinson Microbiology Systems, Cockeysville, MD http://www.bd.com	RPR card test reagents, FITC-labeled rabbit or human anti-*T. pallidum* globulin, FTA-ABS antigen
Biokit, S.A., Barcelona, Spain http://www.biokit.com	TPHA kit
Biologic Products Section, CDC, Atlanta, GA	FITC-labeled monoclonal antibody to *T. pallidum*
Bio-Rad Laboratories, Hercules, CA http://www.bio-rad.com	Syphilis-G EIA kits
Cenogenics Corp., Morganville, NJ http://www.cenogenics.com	VDRL control sera, VDRL test kits
DAYA Medicals, Inc., Boca Raton, FL http://www.dayamed.com	TRUST kit
Diasorin, Inc., Stillwater, MN http://www.diasorin.com	LIAISON Treponema Screen Test
Fisher, Pittsburgh, PA http://www.fishersci.com	RPR card test kit and components, VDRL test control sera
Fujirebio America, Fairfield, NJ http://www.fujirebioamerica.com	TP-PA kit
Globalemed, Alexandria, VA http://www.globalemed.com	Smart Check Syphilis Test
Hardy Diagnostics, Santa Maria, CA http://www.hardydiagnostics.com	Distributor for most reagents
Hemagen Diagnostics, Inc., Columbia, MD http://www.hemagen.com	FTA-ABS kits, antigen slides, sorbent
Immunostics, Inc., Ocean, NJ http://www.immunostics.com	RPR kit
Lab21 Healthcare, Cambridge, United Kingdom http://lab21.com	RPR kit
Lee Laboratories, Grayson, GA http://www.leelabs.com	VDRL test kit, unheated-serum reagin antigen, RPR card test kit, FTA-ABS sorbent and antigen

(continued)

Table 11.5.1–1 *(continued)*

Supplier	Product(s)
New Horizons Diagnostics Corp., Columbia, MD http://www.nhdiag.com	TRUST kits
Phoenix Bio-Tech Corp., Mississauga, Ontario, Canada http://www.phoenixbiotech.com	Trep-Chek EIA (IgG and IgM) kit
Pulse Scientific Inc., Burlington, Ontario, Canada http://www.worldexport.com/pulse/	TRUST kit
Remel Microbiology Products, Lenexa, KS http://www.remel.com	RPR card test kit
SciMedx Corp., Denville, NJ http://www.scimedx.com	FTA-ABS and FTA-ABS DS kits and components
Trinity Biotech, Bray, Ireland http://www.trinitybiotech.com	Syphilis-G EIA kits and FTA-ABS kit
ViroStat, Inc., Portland, ME http://www.virostat-inc.com	Polyclonal anti-*T. pallidum* antibody and conjugate, monoclonal anti-*T. pallidum* antibody
Wampole Laboratories, Cranbury, NJ http://www.wampolelabs.com	Syphilis-G EIA kits, FTA-ABS kits, RPR card test components and kits
Zeus Scientific, Inc., Raritan, NJ http://www.zeusscientific.com	FTA-ABS kit

United States, especially in blood banks where the screening is usually done with either a modified hemagglutination test or an EIA. Unfortunately, by screening with a treponemal test, treated cases of syphilis will also be detected, since about 85% of persons with treated syphilis retain treponemal test reactivity for life. The 15% that lose treponemal test reactivity are usually those who had adequately treated primary syphilis and no history of syphilis (1, 13).

Another concern is the lack of a standard approach to follow-up testing of positive treponemal IgG EIA/negative nontreponemal results (10). This result could also be due to a false-positive treponemal test or seroreversion of nontreponemal antibodies with long-standing infection. In this scenario, treatment history needs to be elicited from the patient, and follow-up testing with a different treponemal test has been recommended (3) to guide appropriate management.

Early syphilis is best diagnosed with an antigen detection test to demonstrate the presence of treponemes in the primary chancre (ulcer). The most frequently used method is dark-field microscopy to detect living, motile spirochetes in lesion exudates (7, 8). This does require a light microscope equipped with a dark-field condenser. Smears also must be read within minutes of collection to ensure the motility of the spirochetes in the sample (8, 9). Alternatively, one can use the DFA-TP technique on an air-dried smear (8). This does not require living, motile spirochetes but does require a monoclonal or polyclonal antibody against *T. pallidum* conjugated to fluorescein isothiocyanate (FITC) and a properly working fluorescent microscope. A modification of the DFA-TP can also be used to look for *T. pallidum* in tissues (8). This modification is especially useful in testing for congenital syphilis, with the umbilical cord being one of the easiest tissue samples to obtain.

PCR-based methods have been reported for detection of *T. pallidum* in primary and secondary lesions as well as other body fluids. However, this technology is not readily available for diagnostic purposes.

There are two types of serologic tests for syphilis: the nontreponemal tests, which use a cardiolipin-based antigen, and the treponemal tests, which use either disrupted *T. pallidum* or cloned antigens. Nontreponemal tests, which detect antibodies produced against cardiolipin in response to lipids in the outer membrane of the syphilis spirochete or against host cardiolipin, are either microscopic or macroscopic flocculation tests. The microscopic tests, the VDRL test and unheated-serum reagin (USR) test, require a microscope to read the test results. The USR test uses a stabilized VDRL antigen, which allows unheated serum to be used in the test. The macroscopic tests, the RPR test and toluidine red unheated-serum test (TRUST), also utilize stabilized VDRL antigen as well as colored particles which get caught in the flocculation matrix and make the reaction visible to the naked eye. The RPR

test uses sized charcoal particles and the TRUST uses an azo red paint pigment. There was an EIA based on VDRL antigen (Reagin II test), but manufacture of that test has recently been discontinued. It is their reactivity to the host cardiolipin, or as one researcher terms it, "Nontreponemal-infection associated phospholipid antibodies" (16), that gives the nontreponemal tests their nonspecificity. These antibodies may be produced in response to damage by the spirochete, autoimmune disease, acute viral infection, recent immunizations, or mechanical damage to the cardiovascular system as occurs in intravenous drug use.

Four treponemal tests are in common use: the FTA-ABS test, FTA-ABS double staining test (FTA-ABS DS), Serodia TP-PA, and EIA. All are available commercially (Table 11.5.1–1). The TP-PA is probably the most commonly used test in clinical laboratories in the United States. The EIA format tests are gaining in popularity, especially in situations where large numbers of tests are performed. The FTA-ABS, FTA-ABS DS, and TP-PA are used only for confirmation of nontreponemal test results. The EIA (IgG only) can be used for confirmation of nontreponemal test results or as a screening test. The IgM EIA is used primarily in diagnosing congenital syphilis. There is also a Western blot test commercially available outside the United States. It can be used to detect either IgG or IgM antibodies. A microhemagglutination test for treponemal antibodies (MHA-TP) had been available in the United States until the end of 1998, when it was replaced by the TP-PA. The TP-PA has been shown to be slightly more sensitive in detecting primary syphilis than the MHA-TP (11). There is a *T. pallidum* hemagglutination assay available outside the United States.

Table 11.5.1–1 lists the manufacturers of various reagents for syphilis diagnosis and their websites. The tests that are discussed below are the DFA-TP, RPR test, and TP-PA. The RPR test and the TP-PA are the most commonly used tests based on the number of laboratories reporting proficiency testing results. The procedures described here are not detailed; readers should refer to *A Manual of Tests for Syphilis* (8) and the manufacturers' product inserts for detailed instructions. All of the nontreponemal tests have similar procedures and measure the same type of antibody. However, because they do have slightly different sensitivities, the tests are not interchangeable and the same test as was used for the baseline titer should be used to monitor treatment efficacy. Regardless of the test used, it is the responsibility of the end user to verify that the test performance meets the laboratory's requirements.

◩ **NOTE:** Because of variations in shipping and storage conditions, the results obtained by the manufacturer or by CDC in the premarket evaluation should not be regarded as the definitive QC results. The end user must determine acceptability of the reagents.

REFERENCES

1. **Augenbraun, M., R. Rolfs, R. Johnson, R. Joesoef, V. Pope, and the Syphilis and HIV Study Group.** 1998. Treponemal specific tests for the serodiagnosis of syphilis. *Sex. Transm. Dis.* **25:**549–552.
2. **Centers for Disease Control.** 1988. Recommendation for diagnosing and treating syphilis in HIV-infected patients. *MMWR Morb. Mortal. Wkly. Rep.* **37:**600–602, 607–608.
3. **Centers for Disease Control and Prevention.** 2006. Sexually transmitted diseases treatment guidelines. *MMWR Morb. Mortal. Wkly. Rep.* **55**(RR-11)**:**1–94.
4. **Hough, M. K., and J. A. Poppe.** 1988. Sexually transmitted diseases; a policymaker's guide and summary of state laws. National Conference of State Legislatures, Denver, CO.
5. **Jaffe, H. W., S. A. Larsen, M. Peters, D. F. Jove, B. Lopez, and A. L. Schroeter.** 1978. Tests for treponemal antibody in CSF. *Arch. Intern. Med.* **138:**252–255.
6. **Joyanes, P., M. V. Borobio, J. M. Arquez, and E. J. Perea.** 1998. The association of false-positive rapid plasma reagin results and HIV infection. *Sex. Transm. Dis.* **25:**569–571.
7. **Larsen, S. A., B. E. McGrew, E. F. Hunter, and E. T. Creighton.** 1984. Syphilis serology and dark-field microscopy, p. 875–888. In K. K. Holmes, P.-A. Mårdh, P. F. Sparling, and P. J. Wiesner (ed.), *Sexually Transmitted Diseases.* McGraw-Hill Book Co., New York, NY.
8. **Larsen, S. A., V. Pope, R. E. Johnson, and E. J. Kennedy, Jr. (ed.).** 1998. *A Manual of Tests for Syphilis*, 9th ed. American Public Health Association, Washington, DC.
9. **Larsen, S. A., B. M. Steiner, and A. H. Rudolph.** 1998. Laboratory diagnosis and interpretation of tests for syphilis. *Clin. Microbiol. Rev.* **8:**1–21.
10. **Peterman, T., J. Schillinger, S. Blank, S. Berman, R. Ballard, D. Cox, R. Johnson, S. Hariri, and N. Selvam.** 2008. Syphilis testing algorithms using treponemal tests for initial screening—four laboratories, New York City, 2005–2006. *MMWR Morb. Mortal. Wkly. Rep.* **57:**872–875.
11. **Pope, V., M. B. Fears, W. E. Morrill, A. Castro, and S. E. Kikkert.** 2000. Comparison of the Serodia *Treponema pallidum* particle agglutination, Captia Syphilis-G, and SpiroTek Reagin II tests with standard test techniques for diagnosis of syphilis. *J. Clin. Microbiol.* **38:**2543–2545.
12. **Rolfs, R. T., M. R. Joesoef, E. F. Hendershot, A. M. Rompalo, M. H. Augenbraun, M. Chiu, G. Bolan, S. C. Johnson, P. French, E. Steen, J. D. Radolf, and S. Larsen for the Syphilis and HIV Study Group.** 1997. A randomized trial of enhanced therapy for early syphilis in patients with and without human immunodeficiency virus infection. *N. Engl. J. Med.* **337:**307–314.
13. **Romanowski, B., R. Sutherland, G. H. Fick, D. Mooney, and E. G. Love.** 1991. Serologic response to treatment of infectious syphilis. *Ann. Intern. Med.* **114:**1005–1009.
14. **Singh, A. E., and B. Romanowski.** 1999. Syphilis: review with emphasis on clinical, epidemiologic, and some biologic features. *Clin. Microbiol. Rev.* **12:**187–209.
15. **Venereal Disease Program.** 1968. Syphilis: a synopsis. U.S. Department of Health, Education and Welfare. National Communicable Disease Center, Atlanta, GA.
16. **Wicher, K., H. W. Horowitz, and V. Wicher.** 1999. Laboratory methods of diagnosis of syphilis for the beginning of the third millennium. *Microbes Infect.* **1:**1035–1049.

11.5.2 Direct Fluorescent-Antibody Test for *Treponema pallidum*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The direct fluorescent-antibody test for *Treponema pallidum* (DFA-TP) (1) can be used in conjunction with dark-field microscopy or in place of it. The test utilizes either monoclonal antibody or polyclonal antibody conjugated to fluorescein isothiocyanate (FITC). The monoclonal conjugate is specific for the pathogenic treponemes and is the conjugate of choice for ulcers that occur in the oral cavity or rectal area. Polyclonal antibodies can be absorbed with nonpathogenic treponemes to remove cross-reacting antibodies. Absorbed polyclonal conjugates and the monoclonal conjugate can be used for the same specimens. Unabsorbed polyclonal conjugate can be used on genital ulcer samples with a fair degree of confidence that the organism identified is a pathogenic treponeme. Because the unabsorbed polyclonal conjugate cross-reacts with nonpathogenic treponemes, it cannot be used on oral or rectal lesions where other spirochetes are part of the normal microbiota. The FITC-labeled antibody reacts with *T. pallidum* antigen present in the samples. Examination of the smear with a fluorescence microscope reveals fluorescing treponemes with typical morphology.

II. SPECIMEN COLLECTION

Observe standard precautions.

Body fluids, lesion or ulcer exudates, tissue impressions, or suspensions of tissue which have been macerated using either scissors to finely mince or a tissue grinder are appropriate samples for DFA-TP. Samples are collected in the same manner as for dark-field microscopy (1, 2). Standard precautions should be observed when collecting samples and preparing slides. Smears are air dried and may be shipped at ambient temperature to an off-site laboratory for staining. However, slides that have been stored frozen should be shipped on dry ice.

III. MATERIALS

A commercial kit is not available, but the conjugates are available commercially (Table 11.5.1–1). In addition, positive and negative control slides are required. Positive control slides can be made from fluorescent treponemal antibody absorption test (FTA-ABS) antigen or impression smears made using infected rabbit testicular tissue (1). Negative slides can be made using smears of washed nonpathogenic Reiter cultures (1). A fluorescent microscope in good working order with the proper filters for FITC conjugates is also required.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. The conjugate must be checked for acceptable performance. The nonreactive control should be negative at 2 doubling dilutions below the working dilution specified by the manufacturer. Treponemes in the reactive control should stain with 3 to 4+ intensity when used at the working dilution recommended by the manufacturer.

IV. QUALITY CONTROL *(continued)*

B. The pH of the mounting medium and phosphate-buffered saline (PBS) must be within the acceptable ranges.

C. Positive and negative controls should be included daily.

V. PROCEDURE

Observe standard precautions.

Prepare smears and allow them to air dry. If the smears will be stained with polyclonal antibody, they should be fixed in acetone for 10 min and air dried prior to staining. If the monoclonal antibody conjugate is going to be used, the slides need to be fixed with 1 or 2 drops of 100% methanol for 10 s, drained, and allowed to air dry. Cover each smear with 30 μl of properly diluted conjugate and incubate in a moist chamber at 35 to 37°C for 30 min. Rinse the slides in PBS and then place on a staining rack, cover with PBS, and allow to sit for 10 min. Rinse off the PBS with distilled water. Blot the areas outside the smear with bibulous paper to remove any residual water. Do not allow the smears to completely dry. Add a small amount of mounting fluid and place a coverslip over the smear. Scan the controls and the test smear using the 45× or 63× objective and the proper filters for FITC conjugates (BG12 or KP490 with K510 or K530 for transmitted light; BG38, K480, KP490, TK510, or K515 for incident light) on the fluorescent microscope. Confirm any treponemes by examining the smear with the 100× oil immersion lens. Ensure that the control slides are satisfactory before interpreting the test results.

POSTANALYTICAL CONSIDERATIONS

VI. INTERPRETATION

Observation of treponemes showing 2+ or greater fluorescence and morphology typical of pathogenic treponemes (2) is considered a positive test result. The absence of treponemes is considered a negative test result; however, a negative result does not rule out syphilis.

VII. LIMITATIONS OF THE PROCEDURE

A. If polyclonal antibody is used and it is not preabsorbed with nonpathogenic treponemes, the test cannot be performed on oral or rectal lesions or ulcers because of other treponemes that are part of the normal microbiota.

B. If there is precipitate in the conjugate, artifacts may interfere with reading the results.

C. If the pH of the mounting medium or PBS is incorrect, fluorescence may be quenched, leading to false-negative results.

D. If smears are too thick, material may wash off the slide, treponemes may not stain, or there may be too much background fluorescence to see treponemes.

E. Care must be taken to ensure that treponemes from the positive control slide do not contaminate patient samples.

F. If the lesion is healing, the sample was collected incorrectly, or the sample contains very few treponemes, one may not be able to demonstrate the presence of treponemes.

G. The test cannot distinguish the treponemes that cause yaws and pinta from those that cause syphilis.

REFERENCES

1. **Larsen, S. A., V. Pope, R. E. Johnson, and E. J. Kennedy, Jr. (ed.).** 1998. *A Manual of Tests for Syphilis*, 9th ed. American Public Health Association, Washington, DC.
2. **Larsen, S. A., B. M. Steiner, and A. H. Rudolph.** 1995. Laboratory diagnosis and interpretation of tests for syphilis. *Clin. Microbiol. Rev.* **8:**1–21.

11.5.3 Rapid Plasma Reagin Test

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The rapid plasma reagin (RPR) 18-mm circle card test is a macroscopic nontreponemal test intended as a screening test for syphilis (4). The antigen is Venereal Disease Research Laboratory (VDRL) antigen that has been stabilized with EDTA and has had choline chloride added to eliminate the need for heat inactivation of the serum sample. Sized charcoal particles have been added to make the reaction visible. Charcoal particles get caught in the lattice formed by the stabilized VDRL antigen and antibodies to cardiolipin that are present in the blood of someone who has syphilis. In the test, unheated serum or plasma is spread within the confines of a circle on a plastic-coated card. A drop of antigen is added, and the card is placed on a rotator at a specified speed for a specified length of time. If antibodies are present, the flocculation reaction takes place. The RPR test measures immunoglobulin G (IgG) and IgM antibodies. These antibodies may be in response to lipoproteins or cardiolipin of the treponeme itself or to host lipoidal antigens exposed by damage from the infection with *Treponema pallidum*. Antilipoidal antibodies may also be produced in response to tissue damage from other causes such as autoimmune disease or intravenous drug use. A fourfold (2 doubling dilutions) decline in titer following syphilis treatment indicates that treatment has been successful; a fourfold increase indicates either treatment failure or reinfection. Failure of the titer to decline following treatment, in the absence of a rise in titer, does not necessarily indicate a treatment failure and must be considered along with the clinical presentation of the patient.

II. SPECIMEN COLLECTION

Observe standard precautions.

When handling serum or plasma, standard precautions must be observed (3). It is recommended that persons who handle blood and body fluids be vaccinated against hepatitis B virus (2).

A. Serum is the preferred sample, especially if testing is going to be delayed for more than 48 h. However, as the name of the test implies, plasma (with EDTA anticoagulant) is a suitable sample if testing is to take place soon after the sample is collected.

B. Blood samples should be collected and processed in such a way as to minimize hemolysis or contamination. Samples that are too hemolyzed, extremely lipemic, or contaminated or that contain particulate matter should not be used since these may interfere with the test.

III. MATERIALS

RPR card test reagents, cards, and control sera are all available commercially (Table 11.5.1–1). In addition to the reagents in the kits, persons performing the test will also need the following reagents and supplies.

A. 0.9% Sodium chloride (0.9 g of NaCl added to 100 ml of distilled water)

B. Diluent
A 1:50 dilution of serum nonreactive for syphilis prepared in 0.9% NaCl. This diluent should be used in the quantitative procedure for dilutions of 1:32 and higher. A calibrated dropper that delivers 50 µl should be used.

C. Safety pipetting device with disposable tip that delivers 50 µl

III. MATERIALS *(continued)*

D. Rotator, either fixed speed or adjustable to 100 ± 2 rpm, circumscribing a 3/4-in.-diameter circle
E. Discard containers and disinfectants
F. Disposable latex or nitrile gloves, safety glasses, and protective clothing

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

It is the responsibility of the laboratory to adhere to Clinical Laboratory Improvement Amendments of 1988 regulations and guidelines. This includes annual calibration of pipettors; daily monitoring of room, water bath, and refrigerator temperatures; calibration of any thermometers used for water baths, etc.; participation and satisfactory results in an approved proficiency testing program; and running and recording controls for new lots of reagents and for daily testing. If the daily controls do not produce expected results, patient results are not valid and cannot be reported.

For lot-to-lot QC, 20 sera of graded reactivity, including minimally reactive, should be tested over a 2-day period using the kit currently in use along with the new kit. For quantitative testing, 6 serum samples of various titers should be tested in parallel on two consecutive work days. For a detailed description of the procedure, refer to *A Manual of Tests for Syphilis* (4).

The revolutions per minute of the rotator, room temperature, and reactivity of the controls should be determined each day that testing is done. In addition, the accuracy of the timer used, whether on the rotator or as a separate timer, should be validated. The needle should be checked each time a new needle is used, when the needle has been dropped or wiped, or when the control pattern is not met.

If the temperature of the room is too warm or cool, or if the reagents and patient samples have not reached room temperature, the accuracy of the test will be compromised (1). Likewise, if the samples or reagents are contaminated, the results may be inaccurate.

V. PROCEDURE

For complete instructions on the test procedure, refer to the manufacturer's product insert or to *A Manual of Tests for Syphilis* (4).

Observe standard precautions.

A. Qualitative test

1. Allow all reagents, controls, and samples to reach room temperature.
2. Place 50 µl of control or patient sample (serum or plasma) onto appropriately labeled circle of the plastic test card. Using the flat end of the Dispenstir or a toothpick, spread the sample to fill the entire circle, taking care not to go outside the circle.
3. Gently shake the antigen-dispensing bottle to resuspend the particles.
4. Holding the dispensing bottle and needle upside down in a vertical position, dispense several drops to clear the needle of air. Add exactly 1 free-falling drop to each circle containing either controls or patient sample. Do not mix.
5. Place the card on the rotator and cover with a humidifying cover. Rotate the card for 8 min at 100 ± 2 rpm.
6. Remove the card from the rotator. Briefly rotate and tilt the card by hand to facilitate differentiation between nonreactive, negative rough, and minimally reactive results.
7. Perform the quantitative test on any samples that had any clumping or roughness.

B. Quantitative test

1. Dilute to endpoint all samples with rough nonreactive and reactive results in the qualitative test.

V. PROCEDURE *(continued)*

2. Place 50 µl of 0.9% saline in circles 2 through 5 in one row of an RPR test card. Do not spread the saline.
3. Using a safety pipetting device, place 50 µl of patient sample (serum or plasma) in circle 1 and 50 µl in circle 2. Mix the saline and sample in circle 2 by drawing the mixture up and down eight times in the safety pipette. Avoid forming bubbles.
4. Transfer 50 µl from circle 2 to circle 3 and mix. Continue making dilutions through circle 5. Discard 50 µl from circle 5.
5. Using the flat end of the Dispenstir or a toothpick, and starting with circle 5, the highest dilution (1:16), spread the serum dilution to fill the circle. Using the same Dispenstir or toothpick, repeat for circles 4 (1:8), 3 (1:4), 2 (1:2), and 1 (undiluted).
6. Gently shake the dispensing bottle to resuspend the antigen and charcoal particles. Hold the dispensing bottle in an inverted vertical position and dispense 1 or 2 drops to clear the needle of air. Add exactly 1 free-falling drop to each circle. Do not mix.
7. Place the card on the rotator. Cover with a humidifying cover and rotate at 100 ± 2 rpm for 8 min.
8. Remove the card from the rotator and gently rotate and tilt the card by hand to and fro several times to aid in differentiating minimally reactive results from nonreactive results.
9. If the 1:16 dilution is still reactive, the sample needs to be diluted further, continuing as follows.
   a. Prepare a 1:50 dilution of nonreactive serum in 0.9% saline to be used for making the 1:32 and high dilutions of the sample to be tested.
   b. Prepare a 1:16 dilution of the test sample by adding 0.1 ml of serum to 1.4 ml of 0.9% saline. Mix well.
   c. Place 50 µl of the 1:50 nonreactive serum dilution in circles 2 through 5 of the test card.
   d. Use a safety pipette to dispense 50 µl of the 1:16 dilution of the test sample in circles 1 and 2.
10. Using the same pipette tip, make twofold serial dilutions. Complete the test as described in steps V.B.4 through 9 above. Use a new pipette tip for each patient sample.
11. At the completion of the day's tests, remove the needle from the dispensing vial, rinse with distilled water, and allow to air dry. Do not wipe the needle, as this removes the silicon coating, which will affect the size of the antigen drop.
12. Recap the dispensing bottle containing the antigen suspension and refrigerate at 2 to 8°C. Do not freeze. Antigen stored in the dispensing bottle will retain its reactivity for 3 months or until the expiration date, whichever occurs first.

POSTANALYTICAL CONSIDERATIONS

VI. INTERPRETATION

A. Qualitative test

1. Read the reaction immediately under a high-intensity incandescent lamp. Do not allow the reaction to start to dry out, which may lead to misinterpretation of results.
2. Any serum or plasma sample that has characteristic clumping ranging from marked and intense (reactive) to slight but definite (minimally to moderately reactive) is considered reactive.
3. Slight roughness or an absence of clumping is considered nonreactive.

Table 11.5.3–1 Reporting quantitative RPR test results[a]

Result with undiluted serum (1:1)	Result at serum dilution of:				Report
	1:2	1:4	1:8	1:16	
Rm or R	N	N	N	N	Reactive, undiluted (1:1)
R	R	N	N	N	Reactive, 2 dilutions or 1:2 or R2
R	R	R	Rm	N	Reactive, 8 dilutions or 1:8 or R8

[a] Abbreviations: N, nonreactive; R, reactive; Rm, reactive minimal.

VI. INTERPRETATION *(continued)*

B. Quantative test

1. Read the reaction immediately under a high-intensity incandescent lamp as for the qualitative test. Do not allow the reaction to start to dry out, which may lead to misinterpretation of results.

2. Report the results of the test as the highest dilution showing any reactivity, including minimally reactive, as shown in Table 11.5.3–1.

VII. LIMITATIONS OF THE TEST

A. False-positive test results can occur in serum or plasma samples from patients who abuse drugs; who have diseases such as lupus erythematosus, human immunodeficiency virus, mononucleosis, malaria, leprosy, viral pneumonia, or hepatitis; or who have recently received antiviral immunizations. Anecdotally, aspirin is thought to cause false-positive reactions, but there is no documented evidence to this effect.

B. Persons with late latent syphilis or late syphilis may have a nonreactive RPR test. Persons who were treated in the late latent or late stage of syphilis or persons who have a history of syphilis infections may maintain low levels of reactivity for life.

C. Prozone reactions may occur in undiluted serum samples. This occurs when antibody levels are high and flocculation cannot occur because of antibody excess. These samples will appear either as rough negatives or as weakly reactive. All samples giving this type of reaction must be quantitated. Additionally, if the clinician suspects syphilis and the nontreponemal test is nonreactive, it should be quantitated to rule out a prozone reaction.

D. The RPR test cannot be used to test CSF for the diagnosis of neurosyphilis. Only the VDRL CSF test can be used for this purpose.

E. The test has to be performed with all reagent, serum sample, and room temperatures between 23 and 29°C (73 to 85°F). Temperatures below this range will cause false negatives and lower titers, while temperatures above have the opposite effect (1).

REFERENCES

1. **Bossak, H. N., A. Harris, and S. Olansky.** 1955. Effect of room temperature on serologic tests for syphilis. *Br. J. Vener. Dis.* **31:**33–36.
2. **Centers for Disease Control.** 1987. Update on hepatitis B prevention. *Morb. Mortal Wkly. Rep.* **36:**353–360, 366.
3. **Centers for Disease Control.** 1988. Update: universal precautions for prevention of transmission of human immunodeficiency virus, hepatitis B virus, and other blood-borne pathogens in health care settings. *Morb. Mortal. Wkly. Rep.* **37:**600–602, 607–608.
4. **Larsen, S. A., V. Pope, R. E. Johnson, and E. J. Kennedy, Jr. (ed.).** 1998. *A Manual of Tests for Syphilis*, 9th ed. American Public Health Association, Washington, DC.

11.5.4 Serodia *Treponema pallidum* Particle Agglutination Test

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The Serodia *Treponema pallidum* particle agglutination test (TP-PA) is a treponemal test for the serologic detection of antitreponemal antibodies (3, 4). Partially purified sonicated *T. pallidum* antigens are used to sensitize gelatin particles. If antibodies against syphilis are found in the patient's serum, an agglutination reaction takes place. The TP-PA is intended to be used as a confirmatory test for reactive results in a nontreponemal test such as the RPR test, for patients with nonreactive nontreponemal tests who have signs or symptoms consistent with late manifestations of syphilis, or for patients for whom clinical history indicates that they may have late latent syphilis. As with all treponemal tests, results of the test remain reactive for life for most patients even when the patients are adequately treated for syphilis.

Serum that contains antibodies to the pathogenic treponemes binds to the antigens on the particles, forming a smooth mat of particles on the bottom of the microtiter plate well. If no antibodies to *T. pallidum* are present, the particles settle to the bottom of the well, forming a compact button. The unsensitized particle control well for each serum sample should also form a compact button on the bottom of the well, indicating an absence of nonspecific agglutination.

II. SPECIMEN COLLECTION

Observe standard precautions.

Serum is the preferred sample, and when handling serum, standard precautions must be observed (2). It is recommended that persons who handle blood and body fluids be vaccinated against hepatitis B virus (1). Blood samples should be collected and processed in such a way as to minimize hemolysis or contamination. Samples that are too hemolyzed or extremely lipemic or that contain microbial contamination should not be used since these may interfere with the test.

III. MATERIALS

A. Reagents

All of the reagents that are required for performance of the test are included in the kit, which is available commercially (Table 11.5.1–1). The user may want to include a minimally reactive control that gives 1 + agglutination for extra QC. Droppers that deliver 25 µl for delivery of sensitized and unsensitized gelatin particles are included in the kit.

B. Additional materials required

1. Disposable 96-well microtiter plates with round-bottom wells
2. Pipette droppers calibrated to deliver 25 µl for dispensing sample diluent
3. 100- and 25-µl automatic safety pipette with disposable tips for adding serum samples and making serum dilutions in the microtiter plate
4. Tray viewer (optional)
5. Automatic vibratory shaker
6. Disposable latex or nitrile gloves, safety glasses, and protective clothing
7. Discard containers and disinfectants

Table 11.5.4–1 Interpretation of agglutination patterns in the TP-PA

Settling pattern of particles	Reading	Interpretation
Smooth mat of particles covering the entire bottom of the well; edges may be folded	4+	Reactive
Smooth mat of particles covering less than the entire bottom of the well and maybe surrounded by faint ring	3+	Reactive
Agglutinated particles uniformly covering the bottom of the well, surrounded by a red circle	2+	Reactive
Particles forming a large ring with an irregular outer margin and peripheral agglutination, surrounded by a red circle	1+	Reactive
Particles concentrated in the shape of a compact ring with smooth edges and a hole in the center	+/−	Inconclusive
Particles concentrated in the shape of a button in the center with a smooth round outer margin, with or without a very small hole in the center	−	Nonreactive

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. All new lots of test kits should be tested in parallel with the lot of the kit currently in use before being placed into use. Results of the two lots should be comparable. Reactive and nonreactive controls plus 10 serum samples of graded reactivity (3 reactive, 4 of 1 to 2+ reactivity, 3 nonreactive) should be used for the comparison. The reactive and nonreactive controls included with the kit should be run daily.

B. Automatic pipettors should be calibrated annually.

V. PROCEDURE

Observe standard precautions.

A. One row of 12 wells is required for the reactive control, four wells are required for the nonreactive control, and four wells are required for all patient samples. Place 100 µl of sample diluent in the first well of each control and patient set of wells. In each additional well per sample or control, add 25 µl of sample diluent.

B. Make 1:5 dilution of sample by adding 25 µl of sample or control to the first well and serial twofold dilutions made either through well 4 (1:40 dilution) for patient samples and the nonreactive control or through well 12 for the reactive control. Discard 25 µl from the last well.

C. Using the droppers included with the kit, add 25 µl of sensitized particles to well 4 (final dilution, 1:80) of each patient sample and the nonreactive control and wells 4 through 12 for the reactive control. Add 25 µl of unsensitized particles to well 3 of the controls and patient samples.

D. Mix the samples by placing on a vibratory shaker for 30 s. Cover with either an empty microtiter plate or microplate cover. Let sit for 2 h at room temperature before reading. The plate may be left overnight to be read the next morning.

E. Read the plate against a white background and read the pattern of agglutination in each well according to Table 11.5.4–1. Alternatively, a plate viewer can be used to aid in visualizing the results. Results are read as either reactive or nonreactive. The unsensitized particle controls should not show any degree of agglutination.

POSTANALYTICAL CONSIDERATIONS

VI. INTERPRETATION

Read the results of agglutination against a white background. Any degree of agglutination (Table 11.5.4–1) in the wells with sensitized particles should be interpreted as reactive. Control sera and patient sera must show nonreactive results in the wells with the unsensitized particles.

A. The titer of the reactive control is read as the last dilution reading 1 + . The titer should not vary more than ± 1 doubling dilution from the endpoint titer established for the reactive control. The nonreactive control should not have any agglutination in the 1:80 dilution with sensitized cells. For any sample that shows agglutination in both the unsensitized and sensitized particles, the manufacturer's procedure for absorption should be followed. A reactive test result indicates past or present infection and the test usually remains reactive for life, even with adequate treatment for syphilis.

B. Any patient sample that shows indeterminate results should be retested or a second sample should be drawn and tested. Repeated inconclusive results should be confirmed with another treponemal test, such as the fluorescent treponemal antibody absorption test.

VII. LIMITATIONS OF THE PROCEDURE

A. All treponemal tests tend to remain reactive for life for persons who have had treponemal infections. Therefore, the TP-PA cannot be used to evaluate response to therapy or in determining relapse or reinfection.

B. Tests for syphilis cannot distinguish syphilis from yaws or pinta or endemic syphilis and may be reactive for persons from areas where these other diseases have been endemic.

C. False-positive reactions may occur in association with other underlying illnesses or conditions such as human immunodeficiency virus infection, rheumatic fever, leprosy, autoimmune diseases, Lyme disease, drug addiction, leprosy, toxoplasmosis, or *Helicobacter pylori* infection.

D. There is a false-positive rate in healthy persons of about 1%. These false-positive reactions are frequently transient and a second serum sample will be nonreactive.

E. Nonspecific agglutination, which rarely occurs in the test, may not be resolved by retesting absorbed serum against the sensitized and unsensitized particles.

REFERENCES

1. **Centers for Disease Control.** 1987. Update on hepatitis B prevention. *MMWR Morb. Mortal. Wkly. Rep.* **36:**353–360, 366.
2. **Centers for Disease Control.** 1988. Update: universal precautions for prevention of transmission of human immunodeficiency virus, hepatitis B virus, and other blood-borne pathogens in health care settings. *MMWR Morb. Mortal. Wkly. Rep.* **37:**377–383, 387–388.
3. **Larsen, S. A., V. Pope, R. E. Johnson, and E. J. Kennedy, Jr. (ed).** 1998. *A Manual of Tests for Syphilis,* 9th ed. American Public Health Association, Washington, DC.
4. **Pope, V., M. B. Fears, W. E. Morrill, A. Castro, and S. E. Kikkert.** 2000. Comparison of the Serodia *Treponema pallidum* particle agglutination, Captia Syphilis-G, and SpiroTek Reagin II tests with standard test techniques for diagnosis of syphilis. *J. Clin. Microbiol.* **38:**2543–2545.

11.6 Detection of *Borrelia burgdorferi* Antibodies

Lyme disease, or Lyme borreliosis (LB), is a multisystem disease caused by *Borrelia burgdorferi* and transmitted through the bite of infected *Ixodes scapularis* ticks (57). It is currently the most frequent vector-borne infectious disease in the United States, with the highest incidence in the northeastern and midwestern states (18). LB is also prevalent in Europe, where evidence of the disease existed in the early 1900s and where it is transmitted by *Ixodes ricinus* complex.

B. burgdorferi is composed of several genospecies that cause different manifestations of LB. The broad group is denominated by *B. burgdorferi* sensu lato; it includes the genospecies *B. burgdorferi* sensu stricto, *Borrelia garinii,* and *Borrelia afzelii. B. burgdorferi* sensu stricto most frequently causes LB in the United States, while *B. garinii* and *B. afzelii* cause disease in Europe. Other *Borrelia* genospecies such as *B. spielmanii, B. valaisiana,* and *B. lusitaniae* have also recently been reported to be associated with LB in Europe (49, 61). While *B. burgdorferi* sensu stricto causes most frequently erythema migrans (EM) and arthritis, *B. garinii* causes neurologic manifestations and *B. afzelii* causes chronic skin infections (60). Published evidence seems to indicate that genetic variability among *B. burgdorferi* strains isolated from skin lesions in North America may explain the different clinical manifestations observed in early disease (62).

B. burgdorferi is motile, helically shaped bacterium with multiple endoflagella and with a complex antigenic composition (10). Genome analysis of the type strain, *B. burgdorferi* sensu stricto B31, indicates that it contains a linear chromosome and 12 linear and 9 circular plasmids (16). Interestingly, *B. burgdorferi* outer surface proteins (OspA through OspF) are plasmid encoded. Expression of outer surface proteins varies depending on environmental conditions, allowing the organisms to adapt to different conditions. OspA is preferentially expressed at 25°C in the unfed tick midgut, while OspC is expressed at 37°C after a blood meal has been obtained by the feeding tick and expressed in the mammal host after transmission has occurred (52, 53). This switch in expression of outer surface proteins is important to understand the development of antibodies in humans as described below. Either certain *B. burgdorferi* sensu lato genes are expressed only in a mammalian host or their expression is significantly up-regulated in that environment; such gene products include VlsE, DbpA (22), BBK3 (27), Erp (40), and Mlp proteins (66).

LB usually occurs a few days to weeks after the bite of an infected tick and presents in about 80% of individuals with a slowly expanding erythematous skin lesion denominated EM (57) (Table 11.6–1).

The diagnosis of LB relies on clinical recognition of the EM lesion during early stages of the disease. When EM is absent, unrecognized, or atypical, other diagnostic modalities are needed. Laboratory tests aimed at detecting the causative agent involve culture, PCR, or microscopy. Unfortunately, these methods are most sensitive in detecting *B. burgdorferi* from EM lesions, for which laboratory tests are theoretically unnecessary (5, 54). *B. burgdorferi* can be cultured in Barbour-Stoenner-Kelly medium, and growth detection can take several weeks of incubation (9). Increased recovery from blood specimens has been obtained for patients with early disease by culturing large volumes of blood (64). PCR has been most useful with synovial fluid of patients with late disease and can be used to determine eradication of infection after treatment (46). The reliability of a urine test to detect *B. burgdorferi* antigens is questionable (33). Due to the lack of sensitivity and the cumbersome nature of the aforementioned tests, the laboratory confirmation of LB relies on indirect means such as antibody detection methods. A review of diagnostic modalities for LB has been published (5). Factors that influence seroreactivity are the duration of disease prior to detection of antibodies, the presence of disseminated versus localized early disease, and the microbiological properties of the *B. burgdorferi* strain causing LB (3, 63, 65).

Several immunodominant antigens are present in *B. burgdorferi.* They include OspC (23 kDa), flagellin (41 kDa), *Borrelia* membrane protein A (BmpA) (39 kDa), and VlsE in early disease. As the disease progresses, antibodies to several other antigens are developed (2, 3, 23, 24, 28). Tremendous knowledge has been gathered in testing for *B. burgdorferi* antibodies since the original assays were developed. First-generation immunoassays used organisms highly passaged in culture that are currently known to be devoid of OspC (20, 21). Immunoglobulin (IgM) antibodies to *B. burgdorferi* appear within 2 weeks of infection and may persist for prolonged periods after antimicrobial treatment (3, 26). IgG antibodies also appear within weeks of infection but reach their peak months after untreated early infection.

Since antigens such as flagellin and high-molecular-weight antigens, including heat shock proteins, are also present in other bacteria, antibody tests using whole-organism preparations may detect cross-reactive antibodies (25). In addition, the facts that available antibody assays have

not been standardized (11) and that most assays are conducted in populations with low probability for Lyme disease (43) have contributed to misdiagnosis of *B. burgdorferi* infection and to the belief that serologic assays for *B. burgdorferi* are unreliable.

In 1994 the CDC, in conjunction with the Association of State and Territorial Public Health Laboratory Directors (ASTPHLD), the FDA, the NIH, the Council of State and Territorial Epidemiologists, and the NCCLS, cosponsored a Conference on the Serological Diagnosis of Lyme Disease. Among the recommendations was that a two-step testing approach was needed for active disease and for a previous infection using a sensitive EIA or indirect immunofluorescent-antibody assay (IFA) as a first step, followed by Western immunoblotting (17). The first step should detect IgG and IgM antibodies to *B. burgdorferi* and when reactive should be followed by separate IgG and IgM immunoblot tests.

A search for better immunodiagnosis in Lyme disease has led to the evaluation and development of assays using peptide antigens such as C6 (invariable region of VlsE) and pepC10 (conserved C-terminal region of OspC) (7, 34, 36). Evaluation of C6 immunoassays has been difficult because of the different origins of such assays. Many investigators have used in-house-developed assays, and others have employed a commercial assay recently approved by the FDA as a first-step assay in the United States (7). Furthermore, some European authors have employed a commercial C6 assay, the QuickC6 assay, not currently approved for use in the United States (38). Due to its high specificity, the C6 assay has been used in Europe as a second step replacing immunoblotting (47). Other studies have found the C6 assay to have a greater sensitivity than two-step testing and have suggested that C6 assays could replace two-step testing (41).

Although C6 was originally reported to be highly cross-reactive, recent studies appear to indicate that differences exist between sequences of this peptide in different *B. burgdorferi* genospecies such that assays based on sequences from a single genospecies may be suboptimal for detecting antibodies in European and American patients with LB (30).

Whether these newly developed assays with single antigens/peptides or combinations will replace the currently recommended two-step testing is not known at present. Further evaluations using sera from patients with well-characterized LB at different stages of the illness are needed.

Besides the matrix-based assays, functional antibody assays have also been used (1, 14). Although these assays are more cumbersome, they have been reported to correlate with disease activity (15). Other types of assays have included specific antibodies bound to immune complexes, reported to be detectable in patients found to be seronegative by standard assays (12, 51). Like the borreliacidal-antibody assays, the immune complex antibody assays are not widely used and are still restricted to a few research laboratories.

Between 1994 and 1997 two recombinant OspA vaccines to prevent Lyme disease were evaluated in the United States, and one such preparation was available for use until 2002 (56). Individuals who participated in those vaccine trials or received the previously commercially available vaccine may still have detectable anti-OspA antibodies, and samples from such persons are expected to react in first-step assays that use whole borrelial cells as antigens, but such reactivity can be distinguished in Western immunoblots (4).

Since Lyme disease in Europe is caused by different *B. burgdorferi* sensu lato genospecies and consensus on serology is being developed (31, 50, 61), the discussion on methods that follows is restricted to detection of *B. burgdorferi* antibodies in the United States.

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

A. First-step testing

More than 70 commercial assays have been cleared by the FDA. Several of these assays are sold under a label different from that under which they were originally marketed, and others are no longer available. The majority of them are ELISAs or modifications of EIAs (19). Since IFAs are less frequently used as a first-step testing, the

Table 11.6–1 Lyme disease stages

Early disease: few days to few weeks after the bite of an infected *I. scapularis* tick (*I. ricinus* in Europe)
Early localized: EM at the site of the tick bite
Early disseminated: EM, accompanied by fever, influenza-like symptoms, and headache, has been considered evidence of dissemination (39)
Multiple skin lesions
Neurologic manifestations (15%): meningitis, cranial nerve palsies, motor or sensory radiculoneuritis
Cardiac manifestations (5%): atrioventricular blocks, myopericarditis
Late disease: months after onset of illness
Arthritis: about 60% of untreated patients develop intermittent attacks of arthritis
Chronic neuroborreliosis in up to 5% of untreated patients: chronic axonal neuropathy, chronic encephalomyelitis (caused by *B. garinii* in Europe), chronic encephalopathy (cognitive disturbances)
ACA[a] chronic skin lesion caused by *B. afzelii* in Europe

[a] ACA, achrodermatitis chronica atrophicans.

I. PRINCIPLE *(continued)*

following description is restricted to ELISA or EIA (for an updated list of FDA-cleared assays, the reader is referred to http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfPMN/pmn.cfm; type "LSR" at product code).

In ELISA, serum is tested for the presence of antibodies reacting with whole-cell sonicates of low-passage *B. burgdorferi* antigens immobilized to a solid surface. Variations to this antigen preparation are the addition of recombinant proteins (Immunowell Borrelia [GenBio] and PreVue *B. burgdorferi* [Chembio Diagnostics Systems]) or purified antigens (GenBio) and the use of synthetic C6 peptide, derived from the VlsE protein of *B. burgdorferi* (Immunetics, Inc.) (34, 36). The serum specimen is diluted (1:20 to 1:400) in a diluent containing buffer solutions, preservatives, and, in some formats, other bacterial antigens to block nonspecific reactions. Several washing steps remove unbound antibodies. Bound antibodies are detected after the addition of enzyme-conjugated anti-human immunoglobulins (IgG, IgM, and IgA, alone or in combination). After removal of unbound conjugate by several washes, a solution containing the substrate for the bound enzyme-conjugate is added. Color reaction (or fluorescence for Vidas [bioMérieux]) is read with a spectrophotometer (or luminometer) and absorbance is converted to a numerical value and translated to categories of reactivity (negative, borderline/equivocal, and positive) based on a ratio of patient specimen absorbance over a calibrator absorbance.

B. Second-step testing: Western blotting

Specimens testing borderline or positive by the first step are assayed in Western blots (immunoblots) where *B. burgdorferi* antigens have been separated electrophoretically by size and transferred (blotted) to membranes. Diluted serum specimens are tested in separate assays for IgG and IgM antibodies to the different antigenic components bound to the membranes, usually in strips. Unbound antibodies are removed by washing steps. Enzyme-conjugated anti-human IgG and IgM are reacted with the two separate strips, and unbound conjugate is removed by washing. After the addition of a substrate for the bound enzyme, colored bands representing reactivity to the different immobilized antigens will develop. Band recording is visually determined by comparison to the reactivity of a band locator control and of a weak intensity control (cutoff control).

II. SPECIMEN COLLECTION

Observe standard precautions.

The most appropriate specimen for testing is serum. Lipemic or hemolyzed specimens should be avoided. Once serum has been separated from whole blood, it should be stored refrigerated (4 to 8°C) for several days or frozen (-20 to -70°C) if testing is delayed beyond a few weeks or for long-term storage.

Since development of antibodies requires a few days to weeks after infection, it is often necessary to test acute- and convalescent-phase sera. The best results are obtained when both specimens are tested in parallel in the same assay under the same conditions. Testing of other types of specimens, such as synovial fluid or CSF, has not yet been standardized.

III. MATERIALS

Several formats are currently available for first-step testing for *B. burgdorferi* antibodies.

A. Antigens

Most commercial first- and second-step assays in the United States use whole-cell sonicates of low-passage *B. burgdorferi* B31, the first isolate of *B. burgdorferi* sensu stricto cultured from an *I. scapularis* tick. Some manufacturers have added the recombinant p39 antigen to the whole-cell antigen preparation. GenBio has produced dot EIAs using recombinant and purified antigens, and Immunetics released an ELISA using the C6 peptide, a synthetic peptide derived from the VlsE antigen of *B. burgdorferi* (36). Recombinant antigens have been evaluated in Europe as well as by several investigators in the United States, but few are commercially available (13, 29, 37, 48).

III. MATERIALS *(continued)*

B. Serum diluent
Due to the presence in *B. burgdorferi* of antigens common to other bacteria, several manufacturers have added bacterial proteins in first-step assays in order to absorb cross-reactive antibodies from patient serum.

C. Other materials required as for any immunoassay
1. Calibrated pipettors
2. Rocking platforms or shakers
3. Timers
4. Serologic pipettes
5. Multichannel incubating trays for immunoblots

ANALYTICAL CONSIDERATIONS

IV. PROCEDURE

Commercially available FDA-cleared kits provide instructions for proper performance of assays.

Observe standard precautions.

A. Recommendations for testing
Special attention should be paid to the following factors.
1. Use kits within their expiration dates.
2. Avoid interchanging reagents from different lot numbers.
3. The following recommendations are particularly applicable for first-step tests such as ELISAs.
   - **a.** Positive, low positive, and negative controls as recommended by the manufacturer should be included in every run.
   - **b.** It is highly recommended that a well-characterized in-house low positive control be included in each run to assess run performance.
   - **c.** Prior to placing a new kit lot in use, it is advisable to run a panel of well-characterized frozen sera (previously tested in the laboratory) that encompasses all the ranges of reactivity. In my experience, differences in detection of reactivity in sera of patients with predominant IgM antibodies exist when different lots from the same manufacturer are used.
4. The following recommendations are applicable to Western immunoblots.
   - **a.** Run respective positive (band locator), weak positive (cutoff control), and negative controls as recommended by the manufacturer. Some recommend running only the weak and negative controls in every run and the positive control only once per new kit.
   - **b.** Inclusion of in-house controls showing bands of significance not present in the band locator is also suggested.
5. Run paired sera in the same run when comparing reactivities of different specimens obtained from the same patient at different time points.

B. Analytical factors
1. Maintain room temperature conditions (20 to 25°C). Day-to-day variations in room temperature affect the reliability and consistency of results. When room temperature exceeds ranges, the assay should be performed in another area of the laboratory (if possible) where conditions are met.
2. Incubation times and use of rotators and shakers should be adhered to as indicated by the manufacturer unless in-house studies have proven them not to affect results.
3. Commercial Western blot kits are also provided with the appropriate instructions for performance. Guidelines for in-house-developed Western blot kits have been published by the NCCLS (now called CLSI) (document M34-A [44]). These guidelines address antigen preparation, electrophoresis, transfer of antigens to the matrix, calibration, and interpretation, among other issues important to this method.

POSTANALYTICAL CONSIDERATIONS

V. INTERPRETATION

A. First-step tests

Interpretation guidelines are provided by the manufacturers. Usually they involve a comparison (ratio) of patient's serum reactivity (absorbance for ELISA) to the reactivity of a calibrator included in the assay (usually a sample testing low positive). This ratio is translated to an index or units such that those samples reacting at about the intensity of the calibrator are labeled as borderline (equivocal) or low positive (reactive). Those with reactivity greater than that of those calibrators are reported as positive. Some in-house tests generate these ratios based on a reactivity that exceeds that of negative controls by 3 standard deviations.

Expected results:

1. Specimens containing cross-reactive antibodies as well as those from patients with early Lyme disease usually test borderline or low positive.
2. Sera from patients with early disseminated disease test low positive to positive (sometimes two to three times above the cutoff).
3. Sera from patients with late disease usually test greater than three times the cutoff.

B. Western immunoblots

If blots are produced in-house, guidelines provided by NCCLS document M34-A are recommended (44). At this writing, two commercial sources of Western immunoblots are available (Marblots, manufactured by MarDx Diagnostics, and Virablots, manufactured by Viramed Biotech), and their package inserts provide sufficient guidelines for interpretation. Although this method offers greater specificity than first-step tests, it suffers limitations due to the subjectivity inherent to visual band scoring. Some investigators have suggested a densitometric analysis of bands, but currently this approach is not widely used (24). Manufacturers have tried to address this issue by including a weak or cutoff control that usually contains antibodies to the 41-kDa antigen (flagellin), 39-kDa protein (BmpA), or 23-kDa protein (OspC) against which individual band intensity is compared.

1. Band scoring
   - **a.** Use the band locator provided by the manufacturer to identify the reactive bands in the patient strip and record the bands.
   - **b.** Bands with intensity similar to or greater than that of the weak (cutoff) control are scored. Adherence to this is particularly important when scoring IgM bands in sera from patients with early disease. IgM reactivities to the 41- and 23-kDa (OspC) proteins usually exceed the intensity of the weak control for these patients.
2. Results
   - **a.** A positive IgM blot shows two of these three significant bands: 23 kDa (OspC) and 39 or 41 kDa (24).
   - **b.** A positive IgG blot shows 5 of the following 10 significant bands: 18, 23 (OspC), 28, 30, 39, 41, 45, 58, 66, and 93 kDa (23).
   - **c.** CDC-ASTPHLD guidelines state that the IgM criteria should be only of diagnostic use during the first 4 weeks of disease but that the IgG criteria can be used to support a clinical diagnosis at any disease stage (17).
   - **d.** Individuals who have received more than two doses of OspA vaccine will show particularly IgG reactivity to the 31-kDa antigen. IgM reactivity is of lesser intensity and duration than the IgG antibodies. In addition, those individuals with high titers of IgG anti-OspA antibodies may show reactivity to other antigens of low molecular size that most likely represent breakdown products of OspA during blot preparation (4, 42).

VI. POSTANALYTICAL RECOMMENDATIONS

A. First-step assays

1. Guidelines established by the manufacturer concerning run acceptability should be followed.
2. Laboratories performing tests for *B. burgdorferi* antibodies should participate in an approved proficiency testing program, such as those offered by different states or the CAP survey.
3. It is important to emphasize that current guidelines recommend that all specimens reactive by first-step tests be referred for second-step IgG and IgM immunoblots and that the final result be that of the immunoblots. Necessary arrangements should be made to refer the specimens to a reference laboratory or establish the Western blot method in the laboratory if the volume of specimens allows it.
4. Absorbance of specimens may vary slightly from run to run; therefore, the numerical values are not as precise as values of chemical blood parameters. This is of particular importance when comparing reactivities of sera obtained from the same individual at different time points. Paired sera should be run in parallel using the same lot of reagents under the same run conditions in order to obtain a meaningful comparison.
5. Since first-step tests are quantitative and the results are derived more objectively than with Western blotting, they better assess the amount of antibodies present. Therefore, these tests are better utilized to determine decline in antibodies than Western immunoblotting.
6. Sensitivity and specificity

   Variable levels of sensitivity are observed with the available first-step assays, as has been demonstrated in proficiency testing of the CAP (8). Most frequently, lack of sensitivity involves the detection of IgM antibodies during early disease (2, 3, 11, 23, 34, 58). For a population of patients with culture-confirmed EM of short duration, IgM antibodies were more frequently detected by immunoblotting than by ELISA. The sensitivity of first-step tests during the acute phase is about 30 to 40%, with a specificity of approximately 90% (2, 3, 23). About 60% or more of patients presenting with a disease of more than 1 week's duration show antibodies by first-step tests (3). Sensitivity increases to about 90% if patients are tested within 2 weeks into convalescence. Due to the lack of specificity of ELISA and other first-step assays, such as ••• (ELFA) and IFA, using whole-cell antigen preparations, a positive test is not indicative of seropositivity. Analysis of results obtained with an automated ELFA found that the specificity was 91% in 559 samples from a control population (32).

B. Western immunoblots

1. It is important to assess the blot reactivities of the entire run as a whole, regardless of whether the run met criteria of acceptability based on the control materials. Intensity of immunoblot bands correlates with reactivity as measured by the first-step tests, and usually the specimens included in a blot run have a spectrum of reactivity by ELISA and blotting. If blot bands are present in most samples and they do not seem to correlate with the ELISA values, the entire run may need to be questioned. Lack of adherence to procedural details, inadequate room temperature, or other factors may have affected the run.
2. If great discrepancies exist between ELISA and immunoblot reactivities, both tests may need to be repeated.
3. Laboratories performing Western immunoblots should participate in an approved proficiency testing program, such as those available through different state health departments or the CAP survey. Although there is not a "gold

VI. POSTANALYTICAL RECOMMENDATIONS
(continued)

standard" method to compare results and materials included in proficiency surveys have been questioned (55), proficiency testing programs have contributed to a better understanding of the performance of available tests (8).

4. When immunoblot testing is recently introduced into the laboratory, the use of a panel of monoclonal antibodies to various antigens may aid in identifying the bands of significance. Such a panel is available through the CDC and consists of monoclonal antibodies to p93, p62, p41, p39, p37 (FlaA), OspB, OspA, OspD, OspC, and p22. Other monoclonal antibodies have also been produced by a number of investigators.
5. Although the OspA vaccine is no longer commercially available, it is advisable to become familiar with the blot reactivities of sera of individuals who have received such preparations. Since patients who have developed natural antibodies after a recent infection with *B. burgdorferi* do not usually develop anti-OspA antibodies, the vaccination reactivity observed in blots is quite characteristic. The presence of reactivity to low-molecular-size antigens in vaccinated individuals is different and should not be confused with reactivities to the significant bands used in the IgG blot criteria (23 kDa [OspC] and 18 kDa) (4).
6. Sensitivity and specificity

   During early stages of Lyme disease the sensitivity of IgM blots is about 40% in the acute phase and 60 to 80% during convalescence. The sensitivity increases for patients with disseminated disease (23).

   Methods used in the detection of antibodies to *B. burgdorferi*, the causative agent of Lyme disease, have many limitations. Contributing factors are the complex antigenic composition of *B. burgdorferi*, the lack of standardization of methods, and their use in unselected patient populations. The American College of Physicians has proposed that antibody tests be restricted to those patients with a pretest probability of Lyme disease of 0.20 to 0.80 (6, 59). Testing of patients with a low probability (<0.20) of Lyme disease will most likely yield false-positive tests. According to their recommendation, patients with a high pretest probability should be treated and not tested (45).

   The current approach to antibody testing involves the use of two steps: a sensitive ELISA or IFA that should detect both IgG and IgM antibodies, followed by separate IgM and IgG immunoblots. Immunoblots should be interpreted according to the CDC-ASTPHLD guidelines (19). The role of these methods is to support the clinical diagnosis of LB, and their indiscriminate use in unselected populations, as well as deviations from the CDC-ASTPHLD guidelines, is strongly discouraged (19).

   Future tests to detect *B. burgdorferi* antibodies most likely will include recombinant or synthetic antigens in a quantitative format. Such an approach seems not so distant, since such tests are already being developed (36). Single-step assays to detect specific antibodies to *B. burgdorferi* are highly desirable.

REFERENCES

1. **Agger, W. A., and K. L. Case.** 1997. Clinical comparison of borreliacidal-antibody test with indirect immunofluorescence and enzyme-linked immunosorbent assays for diagnosis of Lyme disease. *Mayo Clin. Proc.* **72:**510–514.
2. **Aguero-Rosenfeld, M. E., J. Nowakowski, D. F. McKenna, C. A. Carbonaro, and G. P. Wormser.** 1993. Serodiagnosis in early Lyme disease. *J. Clin. Microbiol.* **31:**3090–3095.
3. **Aguero-Rosenfeld, M. E., J. Nowakowski, S. Bittker, D. Cooper, R. B. Nadelman, and G. P. Wormser.** 1996. Evolution of the serologic response to *Borrelia burgdorferi* in treated patients with culture-confirmed erythema migrans. *J. Clin. Microbiol.* **34:**1–9.
4. **Aguero-Rosenfeld, M. E., J. Roberge, C. A. Carbonaro, J. Nowakowski, R. B. Nadelman, and G. P. Wormser.** 1999. Effects of OspA vaccination on Lyme disease serologic testing. *J. Clin. Microbiol.* **37:**3718–3721.

REFERENCES *(continued)*

5. **Aguero-Rosenfeld, M. E., G. Wang, I. Schwartz, and G. P. Wormser.** 2005. Diagnosis of Lyme borreliosis. *Clin. Microbiol. Rev.* **18:**484–509.
6. **American College of Physicians.** 1997. Guidelines for laboratory evaluation in the diagnosis of Lyme disease. Clinical guideline part 1. *Ann. Intern. Med.* **127:**1106–1108.
7. **Bacon, R. M., B. J. Biggerstaff, M. E. Schriefer, R. D. Gilmore, Jr., M. T. Philipp, A. C. Steere, G. P. Wormser, A. R. Marques, and B. J. Johnson.** 2003. Serodiagnosis of Lyme disease by kinetic enzyme-linked immunosorbent assay using recombinant VlsE1 or peptide antigens of *Borrelia burgdorferi* compared with 2-tiered testing using whole-cell lysates. *J. Infect. Dis.* **187:**1187–1199.
8. **Bakken, L. L., S. M. Callister, P. J. Wand, and R. F. Schell.** 1997. Interlaboratory comparison of test results for detection of Lyme disease by 516 participants in the Wisconsin State Laboratory of Hygiene/College of American Pathologists proficiency testing program. *J. Clin. Microbiol.* **35:**537–543.
9. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. *Yale J. Biol. Med.* **57:**521–524.
10. **Barbour, A. G., and S. F. Hayes.** 1986. Biology of *Borrelia* species. *Microbiol. Rev.* **50:**381–400.
11. **Brown, S. L., S. L. Hansen, and J. J. Langone.** 1999. Role of serology in the diagnosis of Lyme disease. *JAMA* **282:**62–66.
12. **Brunner, M., and L. H. Sigal.** 2001. Use of serum immune complexes in a new test that accurately confirms early Lyme disease and active infection with *Borrelia burgdorferi. J. Clin. Microbiol.* **39:**3213–3221.
13. **Burkert, S., D. Rossler, P. Munchhoff, and B. Wilske.** 1996. Development of enzyme-linked immunosorbent assays using recombinant borrelial antigens for serodiagnosis of *Borrelia burgdorferi* infection. *Med. Microbiol. Immunol.* **185:**49–57.
14. **Callister, S. M., R. F. Schell, L. C. L. Lim, D. A. Jobe, K. L. Case, G. L. Bryant, and P. E. Molling.** 1994. Detection of borreliacidal antibodies by flow cytometry. *Arch. Intern. Med.* **154:**1625–1632.
15. **Callister, S. M., D. A. Jobe, W. A. Agger, R. F. Schell, T. J. Kowalski, S. D. Lovrich, and J. A. Marks.** 2002. Ability of the borreliacidal antibody test to confirm Lyme disease in clinical practice. *Clin. Diagn. Lab. Immunol.* **9:**908–912.
16. **Casjens, S., N. Palmer, R. van Vugt, H. W. Mun, B. Stevenson, P. Rosa, R. Lathigra, G. Sutton, J. Peterson, R. J. Dodson, D. Haft, E. Hickey, M. Gwinn, O. White, and M. Fraser.** 2000. A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete *Borrelia burgdorferi. Mol. Microbiol.* **35:**490–516.
17. **Centers for Disease Control and Prevention.** 1995. Recommendations for test performance and interpretation from the second national conference on serologic diagnosis of Lyme disease. *MMWR Morb. Mortal. Wkly. Rep.* **44:**590–591.
18. **Centers for Disease Control and Prevention.** 2007. Lyme disease—United States, 2003–2005. *MMWR Morb. Mortal. Wkly. Rep.* **56:**573–576.
19. **Centers for Disease Control and Prevention.** 2005. Notice to readers: caution regarding testing for Lyme disease. *MMWR Morb. Mortal. Wkly. Rep.* **54:**125.
20. **Coleman, J. L., and J. L. Benach.** 1987. Isolation of antigenic components from the Lyme disease spirochete: their role in early diagnosis. *J. Infect. Dis.* **155:**756–765.
21. **Craft, J. E., D. K. Fischer, G. T. Shimamoto, and A. C. Steere.** 1986. Antigens of *Borrelia burgdorferi* recognized during early Lyme disease. *J. Clin. Investig.* **78:**934–939.
22. **Crother, T. R., C. I. Champion, X. Y. Wu, D. R. Blanco, J. N. Miller, and M. A. Lovett.** 2003. Antigenic composition of *Borrelia burgdorferi* during infection of SCID mice. *Infect. Immun.* **71:**3419–3428.
23. **Dressler, F., J. A. Whalen, B. N. Reinhardt, and A. C. Steere.** 1993. Western blotting in the serodiagnosis of Lyme disease. *J. Infect. Dis.* **167:**392–400.
24. **Engstrom, S. M., E. Shoop, and R. C. Johnson.** 1995. Immunoblot interpretation criteria for serodiagnosis of early Lyme disease. *J. Clin. Microbiol.* **33:**419–427.
25. **Fawcett, P. T., K. M. Gibney, C. D. Rose, S. B. Dubbs, and R. A. Doughty.** 1992. Frequency and specificity of antibodies that cross-react with *Borrelia burgdorferi* antigens. *J. Rheumatol.* **19:**582–587.
26. **Feder, H. M., Jr., M. A. Gerber, S. W. Luger, and R. W. Ryan.** 1992. Persistence of serum antibodies to *Borrelia burgdorferi* in patients treated for Lyme disease. *Clin. Infect. Dis.* **15:**788–793.
27. **Fikrig, E., W. Feng, S. W. Barthold, S. R. Telford III, and R. A. Flavell.** 2000. Arthropod- and host-specific *Borrelia burgdorferi* bbk32 expression and the inhibition of spirochete transmission. *J. Immunol.* **164:**5344–5351.
28. **Gilmore, R. D., Jr., R. L. Murphree, A. M. James, S. A. Sullivan, and B. J. B. Johnson.** 1999. The *Borrelia burgdorferi* 37-kilodalton immunoblot band (P37) used in serodiagnosis of early Lyme disease is the *flaA* gene product. *J. Clin. Microbiol.* **37:**548–552.
29. **Gomes-Solecki, M. J. C., J. J. Dunn, B. J. Luft, J. Castillo, D. E. Dykhuizen, X. Yang, J. D. Glass, and R. J. Dattwyler.** 2000. Recombinant chimeric borrelia proteins for diagnosis of Lyme disease. *J. Clin. Microbiol.* **38:**2530–2535.
30. **Gomes-Solecki, M. J. C., L. Meirelles, J. Glass, and R. J. Dattwyler.** 2007. Epitope length, genospecies dependency, and serum panel effect in the IR6 enzyme-linked immunosorbent assay for detection of antibodies to *Borrelia burgdorferi. Clin. Vaccine Immunol.* **14:**875–879.
31. **Hauser, U., G. Lehnert, and B. Wilske.** 1999. Validity of interpretation criteria for standardized Western blots (immunoblots) for serodiagnosis of Lyme borreliosis based on sera collected throughout Europe. *J. Clin. Microbiol.* **37:**2241–2247.

REFERENCES *(continued)*

32. **Johnson, B. J., B. J. Biggerstaff, R. M. Bacon, and M. E. Schriefer.** 2004. Cost-effectiveness of peptide-antigen immunoassays for Lyme disease. *J. Infect. Dis.* **189:**1962–1964.
33. **Klempner, M. S., C. H. Schmid, L. Hu, A. C. Steere, G. Johnson, B. McCloud, R. Noring, and A. Weinstein.** 2001. Intralaboratory reliability of serologic and urine testing for Lyme disease. *Am. J. Med.* **110:**217–219.
34. **Lawrenz, M. B., J. M. Hardham, R. T. Owens, J. Nowakowski, A. C. Steere, G. P. Wormser, and S. J. Norris.** 1999. Human antibody responses to VlsE antigenic variation protein of *Borrelia burgdorferi*. *J. Clin. Microbiol.* **37:**3997–4004.
35. **Ledue, T. B., M. F. Collins, and W. Y. Craig.** 1996. New laboratory guidelines for serologic diagnosis of Lyme disease: evaluation of the two-test protocol. *J. Clin. Microbiol.* **34:**2343–2350.
36. **Liang, F. T., A. C. Steere, A. R. Marques, B. J. B. Johnson, J. N. Miller, and M. T. Philipp.** 1999. Sensitive and specific serodiagnosis of Lyme disease by enzyme-linked immunosorbent assay with a peptide based on an immunodominant conserved region of *Borrelia burgdorferi* VlsE. *J. Clin. Microbiol.* **37:**3990–3996.
37. **Magnarelli, L. A., J. W. IJdo, S. J. Padula, R. A. Flavell, and E. Fikrig.** 2000. Serologic diagnosis of Lyme borreliosis by using enzyme-linked immunosorbent assays with recombinant antigens. *J. Clin. Microbiol.* **38:**1735–1739.
38. **Marangoni, A., M. Sparacino, and F. Cavrini.** 2005. Comparative evaluation of three different ELISA methods for the diagnosis of early culture-confirmed Lyme disease in Italy. *J. Med. Microbiol.* **54:**361–367.
39. **Massarotti, E. M., S. W. Luger, D. W. Rahn, R. P. Messner, J. B. Wong, R. C. Johnson, and A. C. Steere.** 1992. Treatment of early Lyme disease. *Am. J. Med.* **92:**396–403.
40. **Miller, J. C., K. von Lackum, K. Babb, J. D. McAlister, and B. Stevenson.** 2003. Temporal analysis of *Borrelia burgdorferi* Erp protein expression throughout the mammal-tick infectious cycle. *Infect. Immun.* **71:**6943–6952.
41. **Mogilyansky, E., C. C. Loa, M. E. Adelson, E. Mordechai, and R. C. Tilton.** 2004. Comparison of Western immunoblotting and the C6 Lyme antibody test for laboratory detection of Lyme disease. *Clin. Diagn. Lab. Immunol.* **11:**924–929.
42. **Molloy, P. J., V. P. Verardi, D. H. Persing, and L. H. Sigal.** 1999. Detection of multiple reactive protein species by immunoblotting after recombinant outer surface protein A Lyme disease vaccination. *Clin. Infect. Dis.* **31:**42–47.
43. **Nachamkin, I., D. L. Riddle, M. Feldman, and P. H. Edelstein.** 1996. Utilization of tests for Lyme disease antibody at a university hospital. *Clin. Diagn. Lab. Immunol.* **3:**287–289.
44. **NCCLS.** 2000. *Western Blot Assay for Antibodies to* Borrelia burgdorferi. Approved guideline M34-A, vol. 20, no. 20. NCCLS, Wayne, PA.
45. **Nichol, G., D. T. Dennis, A. C. Steere, R. Lightfoot, G. Wells, B. Shea, and P. Tugwell.** 1998. Test-treatment strategies for patients suspected of having Lyme disease: a cost-effectiveness analysis. *Ann. Intern. Med.* **128:**37–48.
46. **Nocton, J. J., F. Dressler, B. J. Rutledge, P. N. Rys, D. H. Persing, and A. C. Steere.** 1994. Detection of *Borrelia burgdorferi* DNA by polymerase chain reaction in synovial fluid from patients with Lyme arthritis. *N. Engl. J. Med.* **330:**229–234.
47. **Nyman, D., L. Willen, C. Jansson, S. A. Carlsson, H. Granlund, and P. Wahlberg.** 2006. VlsE C6 peptide and IgG ELISA antibody analysis for clinical diagnosis of Lyme borreliosis in an endemic area. *Clin. Microbiol. Infect.* **12:**496–497.
48. **Padula, S. J., F. Dias, A. Sampieri, R. B. Craven, and R. W. Ryan.** 1994. Use of recombinant OspC from *Borrelia burgdorferi* for serodiagnosis of early Lyme disease. *J. Clin. Microbiol.* **32:**1733–1738.
49. **Richter, D., D. Postic, N. Sertour, I. Livey, F. R. Matuschka, and G. Baranton.** 2006. Delineation of *Borrelia burgdorferi* sensu lato species by multilocus sequence analysis and confirmation of the delineation of *Borrelia spielmanii* sp. nov. *Int. J. Syst. Evol. Microbiol.* **56:**873–881.
50. **Robertson, J., E. Guy, N. Andrews, B. Wilske, P. Anda, M. Granstrom, U. Hauser, Y. Moosmann, V. Sambri, J. Schellekens, G. Stanek, and J. Gray.** 2000. A European multicenter study of immunoblotting in serodiagnosis of Lyme borreliosis. *J. Clin. Microbiol.* **38:**2097–2102.
51. **Schutzer, S. E., P. K. Coyle, P. Reid, and B. Holland.** 1999. *Borrelia burgdorferi*-specific immune complexes in acute Lyme disease. *JAMA* **282:**1942–1946.
52. **Schwan, T. G., J. Piesman, W. T. Golde, M. C. Dolan, and P. A. Rosa.** 1995. Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. *Proc. Natl. Acad. Sci. USA* **92:**2909–2913.
53. **Schwan, T. G., and J. Piesman.** 2000. Temporal changes in outer surface proteins A and C of the Lyme disease-associated spirochete, *Borrelia burgdorferi*, during the chain of infection in ticks and mice. *J. Clin. Microbiol.* **38:**382–388.
54. **Schwartz, I., G. P. Wormser, J. J. Schwartz, D. Cooper, P. Weissensee, A. Gazumyan, E. Zimmermann, N. S. Goldberg, S. Bittker, G. L. Campbell, and C. S. Pavia.** 1992. Diagnosis of early Lyme disease by polymerase chain reaction amplification and culture of skin biopsies from erythema migrans lesions. *J. Clin. Microbiol.* **30:**3082–3088.
55. **Seder, R. H., and V. P. Verardi.** 1996. Proficiency testing program for Lyme disease. *Arch. Pathol. Lab. Med.* **120:**323–325. (Letter.)

REFERENCES *(continued)*

56. **Steere, A. C., V. Sikand, F. Meurice, D. L. Parenti, E. Fikrig, R. T. Schoen, J. Nowakowski, C. H. Schmid, S. Laukamp, C. Buscarino, D. S. Krause, and the Lyme Disease Vaccine Study.** 1998. Vaccination against Lyme disease with recombinant *Borrelia burgdorferi* outer surface lipoprotein A with adjuvant. *N. Engl. J. Med.* **339:**209–215.
57. **Steere, A. C.** 2001. Lyme disease. *N. Engl. J. Med.* **345:**115–125.
58. **Trevejo, R. T., P. J. Krause, V. K. Sikand, M. E. Schriefer, R. Ryan, T. Lepore, and D. T. Dennis.** 1999. Evaluation of two-test serodiagnostic method for early Lyme disease in clinical practice. *J. Infect. Dis.* **179:**931–938.
59. **Tugwell, P., D. T. Dennis, A. Weinstein, G. Wells, B. Shea, G. Nichol, R. Hayward, R. Lightfoot, P. Baker, and A. C. Steere.** 1997. Laboratory evaluation in the diagnosis of Lyme disease. *Ann. Intern. Med.* **127:**1109–1123.
60. **Van Dam, A. P., H. Kuiper, K. Vos, A. Widjojokusumo, B. M. De Jongh, I. Spanjaard, A. C. P. Ramselaar, M. D. Kramer, and J. Dankert.** 1993. Different genospecies of *Borrelia burgdorferi* are associated with distinct clinical manifestations of Lyme borreliosis. *Clin. Infect. Dis.* **17:**709–717.
61. **Wilske, B., V. Fingerle, and U. Schultze-Spechtel.** 2007. Microbiological and serological diagnosis of Lyme borreliosis. *FEMS Immunol. Med. Microbiol.* **49:**13–21.
62. **Wormser, G. P., D. Liveris, J. Nowakowski, R. B. Nadelman, F. L. Cavaliere, D. McKenna, D. Holmgren, and I. Schwartz.** 1999. Association of specific subtypes of *Borrelia burgdorferi* with hematogenous dissemination in early Lyme disease. *J. Infect. Dis.* **180:**720–725.
63. **Wormser, G. P., D. Liveris, K. Hanincova, D. Brisson, S. Ludin, V. J. Stracuzzi, M. E. Embers, M. T. Philipp, A. Levin, M. Aguero-Rosenfeld, and I. Schwartz.** 2008. Effect of *B. burgdorferi* genotype on the sensitivity of C6 and 2-tier testing in North American patients with culture-confirmed Lyme disease. *Clin. Infect. Dis.* **47:**910–914
64. **Wormser, G. P., S. Bittker, D. Cooper, J. Nowakowski, R. B. Nadelman, and C. Pavia.** 2001. Yield of large-volume blood cultures in patients with early Lyme disease. *J. Infect. Dis.* **184:**1070–1072.
65. **Wormser, G. P., J. Nowakowski, R. Nadelman, P. Visintainer, A. Levin, and M. E. Aguero-Rosenfeld.** 2008. Impact of clinical variables on *Borrelia burgdorferi*-specific antibody seropositivity in acute-phase sera from patients in North America with culture-confirmed early Lyme disease. *Clin. Vaccine Immunol.* **15:**1519–1522.
66. **Yang, X., T. G. Popova, K. E. Hagman, S. K. Wikel, G. B. Schoeler, M. J. Caimano, J. D. Radolf, and M. V. Norgard.** 1999. Identification, characterization, and expression of three new members of the *Borrelia burgdorferi* Mlp (2.9) lipoprotein gene family. *Infect. Immun.* **67:**6008–6018.

11.7.1 Introduction

Rickettsial diseases in humans result from infection with bacteria of the families *Rickettsiaceae* and *Anaplasmataceae*. The family *Rickettsiaceae* includes the genera *Rickettsia* and *Orientia*, both of which infect endothelial cells and thereby cause vasculitis. *Rickettsia* spp. share lipopolysaccharides and outer membrane protein B (OmpB); however, spotted fever group (SFG) rickettsiae also possess outer membrane protein A (OmpA), whereas typhus group (TG) rickettsiae do not. Since serologic cross-reactivity between groups is common but antibody titers are generally higher within the specific group, distinction between SFG and TG rickettsiae requires paired testing and is not always possible. The *Anaplasmataceae* family includes *Ehrlichia chaffeensis*, which infects monocytes and mononuclear phagocytes, and *Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophila*, *Ehrlichia equi*, and the HGA agent) and *Ehrlichia ewingii*, which infect granulocytes. Serologic cross-reactions occur often (3 to 30%) with *Ehrlichia* and *Anaplasma*, as they do with *Rickettsia*, and differentiation requires comparison of specific antibody titers.

Rocky Mountain spotted fever (RMSF), caused by *Rickettsia rickettsii*, is the second most common vector (tick)-borne infection in North America and has a case fatality rate of up to 8.5%. RMSF is followed in frequency by human granulocytic anaplasmosis (HGA) and human monocytic ehrlichiosis (HME), caused by *A. phagocytophilum* and *E. chaffeensis*, respectively. Reported cases of RMSF, HGA, and HME have been steadily increasing since their identification, with 1,916 cases of RMSF, 834 cases of HGA, and 828 of HME reported in 2007 alone (2, 7, 12). Regional prevalences are as high as 65 human cases of HME per million in Searcy County, AR, and 522 cases of HGA per million in Jackson County, WI (11). *Ehrlichia ewingii* cross-reacts serologically with *Ehrlichia chaffeensis*, and 10% of infections diagnosed as HME may actually be *E. ewingii* infections (3). HME is generally a clinically more severe illness than HGA, and both infections are more severe in immunocompromised persons, including those with human immunodeficiency virus infection. Flea-borne murine typhus, caused by *Rickettsia typhi*, and cat flea typhus, caused by the recently recognized *Rickettsia felis* (6), are particularly prevalent in south Texas and Southern California, but they occur wherever rat and cat fleas exist. Although often a mild illness, murine typhus has a case fatality rate of 2%.

Orientia tsutsugamushi, the agent of scrub typhus, is transmitted by chiggers. Although infections have only been reported from the Asia Pacific triangle (south and Southeast Asia and northern Australia), this region has a population of >2 billion and is a frequent destination of travelers. Serology to confirm scrub typhus is available by referral to public health or reference laboratories. *Coxiella burnetii*, the cause of Q fever, is an obligate intracellular bacterium unrelated phylogenetically to *Rickettsiaceae* or *Anaplasmataceae* and is rarely arthropod transmitted. Serologic diagnosis is available in public health laboratories and is not discussed further here.

Rickettsial infections frequently manifest as fever, headache, myalgias, and malaise. A maculopapular rash is present in approximately 85% of persons with RMSF, 50% with murine typhus, and 30% with HME and is rare in HGA. The nonspecific features make clinical diagnosis difficult. A normal leukocyte count or leukopenia with a left shift, thrombocytopenia, and elevated serum hepatic transaminases are consistent with but not specific for rickettsial infections.

Since the diagnostic standard for rickettsiae remains paired serology (retrospective) and delays in diagnosis and therapy result in increased case severity and fatalities, swift therapy should be initiated based upon clinical suspicion. RMSF may be diagnosed acutely by skin biopsy with demonstration of *R. rickettsii* antigens by direct fluorescent-antibody or immunohistochemical stains (5); PCR is not widely available and remains a research tool (13, 14). Romanowsky-stained peripheral blood or buffy coat smears examined for the presence of intracytoplasmic bacterial clusters called morulae in monocytes (HME) or neutrophils (HGA) are classic but insensitive (10 to 75%) tools. Serology is the most frequently used diagnostic tool now, but PCR promises to be both more sensitive and specific and is applied at the time of active infection.

Classical serologic diagnosis of rickettsial and ehrlichial infections is accomplished with the indirect fluorescent-antibody test. ELISAs offer high throughput and objective determinations but have been less well validated. Only one evaluation of a commercial ELISA for scrub typhus has been published (10), and none have been published for SFG or TG rickettsiae. Other assays, such as solid-phase EIA (dot blot), latex agglutination assay, and immunoblot assays, are not discussed further, since they are rarely used and are not generally commercially available in North America and most of the world. Some countries still use Weil-Felix agglutination of *Proteus* antigens OX-2, OX-19, and OX-K as part of a "febrile aggluti-

nins" panel (8), but poor sensitivity and specificity compromise the clinical value of this test. Alternative serologic tests, such as chimeric antibodies for scrub typhus (9) and monoclonal antibody for RickA (1), and antigen preparations (4) have been reported in the peer-reviewed literature. Tests using recombinant rickettsial and ehrlichial antigens have not been cleared by the FDA for in vitro diagnostic use. Most are not currently commercially available, although some referral laboratories may use them. Current standards do not support reporting results from non-FDA-cleared tests performed at commercial reference laboratories; however, this remains within the purview of each laboratory director.

REFERENCES

1. **Balraj, P., C. Nappez, D. Raoult, and P. Renesto.** 2008. Western-blot detection of RickA within spotted fever group rickettsiae using a specific monoclonal antibody. *FEMS Microbiol. Lett.* **286:**257–262.
2. **Billings, A. N., J. A. Rawlings, and D. H. Walker.** 1998. Tick-borne diseases in Texas: a 10-year retrospective examination of cases. *Tex. Med.* **94:**66–76.
3. **Buller, R. S., M. Arens, S. P. Hmiel, C. D. Paddock, J. W. Sumner, Y. Rikhisa, A. Unver, M. Gaudreault-Keener, F. A. Manian, A. M. Liddell, N. Schmulewitz, and G. A. Storch.** 1999. *Ehrlichia ewingii*, a newly recognized agent of human ehrlichiosis. *N. Engl. J. Med.* **341:**148–155.
4. **Cao, M., H. Guo, T. Tang, C. Wang, X. Li, X. Pan, Z. Jin, and J. Tang.** 2007. Preparation of recombinant antigen of *O. tsutsugamushi* Ptan strain and development of rapid diagnostic reagent for scrub typhus. *Am. J. Trop. Med. Hyg.* **76:**553–558.
5. **Dumler, J. S., W. R. Gage, G. L. Pettis, A. F. Azad, and F. P. Kuhadja.** 1990. Rapid immunoperoxidase demonstration of *Rickettsia rickettsii* in fixed cutaneous specimens from patients with Rocky Mountain spotted fever. *Am. J. Clin. Pathol.* **93:**410–414.
6. **Dumler, J. S., J. P. Taylor, and D. H. Walker.** 1991. Clinical and laboratory features of murine typhus in south Texas, 1980 through 1987. *JAMA* **266:**1365–1370.
7. **Dumler, J. S., and D. H. Walker.** 2005. Rocky Mountain spotted fever—changing ecology and persisting virulence. *N. Engl. J. Med.* **353:**551–553.
8. **Hechemy, K. E., R. W. Stevens, S. Sasowski, E. E. Michaelson, E. A. Casper, and R. N. Philip.** 1979. Discrepancies in Weil-Felix and microimmunofluorescence test results for Rocky Mountain spotted fever. *J. Clin. Microbiol.* **9:**292–293.
9. **Jones, M. L., and R. T. Barnard.** 2007. Use of chimeric antibodies as positive controls in an enzyme-linked immunosorbent assay for diagnosis of scrub typhus (infection by *Orientia tsutsugamushi*). *Clin. Vaccine Immunol.* **14:**1307–1310.
10. **Land, M. V., W. M. Ching, G. A. Dasch, Z. Zhang, D. J. Kelly, S. R. Graves, and P. L. Devine.** 2000. Evaluation of a commercially available recombinant-protein enzyme-linked immunosorbent assay for detection of antibodies produced in scrub typhus rickettsial infections. *J. Clin. Microbiol.* **38:**2701–2705.
11. **McQuiston, J. H., C. D. Paddock, R. C. Holman, and J. E. Childs.** 1999. The human ehrlichioses in the United States. *Emerg. Infect. Dis.* **5:**635–642.
12. **Paddock, C. D., P. W. Greer, T. L. Ferebee, J. Singleton, Jr., D. B. McKechnie, T. A. Treadwell, J. W. Krebs, M. J. Clarke, R. C. Holman, J. G. Olson, J. E. Childs, and S. R. Zaki.** 1999. Hidden mortality attributable to Rocky Mountain spotted fever: immunohistochemical detection of fatal, serologically unconfirmed disease. *J. Infect. Dis.* **179:**1469–1476.
13. **Sexton, D. J., S. S. Kanj, K. Wilson, G. R. Corey, B. C. Hegarty, M. G. Levy, and E. B. Breitschwerdt.** 1994. The use of a polymerase chain reaction as a diagnostic test for Rocky Mountain spotted fever. *Am. J. Trop. Med. Hyg.* **50:**59–63.
14. **Standaert, S. M., T. Yu, M. A. Scott, J. E. Childs, C. D. Paddock, W. L. Nicholson, J. Singleton, Jr., and M. J. Blaser.** 2000. Primary isolation of *Ehrlichia chaffeensis* from patients with febrile illnesses: clinical and molecular characteristics. *J. Infect. Dis.* **181:**1082–1088.

11.7.2 Indirect Fluorescent-Antibody Test

I. PRINCIPLE

Indirect fluorescent antibody (IFA), also called indirect immunofluorescence or microimmunofluorescence, tests are the most frequently used and best characterized serological tests for rickettsial and ehrlichial infections (3, 6, 7, 9, 11, 12, 15, 16). IFA detection of human antibodies to antigens of *Rickettsia*, *Ehrlichia*, and *Anaplasma* species (rickettsial antigens) is accomplished by reacting serum from the patient with rickettsial antigens immobilized onto glass slides. Antibodies, if present in the serum, are detected with a fluorescently labeled anti-human isotype reporter. The reporter signal is detected by UV microscopy using appropriate filters to detect fluorescence that highlights the characteristic morphology of the bacteria. In a patient with a consistent epidemiological history and clinical illness, detection of a high titer (e.g., $\geq$256) of specific antibodies in a single serum sample has historically been considered suggestive of an acute rickettsial infection. However, only demonstration of seroconversion or a fourfold increase in immunoglobulin G (IgG) antibody titer in paired sera enables definitive diagnosis of Rocky Mountain spotted fever (RMSF), human monocytic ehrlichiosis (HME), and human granulocytic anaplasmosis (HGA) (5).

II. SPECIMEN COLLECTION

Observe standard precautions.

Serum for rickettsial serology should be obtained by venipuncture into either standard tubes for serum (red top) or serum separator tubes. Paired samples should be obtained (during acute illness and during convalescence 2 to 4 weeks later). Some commercial assays permit assay of antibodies in heparinized plasma, whole blood, and capillary blood, but the use of serum is strongly recommended. For RMSF, antibody is rarely detected as early as 3 days after onset of illness, but nearly all patients have developed specific antibodies by 21 days (7, 12, 14). For murine typhus, more than 50% of patients are seropositive within the first 6 days of illness, and 100% are seropositive by 13 to 15 days (10). For both HME and HGA, up to 40% of patients are seropositive at presentation, and nearly all are seropositive within 1 month (2, 9). Acute-phase samples should be stored frozen and tested at the same time as convalescent-phase samples. If serum cannot be tested within 24 h, it should be stored at -20°C.

III. MATERIALS

There are few suppliers of high-quality antigen slides or IFA kits for detection of rickettsial and ehrlichial antibodies (Table 11.7.2–1). In addition, the CDC and some public health laboratories provide or use dimethyl sulfoxide-stabilized frozen infected cell antigen suspensions that may be applied to glass slides as needed. Antigen preparations are obtained from whole infected cells or highly purified cell-free bacterial suspensions which are then immobilized onto multiwell, Teflon-coated glass slides. Some include two or more rickettsial or ehrlichial antigens within a single well on the slide or two species (e.g., *Rickettsia rickettsii* and *Rickettsia typhi*) in adjacent wells, the latter of which allows for comparison of titers by species. In addition, some kits include antigen slides, conjugates, positive control sera, negative control sera, IgM removal diluent, mounting medium, and buffered diluent. Not supplied are additional supplies such as coverslips for the antigen

Table 11.7.2–1 Manufacturers of rickettsial and ehrlichial serodiagnostic kits and reagents[a]

Antigen (disease)	Kit type according to manufacturer		
	Focus Diagnostics[b]	PanBio Diagnostics (not available in the United States)[c]	SciMedx Corp.[d]
Anaplasma phagocytophilum (HGA)	IFA	IFA (IgG only)	
Ehrlichia canis		IFA	IFA
Ehrlichia chaffeensis (HME)	IFA	IFA	IFA
Orientia tsutsugamushi (scrub typhus)		ELISA	IFA
Rickettsia prowazekii (epidemic typhus)		IFA	IFA
Rickettsia typhi (murine typhus)	IFA	IFA	IFA
Rickettsia conorii (Mediterranean spotted fever)	IFA		IFA
Rickettsia rickettsii (RMSF)	IFA	IFA, ELISA	IFA
Rickettsia spotted fever group IgG and IgM	IFA	IFA (IgM)	

[a] Check with each manufacturer regarding regional availability.

[b] Focus Diagnostics, 10703 Progress Way, Cypress, CA 90630-4717. Telephone: (714) 220-1900, (800) 445-0185. Fax: (714) 220-1683. URL: http://www.focusdx.com/focus/0-home/index.asp.

[c] PanBio, Limited—Australia, 116 Lutwyche Rd., Windsor, Brisbane 4030, Queensland, Australia. Telephone: 61 (0) 7 3363 7100. Fax: 61 (0) 7 3363 7199. E-mail: PanBio@PanBio.com. URL: http://www.panbio.com.

[d] SciMedx Corporation, 100 Ford Rd., Suite 100-08, Denville, NJ 07834. Telephone: (800) 221-5598 or (international) 1-973-625-8822. Fax: 1-973-625-8796. E-mail: info@scimedx.com. URL: http://www.scimedx.com/products/ifa_infectious.htm.

III. MATERIALS *(continued)*

slides, test tubes and racks for making serum dilutions, a humidified chamber for incubation of slides, and a timer. A fluorescent microscope with appropriate excitation filters is required to examine stained slides.

IV. PROCEDURE

In general, IFA procedures for the various species are similar and have several steps. One should record the placement of each serum or control sample into specific wells first.

Observe standard precautions.

A. Slides should be warmed to room temperature.

B. Add a small volume (usually 10 to 25 µl) of each appropriate dilution of the positive control, negative control, patient sera, and patient sera depleted of IgG (for IgM detection) to the appropriate well on a multiwell antigen slide. Some manufacturers supply control sera in a prediluted format. Dilutions are determined by the manufacturers, but most agree that a screening dilution of 1:64 should be used for IgG and polyvalent tests; dilutions for IgM tests may vary.

C. Incubate slides as required by the manufacturer (generally 30 min to 1 h at ambient temperature or at 37°C). A humidified chamber may be needed to avoid evaporation.

D. Rinse and wash slides by one or more immersions in buffer, and then remove buffer salts by rinsing briefly with distilled water. Next, air dry slides.

E. Add an appropriate volume (usually 10 to 25 µl) of isotype-specific conjugate to each well.

F. Repeat the incubation and wash steps (steps C and D). Some protocols include a step for counterstaining with reagents such as Evan's blue or Eriochrome black prior to or during the final wash step.

G. After the slides are dry, add a small volume (10 to 25 µl) of mounting medium, usually a glycerol–phosphate-buffered saline mixture, to each well.

H. Place coverslips over the wells on the slides without trapping air bubbles. Do not use excessive mounting medium, since it will seep from under the coverslip and contaminate the microscope objectives or emersion oil (if used). Excess mounting medium may be removed by capillary action with absorbent blotting paper.

IV. PROCEDURE *(continued)*

I. Examine slides at a final magnification of between ×200 and ×1,000 using a fluorescence microscope with appropriate filters to allow excitation and emission for fluorescein isothiocyanate.

J. If the slides cannot be viewed immediately, keep them at 4°C in the dark for 24 h.

Since a fourfold change in titer offers the most sensitive seroconfirmation, sera that react at the screening dilution should be titrated. To do this, dilute sera serially (after removing IgG for IgM titer determinations) in doubling dilutions starting at the screening dilution (usually 1:64) until the end point (where no definite fluorescent bacteria are identified) is reached.

A reactive sample allows definitive fluorescent visualization of the bacterial morphology. *Rickettsia* spp. appear as small (~0.3 by 1.5-μm) bacilli or coccobacilli, whereas *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* appear as tiny (0.2- by 1.0-μm) coccobacilli and cocci. Some antigen preparations use bacteria free of host cells, which are prepared in chicken embryo yolk sacs or by density gradient purification from infected tissue culture cells. When bacteria are purified from yolk sac, a diluent that includes normal yolk sac as a 10% suspension in diluting buffer should be used to absorb any antibodies reactive with yolk sac antigens and decrease background staining. Antigen preparations derived from whole infected cells (*R. rickettsii* or *R. typhi* grown in Vero or other cell lines, *E. chaffeensis* grown in DH82 or Vero cells, or *A. phagocytophilum* grown in HL-60 cells) may have appearances different from those in cell-free preparations. In general, it is best to only interpret bacteria that are within cells in these preparations. With *R. rickettsii* and *R. typhi*, short bacilli will be found within the host cell's cytoplasm (or occasionally in the nucleus for *R. rickettsii*). With *E. chaffeensis* and *A. phagocytophilum*, clusters or inclusions of coccobacilli and cocci will be found in the cytoplasm of the host cells.

Examine slides for the presence of fluorescent bacteria in several locations, as staining intensity may vary considerably even in a single well. Bacteria at the periphery of the well should not be evaluated. Various intensities of fluorescence may be observed, and many manufacturers suggest recording the intensity in a semiquantitative manner (0 to 4+). However, the best practice is to examine the well of each patient sample to define the morphology, distribution, and quantity of intracellular bacteria compared with the positive and negative control sample wells. This is often best accomplished at high magnifications, such as ×1,000. The positive control sample will provide the approximate distribution and an appropriate comparative morphology, whereas the negative control will provide a meaningful level of background fluorescence. Fluorescence of bacterial morphology in samples that does not exceed that identified on the negative control should be considered negative. Optimally, fluorescent bacteria should not appear in negative control samples. If they do, the test should be repeated.

V. INTERPRETATION

IFA testing to detect antibodies to *R. rickettsii, R. typhi, E. chaffeensis,* and *A. phagocytophilum* is the most sensitive diagnostic method (Table 11.7.2–2). In general, it is a good practice to establish appropriate screening and cutoff titers for positive samples in each laboratory. This situation is not possible for most clinical laboratories owing to the lack of sufficient numbers of well-characterized samples. Thus, most laboratories and laboratory directors rely upon the validation performed by the manufacturer. In most cases, the CDC case definition provides a serologic cutoff that is widely accepted and corroborated by manufacturers of rickettsial and ehrlichial serodiagnostic tests. For IFA testing, the current CDC seroepidemiological criterion for diagnostic confirmation of suspected RMSF (5) is a fourfold

Table 11.7.2–2 Sensitivity and specificity of serologic tests for confirmation of RMSF, murine typhus, HME, and HGA

Disease	Serologic assay	Sensitivity (%)	Specificity (%)
RMSF	IFA IgG	89–100	99–100
	IFA IgM	83–85	100
Murine typhus	IFA IgG	≥83	≥93
	IFA IgM	53–85	99
Scrub typhus	IFA IgG	91	96
	IFA IgM	85	98
	ELISA IgG	97	89–92
	ELISA IgM	93–94	91–94
HME	IFA IgG	88	Not determined
	IFA IgM	86	Not determined
HGA	IFA IgG	82–100	82–100
	IFA IgM	27–37	83–100

V. INTERPRETATION *(continued)*

change in titer of antibody to *R. rickettsii* antigen; for a serologic test that screens samples at a dilution of 1:64, this implies a minimum titer of 128 if the acute-phase titer is <64. Similar CDC laboratory criteria exist for confirmation of the diagnosis of HME and HGA in clinically suspect cases (5). Some suppliers recommend that initial dilutions of sera begin at 1:8, and a fourfold increase would occur with a minimum titer of 16 or 32. However, prior studies suggest that dilutions lower than 1:64 often result in nonspecific fluorescence; thus, a minimum titer of 64 would be required before any sample is considered specifically reactive with *R. rickettsii*, *E. chaffeensis*, or *A. phagocytophilum.*

Since antibodies are rarely detected in patients during the early phases of RMSF and murine typhus, occur infrequently (≤30% of patients) in those during early phases of HME or HGA, and could represent old infection if present, a convalescent-phase serum sample (obtained 2 to 4 weeks later) should be tested with the acute-phase sample as paired sera (2, 9, 12, 14). More than 99% of patients with HGA and HME are IgG seropositive at 1 month postinfection; however, IgG or polyvalent reactions may persist for years. IgM antibodies reportedly decline 45 to 60 days after infection and are, therefore, often used to support a diagnosis of acute infection; however, IgM antibodies may persist for a year or more, and seroconversion or a fourfold rise in IgG is required to confirm acute infection.

Overall, serologic testing for antibodies reactive with *R. rickettsii, R. typhi, E. chaffeensis,* and *A. phagocytophilum* should be conducted only when a reasonable level of clinical suspicion exists. Although the specificity is relatively high (≥92%), the predictive value of a positive test is only moderate unless the prevalence of the infection or the pretest probability of infection is relatively high. Likewise, since sensitivity is high in all of these tests (88 to 100%), the predictive value of a negative test is very high when paired acute- and convalescent-phase samples are compared. The predictive value of a negative test is low with acute infection (generally <40%). Since more patients will have serologic evidence of infection by *R. rickettsii, E. chaffeensis,* and *A. phagocytophilum* than report clinical disease, examination of paired (acute- and convalescent-phase) sera is preferred. Whether high seroprevalence reflects subclinical infections or antigenic stimulation with cross-reactive antigens or "nonpathogenic" species is undetermined. Recently, *Rickettsia parkeri* was recognized as a cause of tick-transmitted spotted fever group infection with clinical manifestations milder than those of RMSF (13). The true incidence of *R. parkeri* infection and potentially other mild spotted fever group rickettsioses that could stimulate serodiagnostic levels of RMSF titers is not known and is under active study.

V. INTERPRETATION
(continued)

Many preexisting conditions may predispose patients to false-positive serologic reactions for *Rickettsia*, *Ehrlichia*, and *Anaplasma* spp. (Table 11.7.2–3). *R. rickettsii* IFA testing detects antibodies broadly reactive with spotted fever group rickettsiae and more weakly reactive with typhus group rickettsiae; the converse is true for reactions with *R. typhi.* Thus, seroconfirmation of RMSF or murine typhus is dependent upon a strong clinical history consistent with a geographical prevalence of infection, or upon more specific serologic tests conducted in reference and public health laboratories. A beneficial aspect of these nonspecific serologic tests is the ability to use them to detect antibodies reactive with other spotted fever group or typhus group rickettsiae. Thus, these assays can be useful for diagnosis of rickettsial infections that are domestically rare (*Rickettsia akari*, *R. parkeri*, or *R. felis*) or may have been acquired during international travel, including such agents as *Rickettsia conorii* (Mediterranean spotted fever), *Rickettsia africae* (African tick bite fever), or *Rickettsia prowazekii* (epidemic typhus), among many. Occasional cross-reactions with other infectious agents are also observed, but under ordinary circumstances, the cross-reactive titers are at least fourfold lower than the titer for the homologous rickettsial infectious agent.

Similarly, serologic tests for *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* may yield cross-reactions with each other and other related species (8, 16). For example, *Ehrlichia ewingii* induces antibodies reactive with *E. chaffeensis*, as do veterinary infections with other *Ehrlichia* spp. (*E. canis*, *E. muris*, and *E. ruminantium*) and some *Anaplasma* spp. (*A. phagocytophilum*) (4). In general, most infections result in a fourfold-higher titer of IFA to the homologous agent; however, a high degree of clinical suspicion is required for the highest predictive value, since

Table 11.7.2–3 Selected conditions that may lead to false-positive serologic tests for rickettsial diseases

Condition	Agent for which condition is confounding			
	R. rickettsii	*R. typhi*	*E. chaffeensis*	*A. phagocytophilum*
Spotted fever		+	+	±
Typhus	+		+	±
HME				+
HGA			+	
Syphilis	+			
Lyme disease			+	
Leptospirosis	+			
Q fever			+	+
Brucella infection			+	
Bartonella sp. infection	+			
Cholera	+			
Typhoid		+		
Shigellosis	+	+		
Malaria	+	+		
Epstein-Barr virus infection			+	+
Dengue	+			
Rheumatoid factor	+	+	+	+
ANA[a]	+	+		+
ANCA[b]				+
Antiplatelet antibody				+
Pregnancy	+			

[a] ANA, antinuclear antibody.

[b] ANCA, antineutrophil cytoplasmic antibody.

V. INTERPRETATION
(continued)

antigens are not available for all *Ehrlichia* and *Anaplasma* spp. Approximately 3 to 30% of patients with HGA will develop antibodies reactive with *E. chaffeensis*, but the diagnosis of a few (<10%) will not be resolved by comparison of titers.

Sera from patients with HGA and HME will also react in some serologic tests for Lyme disease (17, 19). Cases of *Borrelia burgdorferi-A. phagocytophilum* coinfection have also been described. Thus, *B. burgdorferi* and ehrlichial serologies must be carefully interpreted in the context of the epidemiological history and clinical manifestations.

Since *A. phagocytophilum*-infected HL-60 cells of human derivation are often used, whole-cell-infected antigen preparations for serologic diagnosis of *A. phagocytophilum* may yield false-positive results for patients with autoimmune disorders, including those with antinuclear, antiplatelet, and anti-smooth muscle antibodies (18). False-positive tests can be minimized by adherence to careful morphological criteria for interpretation and examination at a high magnification (usually $\times$1,000).

VI. QUALITY ASSURANCE

Include QC information on reagent container and in QC records.

A positive and negative control serum sample should be tested in parallel with all patient specimens. Most commercially available kits will provide both and will give specific instructions about their preparation and use and interpretation of related results. In general, positive control samples have been obtained from individual patients who have had a rickettsial infection or from pools of sera of such patients; separate controls may be provided for IgG and IgM analyses. Negative control samples are generally derived from sera of healthy individuals without a history of a rickettsial infection and may be supplied as either individual samples or pools from a number of individuals. Controls should be diluted and tested in parallel with clinical samples. If prediluted control sera from the manufacturer are used, additional positive and negative control sera of established titer should be used to control for problems with diluting or dilution buffers. Each positive control serum provided by commercial manufacturers will have a target titer that should be confirmed at least with each new lot of antigen slides and new lot of conjugate. Manufacturers' instructions should be followed and often suggest replication of stated titers within twofold dilutions. Each new lot of conjugate should be tested for reactivity at the recommended dilution in the absence of any primary serum to ensure that direct reaction with the antigen slides is not occurring.

Should positive control serum not react or not achieve the stated range of titer, or if negative control serum should become reactive, clinical test results should not be reported and the tests instead should be repeated after appropriate troubleshooting. Periodic review of clinical records to confirm the plausibility of positive results is desirable, so that the laboratory director can promptly identify and investigate aberrant testing practices or results. Currently, no proficiency testing program is available from the CAP.

REFERENCES

1. **Aguero-Rosenfeld, M. E., F. Kalantarpour, M. Baluch, H. W. Horowitz, D. F. McKenna, J. T. Raffalli, T. Hsieh, J. Wu, J. S. Dumler, and G. P. Wormser.** 2000. Serology of culture-confirmed cases of human granulocytic ehrlichiosis. *J. Clin. Microbiol.* **38:**635–638.
2. **Bakken, J. S., I. Haller, D. Riddell, J. J. Walls, and J. S. Dumler.** 2002. The serological response of patients infected with the agent of human granulocytic ehrlichiosis. *Clin. Infect. Dis.* **34:**22–27.
3. **Brouqui, P., E. Salvo, J. S. Dumler, and D. Raoult.** 2001. Diagnosis of granulocytic ehrlichiosis in humans by immunofluorescence assay. *Clin. Diagn. Lab. Immunol.* **8:**199–202.
4. **Buller, R. S., M. Arens, S. P. Hmiel, C. D. Paddock, J. W. Sumner, Y. Rikhisa, A. Unver, M. Gaudreault-Keener, F. A. Manian, A. M. Liddell, N. Schmulewitz, and G. A. Storch.** 1999. *Ehrlichia ewingii*, a newly recognized agent of human ehrlichiosis. *N. Engl. J. Med.* **341:**148–155.

REFERENCES *(continued)*

5. **Centers for Disease Control and Prevention.** 2009, posting date. Case Definitions. ••••
6. **Childs, J. E., J. W. Sumner, W. L. Nicholson, R. F. Massung, S. M. Standaert, and C. D. Paddock.** 1999. Outcome of diagnostic tests using samples from patients with culture-proven human monocytic ehrlichiosis: implications for surveillance. *J. Clin. Microbiol.* **37:**2997–3000.
7. **Clements, M. L., J. S. Dumler, P. Fiset, C. L. Wisseman, Jr., M. J. Snyder, and M. M. Levine.** 1983. Serodiagnosis of Rocky Mountain spotted fever: comparison of IgM and IgG enzyme-linked immunosorbent assays and indirect fluorescent antibody test. *J. Infect. Dis.* **148:**876–880.
8. **Comer, J. A., W. L. Nicholson, J. G. Olson, and J. E. Childs.** 1999. Serologic testing for human granulocytic ehrlichiosis at a national referral center. *J. Clin. Microbiol.* **37:**558–564.
9. **Dawson, J., D. Fishbein, T. Eng, M. Redus, and N. Greene.** 1990. Diagnosis of human ehrlichiosis with the indirect fluorescent antibody test: kinetics and specificity. *Ann. N. Y. Acad. Sci.* **590:**308.
10. **Dumler, J. S., J. P. Taylor, and D. H. Walker.** 1991. Clinical and laboratory features of murine typhus in south Texas, 1980 through 1987. *JAMA* **266:**1365–1370.
11. **Magnarelli, L. A., J. W. Ijdo, J. S. Dumler, R. Heimer, and E. Fikrig.** 1998. Reactivity of human sera to different strains of granulocytic ehrlichiae in immunodiagnostic assays. *J. Infect. Dis.* **178:**1835–1838.
12. **Newhouse, V. F., C. C. Shepard, M. D. Redus, T. Tzianabos, and J. E. McDade.** 1979. A comparison of the complement fixation, indirect fluorescent antibody, and microagglutination tests for the serological diagnosis of rickettsial diseases. *Am. J. Trop. Med. Hyg.* **28:**387–395.
13. **Paddock, C. D., R. W. Finley, C. S. Wright, H. N. Robinson, B. J. Schrodt, C. C. Lane, O. Ekenna, M. A. Blass, C. L. Tamminga, C. A. Ohl, S. L. McLellan, J. Goddard, R. C. Holman, J. J. Openshaw, J. W. Sumner, S. R. Zaki, and M. E. Eremeeva.** 2008. *Rickettsia parkeri* rickettsiosis and its clinical distinction from Rocky Mountain spotted fever. *Clin. Infect. Dis.* **47:**1188–1196.
14. **Philip, R. N., E. A. Casper, R. A. Ormsbee, M. G. Peacock, and W. Burgdorfer.** 1976. Microimmunofluorescence test for the serological study of Rocky Mountain spotted fever and typhus. *J. Clin. Microbiol.* **3:**51–61.
15. **Walker, D. H.** 2000. Diagnosing human ehrlichiosis: current status and recommendations. *ASM News* **66:**287–290.
16. **Walls, J. J., M. Aguero-Rosenfeld, J. S. Bakken, J. L. Goodman, D. Hossain, R. C. Johnson, and J. S. Dumler.** 1999. Inter- and intralaboratory comparison of *Ehrlichia equi* and human granulocytic ehrlichiosis (HGE) agent strains for serodiagnosis of HGE by the immunofluorescent-antibody test. *J. Clin. Microbiol.* **37:**2968–2973.
17. **Wong, S. J., G. S. Brady, and J. S. Dumler.** 1997. Serological responses to *Ehrlichia equi*, *Ehrlichia chaffeensis*, and *Borrelia burgdorferi* in patients from New York State. *J. Clin. Microbiol.* **35:**2198–2205.
18. **Wong, S. J., and J. A. Thomas.** 1998. Cytoplasmic, nuclear, and platelet autoantibodies in human granulocytic ehrlichiosis patients. *J. Clin. Microbiol.* **36:**1959–1963.
19. **Wormser, G. P., H. W. Horowitz, J. S. Dumler, I. Schwartz, and M. Aguero-Rosenfeld.** 1996. False-positive Lyme disease serology in human granulocytic ehrlichiosis. *Lancet* **347:**981–982.

11.7.3 ELISA

I. PRINCIPLE

Circulating rickettsial antibodies can also be detected using rickettsial antigens bound to plastic on a 96-well plate (1, 4). Antibodies are detected by measuring a reporter signal generated in a substrate solution using a specialized spectrophotometer (ELISA reader). Current commercially available ELISAs are not quantitative and therefore cannot be used to assess the fourfold change in immunoglobulin G (IgG) titer required to confirm a diagnosis of acute infection. Detecting elevated IgG or IgM antibodies by ELISA only suggests acute infection, since they may not be specific for the agent or may represent a persistent response to an infection months earlier. The strengths of ELISA are the objective readout (based upon a positive cutoff value) and the ability to rapidly screen large numbers of samples (high throughput).

II. SPECIMEN COLLECTION

Observe standard precautions.

Serum samples are obtained for ELISA as recommended for immunofluroscence.

III. MATERIALS

ELISA kits contain needed reagents, including antigen-coated microwell strips, buffers, color-coded prediluted reagents, positive and negative control sera, cutoff calibrator serum, substrate solution, and stop solution. Additional required supplies include a 37°C incubator, pipettors, and a microplate ELISA reader that measures optical density (OD) at 450 nm or other wavelengths specified by the manufacturer; a multiwell plate washer is recommended.

IV. PROCEDURE

Observe standard precautions.

Specific instructions from the manufacturer should be followed precisely.

A. Prepare sufficient numbers of break-apart well strips for patient identification.

B. Add serum to the provided diluent to achieve a 1:100 dilution. Add 100 µl of diluted serum to each well and incubate for 30 min at 37°C.

C. Wash wells six times with wash buffer and then remove excess wash buffer, add 100 µl of prediluted conjugate to each well, and reincubate wells at 37°C for 30 min.

D. After six more washes, add 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) or appropriate substrate to each well and incubate for 10 min at room temperature. Then, add 100 µl of stop solution to halt the reaction.

E. Use an automated ELISA microplate reader capable of measuring OD at 450 nm or other recommended wavelengths to detect color in each well. The OD in each well is adjusted for background color in control wells with only diluting buffer to yield a net OD. Average the results for duplicate or triplicate wells, if used.

V. INTERPRETATION

In commercially available kits, a calibrator cutoff serum that is run in triplicate is provided; an OD between 9 and 11 times the average calibrator cutoff value is considered equivocal, and one >11 is considered reactive. Each run should include a positive and negative control. Serum yielding an equivocal result should be retested, or, ideally, a convalescent-phase sample should be tested simultaneously. Although the net OD has been used by some to approximate the titer by indirect fluorescent-antibody (IFA) testing (4, 5), there is not a precise correlation; therefore, ELISA results should be considered qualitative.

ELISA has been broadly applied as a seroepidemiological tool and occasionally as a serodiagnostic tool for patients with spotted fever group rickettsiosis, murine or epidemic typhus, and scrub typhus, although reports with careful correlation with the standard IFA test have been infrequent (2–5). Key findings are a relative correlation of ELISA OD with IFA titer and the detection of both IgM and IgG antibodies earlier by ELISA than by IFA. Sensitivities and specificities are similar (5).

VI. QUALITY ASSURANCE

Include QC information on reagent container and in QC records.

QA practices are identical to those for IFA testing, except for the use of calibrator cutoff sera. If positive control and calibrator cutoff sera do not fall within the stated acceptable range, the test is considered invalid, results should not be reported, and the test should be repeated.

REFERENCES

1. **Clements, M. L., J. S. Dumler, P. Fiset, C. L. Wisseman, Jr., M. J. Snyder, and M. M. Levine.** 1983. Serodiagnosis of Rocky Mountain spotted fever: comparison of IgM and IgG enzyme-linked immunosorbent assays and indirect fluorescent antibody test. *J. Infect. Dis.* **148:**876–880.
2. **Jang, W. J., M. S. Huh, K. H. Park, M. S. Choi, and I. S. Kim.** 2003. Evaluation of an immunoglobulin M capture enzyme-linked immunosorbent assay for diagnosis of *Orientia tsutsugamushi* infection. *Clin. Diagn. Lab. Immunol.* **10:**394–398.
3. **Keysary, A., and C. Strenger.** 1997. Use of enzyme-linked immunosorbent assay techniques with cross-reacting human sera in diagnosis of murine typhus and spotted fever. *J. Clin. Microbiol.* **35:**1034–1035.
4. **Land, M. V., W. M. Ching, G. A. Dasch, Z. Zhang, D. J. Kelly, S. R. Graves, and P. L. Devine.** 2000. Evaluation of a commercially available recombinant-protein enzyme-linked immunosorbent assay for detection of antibodies produced in scrub typhus rickettsial infections. *J. Clin. Microbiol.* **38:**2701–2705.
5. **Newhouse, V. F., C. C. Shepard, M. D. Redus, T. Tzianabos, and J. E. McDade.** 1979. A comparison of the complement fixation, indirect fluorescent antibody, and microagglutination tests for the serological diagnosis of rickettsial diseases. *Am. J. Trop. Med. Hyg.* **28:**387–395.

11.8 Immunoassay Detection of Shiga Toxin-Producing *Escherichia coli*

Shiga toxin-producing *Escherichia coli* (STEC) organisms are responsible for a broad range of gastrointestinal illnesses, from mild watery diarrhea to severe hemorrhagic colitis associated with hemolytic-uremic syndrome (HUS) (1, 5, 21, 30, 34, 36). These organisms cause disease primarily through elaboration of one or more Shiga toxins (Stx1, Stx1 variants, Stx2, and Stx2 variants) encoded by genes carried on lambda bacteriophages (10, 14, 34, 36). Research supports the fact that hemorrhagic colitis and HUS likely result from the action of these toxins on vascular endothelium since Stx targets digalactosyl receptors on microvascular endothelial cells in the gut, kidneys, and brain (30, 34, 36). *E. coli* organisms that produce Stx2 alone have been found to be more virulent than isolates that have Stx1 alone or that produce Stx1 and Stx2 in combination (34). Enhanced virulence may also be related to host factors and genetic differences among strains. Some investigators have reported differences in the frequency and distribution of Stx genes in the type of clinical disease reported (24, 30). Hence, genotyping may be important not only in characterizing particular outbreaks but also in predicting disease severity and treatment (18, 24, 30). Among other organism virulence factors important for pathogenesis are an adhesin, intimin, encoded by an *eae* gene, and enterohemorrhagic *E. coli* (EHEC) hemolysin (*ehxA* gene), a potent cytolysin (34, 36).

E. coli O157:H7 is the major serotype associated with illness in North America. In the United States, in 2007, the reported incidence of *E. coli* O157:H7 was approximately 1.20 cases/100,000 population; for STEC non-O157, it was 0.57 case/100,000 population (8). Although the number of laboratories routinely testing for these organisms has increased over the last decade, these values are still likely to be underestimates, since many laboratories do not routinely evaluate for STEC.

STEC incidence is higher in rural than in urban communities (36). O157:H7 is more prevalent in certain geographic areas such as the western, midwestern, and northern regions of the United States. In the 1980s, outbreaks of O157:H7 were associated primarily with fast-food restaurants and undercooked hamburger meat (5, 36). Since then, cases have more often been sporadic. A variety of other foods, such as spinach, sprouts, apple cider, and dairy products, and water sources have been reported as vehicles of transmission and have been implicated in outbreaks (5, 7, 8, 34, 36). Most STEC infections occur in the summer and fall. Person-to-person spread and contact with animals have emerged as important methods of spread (5, 34, 36). Children and the elderly are the individuals most at risk for development of hemorrhagic colitis and HUS (1, 34, 36).

Over 100 non-O157 serotypes of *E. coli* produce one or both Shiga toxins, and over 50 serotypes have been associated with hemorrhagic colitis or HUS (34, 36). In Europe and Australia, non-O157 serotypes predominate (34). Small outbreaks of non-O157 serotypes have been reported in the United States, and the reported incidence of non-O157 serotypes has increased as more laboratories have added testing using Stx EIAs. Between 1983 and 2002, the CDC conducted national surveillance for non-O157 STEC and found that the most common serogroups in the United States are STEC O26 (22%), O111 (16%), O103 (12%), O121 (8%), and O45 (7%) (5). Those laboratories that have surveyed for them routinely have found that non-O157 STEC may cause disease equal in severity to illness caused by O157 (5, 18, 36). More recent systematic surveys have demonstrated that the non-O157 strains tend to be more heterogeneous with respect to possession or expression of virulence genes and in their propensity to cause bloody diarrhea and HUS (18, 21, 34, 36).

E. coli O157:H7 can be detected on routine stool screening using sorbitol-MacConkey agar (SMAC) or other selective and differential media that readily take advantage of the phenotypic differences between O157 (non-sorbitol fermenting) and other *E. coli* serotypes, which are sorbitol fermenting. However, non-O157 STEC organisms that cause disease cannot be readily detected using SMAC (13, 20, 21, 29).

There are several methods available to laboratories for toxin detection. These include cell culture techniques, commercial EIAs, rapid immunochromatographic assays, passive latex agglutination methods, and amplification technologies (2, 6, 10, 15, 19, 20, 22, 23, 25, 26, 29, 31, 32, 35). Commercial kits can be used to detect toxins directly in the stool samples, in broth-enhanced stool cultures, and from colony sweeps of confirmed *E. coli* organisms growing on agar media (2, 6, 10, 12, 15, 19, 20, 22, 23, 25, 26, 28, 29, 31–33, 35).

The advantage of using a method that detects toxins responsible for disease is that theoretically all serotypes associated with hemorrhagic colitis and HUS could readily be detected. Such methods are also rapid, allowing a prompt distinction between STEC diarrhea and diarrhea attributable to other infectious etiologies and noninfectious syndromes. The disadvantage of this procedure when used alone is that an organism is not available for ser-

otyping and additional epidemiological studies, such as tracing the source of outbreaks (36). In addition, false-positive results have led to failure to appropriately characterize community outbreaks of diarrhea (7, 9). The CDC recommends simultaneous use of both culture on selective media and a test for toxin by nonculture methods routinely by all laboratories that test patients with acute diarrhea (7). However, an issue for laboratories is cost. Commercial kits for the detection of toxins are severalfold more expensive than culture-based screening. Clinical microbiologists and clinicians need to decide whether local prevalence justifies routine testing of all stool samples submitted (15, 20).

I. PRINCIPLE

Commercial immunoassays for the detection of Stx1 and Stx2 consist of the following methods: (i) EIAs, (ii) immunochromatographic rapid tests, (iii) optical immunoassays, and (iv) latex agglutination. The classic EIAs are performed in a standard microplate format. Available kits use either monoclonal or affinity-purified polyclonal anti-Shiga toxin capture antibodies bound to microwells to bind, or capture, the toxin present in stool specimens or culture systems. After incubation at room temperature, unbound material is washed from the microtiter wells. A secondary enzyme-conjugated antibody is added, followed by incubation at room temperature and washing to remove unbound conjugate. Substrate for the enzyme is added. If toxin is present, a colorimeter reaction ensues as the antibody-enzyme complex converts the substrate to a reaction product. The color development can be detected visually or spectrophotometrically (28, 33). A more rapid version of the immunoassay is offered in single-use device immunochromatographic tests. These consist of a plastic holder with a well into which stool or broth samples are placed. The device holds a nitrocellulose membrane that contains colloidal-gold-labeled monoclonal antibodies to both Stx1 and Stx2 (27, 31) or to *E. coli* O157 (22, 35). Depending upon the test, antigen or toxin complexes with antibodies in the reaction zones as the test sample migrates over a membrane, forming a sandwich complex with immobilized antibody to antigen or toxins. A colorimetric reaction occurs in the presence of antigen or toxin that is compared to an internal control or a chart provided by the manufacturer (22, 31). These assays can be used directly on stool samples or on broth specimens. An assay for the detection of Stx1 and Stx2 is manufactured by Merck and marketed as both the ImmunoCard STAT! EHEC assay (Meridian Bioscience, Inc., Cincinnati, OH), an FDA-cleared assay, and the Duopath verotoxin assay (Merck, Darmstadt, Germany), which is not FDA cleared for use on clinical samples (16, 25, 27, 31). The ImmunoCard STAT! *E. coli* O157 Plus is FDA approved for the detection of *E. coli* O157:H7 (22, 26). Optical immunoassay technology allows visual detection due to a physical change in the optical thickness of a thin silicon wafer coated with heterologous anti-Shiga toxin antibodies. Specimen potentially containing toxin is mixed with a conjugate (rabbit antibodies conjugated to horseradish peroxidase) in the reaction tube and then placed into the test device. After 10 min of incubation, the surface is washed vigorously with the wash solution, providing a clean test surface. The test device is then closed, allowing for the removal of residual moisture from the test surface. A substrate (tetramethylbenzidine and hydrogen peroxide) is added, and the specimen is incubated for 5 min before a final wash. If toxin is present, a change in optical thickness produces a visible color change (17, 37).

Reverse passive latex agglutination uses latex particles sensitized with antibodies against Stx1 and Stx2. Test samples are mixed with polymyxin B to enhance toxin release and then added to the latex particles. These particles agglutinate and settle diffusely over the base of a microtiter plate well when mixed with specimens containing toxin. If no toxins are present, the latex particles will settle to form a tight button (6, 10, 12).

II. SPECIMEN TYPES, COLLECTION, AND TRANSPORT

Observe standard precautions.

Patients should be instructed to excrete directly into a clean, dry container and not to take a specimen from the toilet water. The specimen should be free of urine. Stool specimens should be transported to the laboratory as quickly as possible at 2 to 8°C (refrigeration temperatures). Diarrheal stool specimens are preferable to rectal swabs. The latter, if collected, should have visible fecal matter and should be placed immediately into transport media. Specimens that will also be cultured for

II. SPECIMEN TYPES, COLLECTION, AND TRANSPORT *(continued)*

enteric pathogens should be placed in Cary-Blair or another enteric transport medium if transport time exceeds 2 h.

Testing can be performed directly on stool samples, on broth samples after overnight incubation, and on culture plates after incubation at 16 to 24 h at 37°C. For EIAs, stool samples may be stored at 2 to 8°C for up to 7 days. If testing is not to be performed within that time frame, samples should be frozen at −20 to −70°C. Repeated freeze-thawing of samples should be avoided because it leads to degradation of toxin.

III. MATERIALS

Commercial monoclonal and polyclonal reagents used in the immunoassays typically come packaged as kits or one-time-use devices. All reagents necessary for test performance are included. Examples of additional reagents supplied include positive and negative control materials, specimen diluent, wash buffers, color substrates, and stop solution. Often there are additional supplies required but not provided by the manufacturers. These include, but are not limited to, wooden applicator sticks, pipettes, tubes needed for dilutions, deionized water, a squirt bottle, a graduated cylinder, and an EIA reader capable of reading absorbance at 450 or 630 nm.

IV. QUALITY ASSURANCE

Include QC information on reagent container and in QC records.

At the time of testing, all reagents should be examined for leakage, contamination, and stability. Reagents from different lot numbers should not be mixed or interchanged. Each time an immunoassay is performed, a positive and negative control should be included. Commercial kits contain a set of QC reagents, and each manufacturer defines specific limits for the positive and negative control values. Ideally, previously positive and negative patient specimens should be included as controls, if not with every run, then certainly when changing to a new lot of reagents and when training new personnel.

When reading the EIA results visually, it is particularly important to adhere to the manufacturer's guidelines. In the case of colorimetric reactions, an intense color is required for a definitive positive result. A pale color should be considered indeterminate and the assay should then be repeated or, alternatively, the sample can be read using a spectrophotometer. Similarly, when using a spectrophotometer, a result should be within the optical density (OD) cutoff for positivity or negativity. Any indeterminate assay should be repeated. In general, if a laboratory is experiencing a significant number of indeterminate or low positives (~5% of specimen results), the most common reason is insufficient washing of the microwells. As mentioned above, automated plate washers should be avoided, as sufficient washing cannot be achieved (personal observations).

Rapid immunochromatographic test devices always include a positive control line which should react regardless of the presence or absence of antigen. A lack of appearance of a pink-red control line indicates an invalid result.

The optical immunoassay is considered positive if a solid blue-purple reaction circle of any intensity appears surrounding the built-in positive internal control dot. The optical immunoassay includes positive and negative external controls. Each test device also includes an internal positive control, Stx1 toxoid, which serves as a reagent check for each test device. Insufficient washing may result in leftover debris causing the appearance of a colored ring or spots surrounding the internal control dot; this should not be interpreted as a positive result (17).

For interpretation of the latex agglutination results, the agglutination reaction in the control latex wells is compared to that in the sensitized latex wells. Specimens with indeterminate results should be retested, preferably by a culture method.

With both EIA and latex agglutination methods, direct stool testing leads to more frequent observations of nonspecific results possibly related to interfering sub-

IV. QUALITY ASSURANCE *(continued)*

stances. Specificity is enhanced when testing is performed on broth cultures or colonies picked from culture plates.

Since non-*E. coli* pathogens such as *Enterobacter* or *Shigella dysenteriae* may also produce verocytotoxins, it is suggested that attempts be made to confirm the source of toxin as one of the serotypes of *E. coli* associated with disease. This may require simultaneous culture with identification of the enterics as *E. coli* and subsequent testing of isolates for toxin production. Alternatively, sending positive stool samples or toxin-positive enrichment broths to a public health or reference laboratory for confirmation is an option.

V. PROCEDURES

It is imperative that these cultures be handled in a biological safety cabinet.

Instructions on test performance are provided with each FDA-cleared kit. Most commercially available assays have procedures for direct stool testing, broth culture, and plate culture methods. A brief overview of EIA methods follows (28, 32, 33).

A. Specimen preparation for EIA methods

1. Direct stool testing
   - **a.** The stool sample should be mixed thoroughly prior to pipetting.
   - **b.** For liquid samples, a defined aliquot is added to the manufacturer's specimen diluent using a scored transfer pipette. The volume of stool and the amount of specimen diluent are specified by each manufacturer.
   - **c.** For solid specimens (nonpipettable), a 3- to 4-mm-diameter portion (pellet sized) is added to specimen diluent and then emulsified using a wooden applicator stick. Samples added to the specimen diluent should be mixed using a vortex.
   - **d.** Patient samples transported in Cary-Blair medium can be added directly to the microplate without further dilution.
2. Broth methods
   - **a.** The broth-enhanced method requires the addition of fresh stool or stool in Cary-Blair medium to be added to 5 ml of MacConkey broth, TSB, modified TSB, GN broth, or E-Z Coli broth (Difco Laboratories, Detroit, MI).
   - **b.** The sample is incubated overnight at 37°C.
   - **c.** A defined volume of broth growth is then added to specimen diluent prior to EIA testing.
3. Culture plate methods

   After the stool sample has been plated to MAC or a SMAC plate and incubated for 16 to 24 h at 37°C, a colony sweep or individual colonies can be removed with a loop and placed in the specimen diluent for EIA testing.

Observe standard precautions.

B. Addition of specimen to microtiter plates

1. All reagents and microtiter wells should be allowed to reach room temperature (20 to 25°C). Reagents should be thoroughly mixed.
2. The required number of microwells, enough for each patient and the positive and negative controls, should be removed from the pouch.
3. Add a negative control to a test well and a positive control to a second well.
4. Using a transfer pipette, add a measured amount of dilute specimen or undiluted stool in Cary-Blair medium to each well. Care should be taken not to splash into adjacent wells. This can be accomplished by inserting the pipette tip no more than halfway into the well and allowing the sample to slowly run down into the well.
5. Seal or cover the microwells and incubate at room temperature for 1 h.

C. Washing unbound material

1. After incubation, the contents of the wells should be aspirated or shaken out into a biohazard container.

V. PROCEDURES *(continued)*

2. Each well is washed with a buffered solution using a squirt bottle. After the wells are filled with buffer, they are aspirated or inverted, and then the plates are forcefully tapped on clean paper towels. This process is critical and should be repeated three or four times as suggested by the manufacturer. The use of an automated plate washer is not recommended (personal observations).

Observe standard precautions.

D. Addition of detection antibody-enzyme conjugate

1. Depending upon which manufacturer's kit is used, the addition of detection antibody (usually a few drops) as specified by the manufacturer, followed by enzyme conjugate or enzyme conjugate alone, occurs at this step.

2. The microwells are incubated at room temperature (22 to 27°C) and then washed as in step V.C.2 above.

E. Addition of color substrate

Two free-falling drops of color substrate are added, followed by a short incubation at room temperature.

Observe standard precautions.

F. Addition of stop solution

One or two drops of stop solution are added to each well. The microwells should be read for the colorimetric reaction either visually or spectrophotometrically within 10 to 30 min after the addition of the stop solution, depending upon the manufacturer.

Observe standard precautions.

G. Immunochromatographic assays

1. Specimen preparation

a. Direct stool specimens
Sample diluent should be mixed with a measured amount of liquid or solid stool in a test tube and vortexed.

b. Broth enrichment or Cary-Blair specimens
Sample diluent should be mixed with a manufacturer's determined measured amount of patient sample and vortexed.

c. Plate culture method
A heavy suspension of colonies should be made in the sample diluent or a dilution of polymyxin B, depending on the assay, and mixed well.

2. Test procedure

a. Bring all samples and test devices to room temperature (20 to 25°C).

b. Add a measured amount of prepared specimen to the sample port on the test device.

c. Incubate at room temperature for 10 min, during which time the sample must migrate past the control line.

3. Reading results
After the 10-min incubation is completed, visually read control and test zones for the presence or absence of a red line.

Observe standard precautions.

H. Optical immunoassay (17)

1. Specimen preparation

a. Liquid specimens (direct liquid stool, stool in transport medium, or broth culture) should be mixed thoroughly and transferred to the reaction tube using the manufacturer's provided transfer pipette. Two drops of liquid specimen are used in the assay.

b. Semisolid specimens should be gently mixed with the swab provided by the manufacturer, allowing the sample to absorb onto the swab. Excess fecal matter should be removed from the swab by rotating the swab against the wall of the sample container. Two drops of diluent or negative control are added to the reaction tube.

c. Colony growth on plates should be collected by using the manufacturer's provided swab to wipe across the first and second quadrants of growth.

V. PROCEDURES *(continued)*

2. Reacting with conjugate
   - **a.** Add 3 drops each of reagent 1 (anti-Stx1 antibody) and reagent 2 (anti-Stx2 antibody) to the provided reaction tube.
   - **b.** Add 2 drops of liquid specimens or swab from other sample types to the reaction tube, and mix well by squeezing the sides of the tube.
3. Reacting with optical surface
   - **a.** Using a transfer pipette, place 1 or 2 drops of mixture onto the center of the test surface.
   - **b.** Wait for 10 min.
   - **c.** Wash vigorously with wash solution.
   - **d.** With the blotter placed appropriately in the test device, close the lid of the device for 10 s, allowing removal of residual moisture.
4. Reacting with substrate
   - **a.** Open the device lid and move the blotter.
   - **b.** Apply substrate to the center of each device.
   - **c.** Wait for 5 min.
   - **d.** Wash vigorously with wash solution.
5. Reading result
   - **a.** Close the device for 10 s.
   - **b.** Open the device and examine for color changes of the test surface.

Observe standard precautions.

I. Latex agglutination (12)

1. Specimen preparation

   Direct stool testing is currently not recommended by the manufacturer.
   - **a.** Colony sweep testing
     - (1) After overnight culture of the stool sample on SMAC, a volume of cells equal to a large matchstick head is placed into a microcentrifuge tube which contains 0.1 ml of the polymyxin B solution.
     - (2) The sample is incubated at 37°C (35 to 39°C) for 30 min, without shaking.
     - (3) A total of 0.1 ml of 25% kaolin suspension in phosphate-buffered saline is added, and the solution is mixed again and centrifuged at 12,000 rpm for 10 min. The supernatant is used for testing.
   - **b.** BHI agar enrichment method
     - (1) An aliquot of stool is inoculated onto SMAC or another selective medium and incubated overnight at 37°C.
     - (2) Bacterial cells from a dense area of colonies are picked up with a sterilized cotton swab and are inoculated onto a BHI agar plate. The entire plate is inoculated.
     - (3) The plate is incubated at 37°C for 6 h.
     - (4) A volume of growth equivalent to a paper matchstick head is suspended in 0.1 ml of polymyxin solution.
     - (5) The suspension is incubated at 37°C for 30 min, with intermittent shaking (every 5 to 10 min). This ensures optimal extraction of verotoxins.
     - (6) A total of 0.1 ml of kaolin suspension is added.
     - (7) The suspension is mixed briefly and centrifuged at 12,000 rpm for 10 min.
     - (8) The supernatant is used for the test specimen.
   - **c.** Stationary culture from BHI agar
     - (1) A test isolate of *E. coli* is inoculated onto BHI agar and incubated for 16 to 20 h at 37°C.
     - (2) A volume of cells equivalent to three paper matchstick heads is suspended in 0.5 ml of polymyxin solution using a microbiological loop or cotton swab.

V. PROCEDURE *(continued)*

(3) The suspension is incubated at 37°C for 30 min, with intermittent shaking (every 5 to 10 min). This ensures optimal extraction of verotoxins.
(4) The suspension is centrifuged at 12,000 rpm for 10 min.
(5) The supernatant is used for the test specimen.

d. Shaking broth method
(1) An *E. coli* test isolate is inoculated into 5 ml of Casamino Acid yeast extract broth in a test tube.
(2) The broth is incubated aerobically, with shaking (120 to 150 strokes/min), at 37°C for 16 to 20 h.
(3) The broth is then centrifuged at 900 × *g* for 15 to 20 min.
(4) The supernatant is used for the test specimen.

2. Reverse passive latex agglutination
a. The microtiter plate is arranged so that each specimen is allotted two wells, one for testing with the sensitized latex reagent and the other for testing with the control latex reagent. The positive control and the reagent control (negative control) are two and one well each, respectively.
b. A total of 25 μl of the supernatant (test specimen; *see* above) is added to each of the two allotted wells.
c. A total of 25 μl of sensitized latex is added to the first well and 25 μl of the control latex is added to the second well for each specimen to be tested.
d. For the positive control, 25 μl is added to two wells and 25 μl each of sensitized and control latex is added to the respective wells. For the negative reagent control, 25 μl of diluent and 25 μl of sensitized latex are added to the allotted well.
e. The plates are mixed well using a plate mixer or by carefully tapping by hand.
f. The plates are sealed with a lid and then incubated for 16 h at room temperature.
g. Agglutination patterns are observed by placing the plate over a black surface.

VI. INTERPRETATION

Before patient specimen results are read, the positive and negative control wells should be examined. Any color in the negative control well with the EIA method constitutes an invalid result, and the entire run should be repeated. Immunochromatographic tests are invalid if the control line does not appear or if the assay is not read during the appropriate time frame. The optical immunoassay has a built-in positive control. An invalid test result occurs if it does not react appropriately, and testing should be repeated. For reverse passive latex agglutination, if agglutination in the negative control well is seen, this constitutes an invalid result and the kit should not be used.

A. EIA methods (28, 32, 33)

As indicated above, results for an EIA can be read either visually or spectrophotometrically. It is imperative that the manufacturer's guidelines be followed implicitly. A negative result is colorless and will yield a low OD reading. A definitive positive is characterized by a strong (usually yellow) color. A positive will demonstrate an OD reading usually well above the cutoff. Some manufacturers have created an indeterminate value or range. Results that fall into this category typically have a pale or faint color or an OD reading that falls somewhere between the positive and negative cutoffs. Samples with indeterminate

VI. INTERPRETATION
(continued)

results should be retested (*see* item IV above). A positive result indicates the presence of Stx1 or Stx2.

Currently there are two FDA-cleared EIA kits available: the Premier EHEC kit (Meridian Diagnostics, Inc.) and the ProSpecT Shiga toxin *E. coli* (STEC) microplate assay (Remel Inc.) (Table 11.8–1). The Premier EHEC assay has been extensively reviewed in the literature (13, 15, 18, 19). Published reports indicate sensitivity in the range of 82 to 100% and specificity in the range of 99 to 100%, depending upon the comparative methods and whether O157:H7 alone or all STEC organisms are considered (13, 19, 21, 23, 29). These reported sensitivities are significantly better than SMAC culture results reported in the same studies (50 to 60%), even when only O157:H7 detection is considered (7, 11, 23, 28). In one study the ProSpecT assay had 100% sensitivity in detecting *E. coli* O157 and was comparable to the Premier EHEC assay in overall performance (15). Both commercial assays have better sensitivity and specificity when testing is performed on broth-enhanced cultures as opposed to direct stool samples (28, 33). A commercially available non-FDA-cleared assay, RIDA-

Table 11.8–1 Commercial EIA reagents

Assay and manufacturer	Method	Notes	FDA cleared?
Premier EHEC; Meridian Bioscience, Inc., Cincinnati, OH	Microwell EIA	Monoclonal capture antibody; polyclonal rabbit antibodies specific for Shiga toxin; goat anti-rabbit antibody conjugated to horseradish peroxidase	Yes; approved for direct stool testing and broth and plate cultures
ProSpecT Shiga Toxin *E. coli* (STEC) microplate assay; Remel Inc., Lenexa, KS	Microplate EIA	Rabbit polyclonal anti-Stx1 and anti-Stx2 capture antibodies; monoclonal mouse anti-Stx1 and anti-Stx2 antibody conjugated to horseradish peroxidase for detection	Yes; approved for direct stool testing and broth cultures
RIDASCREEN Verotoxin; R-Biopharm, Darmstadt, Germany	Microplate EIA	Monoclonal mouse anti-Stx1 and anti-Stx2 capture antibodies; monoclonal anti-Stx1 and anti-Stx2 antibodies conjugated to horseradish peroxidase	No; manufacturer recommends testing broth enrichment culture with mitomycin C
BioStar OIA SHIGATOX; Inverness Medical Professional Diagnostics, Louisville, CO	Optical immunoassay	Rabbit polyclonal anti-Stx1 and anti-Stx2 capture antibodies; rabbit anti-Stx1 and anti-Stx2 antibodies conjugated to horseradish peroxidase	Yes; direct stool testing, broth and plate cultures
ImmunoCard STAT! *E. coli* O157 Plus; Meridian Bioscience, Inc., Cincinnati, OH	Immunochromatographic	Anti-H7 capture antibody; anti-O157 conjugated to colloidal gold	Yes; direct stool testing, broth and plate cultures
ImmunoCard STAT! EHEC; Meridian Bioscience, Inc., Cincinnati, OH	Immunochromatographic	Anti-Stx1 and anti-Stx2 capture and colloidal-gold-conjugated antibodies	Yes; broth or plate cultures
Duopath Verotoxin; Merck, Darmstadt, Germany	Immunochromatographic	Anti-Stx1 and anti-Stx2 capture and colloidal-gold-conjugated antibodies	No; broth or plate cultures
VTEC-Screen (II) Seiken; Denka Seiken Co. Ltd., Tokyo, Japan	Reverse passive latex agglutination	Polymyxin B and kaolin are added to stool samples to increase toxin release and to enhance specificity, respectively. Latex particles sensitized with Stx1 and Stx2 rabbit polyclonal antibodies.	No

VI. INTERPRETATION
(continued)

SCREEN Verotoxin (R-Biopharm, Darmstadt, Germany), detects Stx1 and Stx2 in broth enrichment cultures that have been incubated with mitomycin C (3, 32). When compared to the Vero cell assay, the RIDASCREEN was found to have a relative sensitivity and specificity of 95.7 and 98.7%, respectively (3). Clearly, the major advantage of these assays is improved detection of non-O157 serotypes. On the other hand, there have been problems with false-positive results leading to unnecessary public health concerns (7, 9). The Council of State and Territorial Epidemiologists adopted a position paper calling for culture confirmation of positive results from immunoassays (11).

B. Immunochromatographic assays

Available commercial assays detect the presence of *E. coli* O157:H7 (ImmunoCard STAT! *E. coli* O157:H7) or the presence of Stx1 and Stx2 (ImmunoCard STAT! EHEC and Duopath Verotoxin [25–27]). When the ImmunoCard STAT! *E. coli* O157:H7 assay was compared to culture, a prospective analysis revealed a sensitivity and specificity of 100% if both direct testing and enrichment incubation were used (22). One positive (1 of 14) was missed by each direct and enrichment testing method (22). Retrospective analysis revealed a sensitivity of 81% and a specificity of 95% when stored frozen stool was tested (22). The Duopath assay was compared to the Premier EHEC assay and found to be 100% sensitive and specific (31).

C. Optical immunoassay

A commercial FDA-cleared kit, the BioStar OIA SHIGATOX (Inverness Medical, Louisville, CO), detects the presence of Stx1 and Stx2. A change in OD resulting from the binding of Shiga toxin causes a detectable change in color. A single multicenter study determined a higher sensitivity of toxin detection than for the Premier EHEC (96.8%, versus 83.9%) when testing directly on fresh stool specimens. The two assays performed equally well when testing frozen and broth-enriched samples (37).

D. Reverse passive latex agglutination

A commercial non-FDA-cleared kit, the VTEC-Screen Seiken (Denka Seiken Co. Ltd., Tokyo, Japan), detects the presence of Stx1 and Stx2. The agglutination reactions in the control latex wells are compared to those in the sensitized latex wells.

Preliminary studies (2, 4, 6, 10) indicate a sensitivity slightly less than that of the traditional verocytotoxin assay. One study on 239 strains, representing 50 human and animal serotypes, noted a clinical sensitivity of 100% and a specificity of 97% compared to the verocytotoxin assay and PCR methods to detect toxin genes (2). A multicenter study performed in the United States found a 99% agreement between the Meridian EHEC test and the VTEC-Screen when testing 554 clinical diarrheal stool samples (6).

REFERENCES

1. **Banatvala, N., P. M. Griffin, K. D. Greene, T. J. Barrett, W. F. Bibb, J. H. Green, J. G. Wells, and the Hemolytic Uremic Syndrome Study Collaborators.** 2001. The United States National Prospective Hemolytic Uremic Syndrome Study: microbiologic, serologic, clinical and epidemiologic findings. *J. Infect. Dis.* **183:**1063–1070.
2. **Bettelheim, K. A.** 2001. Development of a rapid method for the detection of verocytotoxin-producing *Escherichia coli* (VTEC). *Lett. Appl. Microbiol.* **32:**1–5.
3. **Beutin, L., H. Steinruck, G. Krause, K. Steege, S. Haby, G. Hultsch, and B. Appel.** 2006. Comparative evaluation of the Ridascreen Verotoxin enzyme immunoassay for detection of Shiga-toxin producing strains of *Escherichia coli* (STEC) from food and other sources. *J. Appl. Microbiol.* **102:**630–639.
4. **Beutin, L., S. Zimmermann, and K. Gleier.** 1996. Rapid detection and isolation of Shiga-like toxin (Verocytotoxin)-producing *Escherichia coli* by direct testing of individual enterohemolytic colonies from washed sheep blood agar plates in the VTEC-RPLA assay. *J. Clin. Microbiol.* **34:**2812–2814.
5. **Brooks, J. T., E. G. Sowers, J. G. Wells, K. D. Greene, P. M. Griffin, R. M. Hoekstra, and N. A. Strockbine.** 2005. Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983–2002. *J. Infect. Dis.* **192:**1422–1429.

REFERENCES *(continued)*

6. **Carroll, K. C., K. Adamson, K. Korgenski, A. Croft, R. Hankemeier, J. Daly, and C. H. Park.** 2003. Comparison of a commercial reversed passive latex agglutination assay to an enzyme immunoassay for the detection of Shiga toxin-producing *Escherichia coli*. *Eur. J. Clin. Microbiol. Infect. Dis.* **22:**689–692.
7. **Centers for Disease Control and Prevention.** 2006. Importance of culture confirmation of Shiga toxin-producing *Escherichia coli* infection as illustrated by outbreaks of gastroenteritis—New York and North Carolina, 2005. *MMWR Morb. Mortal. Wkly. Rep.* **29:**1042–1045.
8. **Centers for Disease Control and Prevention.** 2008. Preliminary FoodNet Data on the incidence of infection with pathogens transmitted commonly through food—10 states, 2007. *MMWR Morb. Mortal. Wkly. Rep.* **57:**366–370.
9. **Centers for Disease Control and Prevention.** 2001. University outbreak of calicivirus infection mistakenly attributed to Shiga toxin-producing *Escherichia coli* O157:H7—Virginia, 2000. *MMWR Morb. Mortal. Wkly. Rep.* **50:**489–491.
10. **Chart, H., G. A. Willshaw, and T. Cheasty.** 2001. Evaluation of a reversed passive latex agglutination test for the detection of verocytotoxin (VT) expressed by strains of VT-producing *Escherichia coli*. *Lett. Appl. Microbiol.* **32:**370–374.
11. **Council of State and Territorial Epidemiologists.** 2001. Position Statements 2000-ID-#1. http://www.cste.org.
12. **Denka Seiken Co. Ltd.** 2001. VTEC-Screen (II) Seiken product package insert, p. 1–6. Denka Seiken Co. Ltd., Tokyo, Japan.
13. **Fey, P. D., R. S. Wickert, M. E. Rupp, T. J. Safranek, and S. H. Hinrichs.** 2000. Prevalence of non-O157:H7 Shiga toxin-producing *Escherichia coli* in diarrheal stool samples from Nebraska. *Emerg. Infect. Dis.* **6:**530–533.
14. **Friedrich, A. W., M. Bielaszewska, W. L. Zhang, M. Pulz, T. Kuczius, A. Ammon, and H. Karch.** 2002. *Escherichia coli* harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. *J. Infect. Dis.* **185:**74–84.
15. **Gavin, P. J., L. R. Peterson, A. C. Pasquariello, J. Blackburn, M. G. Hamming, K. J. Kuo, and R. B. Thomson.** 2004. Evaluation of performance and potential clinical impact of ProSpecT Shiga toxin *Escherichia coli* Microplate assay for detection of Shiga toxin-producing *E. coli* in stool samples. *J. Clin. Microbiol.* **42:**1652–1656.
16. **Grif, K., D. Orth, M. P. Dierich, and R. Wurzner.** 2007. Comparison of an immunochromatographic rapid test with enzyme-linked immunosorbent assay and polymerase chain reaction for the detection of Shiga toxin from human stool samples. *Diagn. Microbiol. Infect. Dis.* **59:**97–99.
17. **Inverness Medical Professional Diagnostics.** 2007. BioStar OIA SHIGATOX package insert. Inverness Medical Professional Diagnostics, Louisville, CO.
18. **Jelacic, J. L., T. Damrow, G. S. Chen, S. Jelacic, M. Bielaszewska, M. Ciol, H. M. Carvalho, A. R. Melton-Celsa, A. D. O'Brien, and P. I. Tarr.** 2003. Shiga toxin-producing *Escherichia coli* in Montana: bacterial genotypes and clinical profiles. *J. Infect. Dis.* **188:**719–729.
19. **Kehl, K. S., P. Havens, C. E. Behnke, and W. K. Acheson.** 1997. Evaluation of the Premier EHEC assay for detection of Shiga toxin-producing *Escherichia coli*. *J. Clin. Microbiol.* **35:**2051–2054.
20. **Kehl, S. C.** 2002. Role of the laboratory in the diagnosis of enterohemorrhagic *Escherichia coli* infections. *J. Clin. Microbiol.* **40:**2711–2715.
21. **Klein, E. J., J. R. Stapp, C. R. Clausen, D. R. Boster, J. G. Wells, X. Qin, D. L. Swerdlow, and P. I. Tarr.** 2002. Shiga toxin-producing *Escherichia coli* in children with diarrhea: a prospective point-of-care study. *J. Pediatr.* **141:**172–177.
22. **Mackenzie, A., E. Orrbine, L. Hyde, M. Benoit, F. Chan, C. Park, J. Alverson, A. Lembke, D. Hoban, and W. Kennedy.** 2000. Performance of the ImmunoCard STAT! E. coli O157:H7 test for detection of *Escherichia coli* O157:H7 in stools. *J. Clin. Microbiol.* **38:**1866–1868.
23. **Mackenzie, A. M. R., P. Lebel, E. Orrbine, P. C. Rowe, L. Hyde, F. Chan, W. Johnson, P. N. McLaine, and The SYNSORB Pk Study Investigators.** 1998. Sensitivities and specificities of Premier *E. coli* O157 and Premier EHEC enzyme immunoassays for diagnosis of infection with verotoxin (Shiga-like toxin)-producing *Escherichia coli*. *J. Clin. Microbiol.* **36:**1608–1611.
24. **Manning, S. D., A. S. Motiwala, A. C. Springman, W. Qi, D. W. Lacher, L. M. Ouellette, J. M. Mladonicky, P. Somsel, J. T. Rudrik, S. E. Dietrich, W. Zhang, B. Swaminathan, D. Alland, and T. S. Whittam.** 2008. Variation in virulence among clades of *Escherichia coli* O157:H7 associated with disease outbreaks. *Proc. Natl. Acad. Sci. USA* **105:**4868–4873.
25. **Merck.** Duopath verotoxins package insert. Merck, Darmstadt, Germany.
26. **Meridian Bioscience, Inc.** 2003. ImmunoCard STAT! *E. coli* O157 Plus, p. 1–5. Meridian Bioscience, Inc., Cincinnati, OH.
27. **Meridian Bioscience, Inc.** 2007. ImmunoCard STAT! EHEC. Meridian Bioscience, Inc., Cincinnati, OH.
28. **Meridian Bioscience, Inc.** 2007. Premier EHEC product package insert, p. 1–7. Meridian Bioscience, Inc., Cincinnati, OH.
29. **Novicki, T. J., J. S. Daly, S. L. Mottice, and K. C. Carroll.** 2000. Comparison of sorbitol MacConkey agar and a two-step method which utilizes a chromogenic agar to detect and isolate enterohemorrhagic *Escherichia coli*. *J. Clin. Microbiol.* **38:**547–551.

REFERENCES *(continued)*

30. **Orth, D., K. Grif, A. B. Khan, A. Naim, M. P. Dierich, and R. Wurzer.** 2007. The Shiga toxin genotype rather than the amount of Shiga toxin or the cytotoxicity of Shiga toxin in vitro correlates with the appearance of the hemolytic uremic syndrome. *Diagn. Microbiol. Infect. Dis.* **59:**235–242.
31. **Park, C. H., H. J. Kim, D. L. Hixon, and A. Bubert.** 2003. Evaluation of the Duopath verotoxin test for detection of Shiga toxins in cultures of human stools. *J. Clin. Microbiol.* **41:**2650–2653.
32. **R-Biopharm.** 2006. RIDASCREEN Verotoxin package insert. R-Biopharm, Darmstadt, Germany.
33. **Remel Inc.** 2003**.** ProSpecT Shiga toxin *E. coli* (STEC) microplate assay package insert. Remel Inc., Lenexa, KS.
34. **Serna, A., IV, and E. D. Boedecker.** 2008. Pathogenesis and treatment of Shiga toxin-producing *Escherichia coli* infections. *Curr. Opin. Gastroenterol.* **24:**38–47.
35. **Stapp, J. R., S. Jelacic, Y. L. Yea, E. J. Klein, M. Fischer, C. R. Clausen, X. Qin, D. L. Swerdlow, and P. I. Tarr.** 2000. Comparison of *Escherichia coli* O157:H7 antigen detection in stool and broth cultures to that in sorbitol-MacConkey agar stool cultures. *J. Clin. Microbiol.* **38:**3404–3406.
36. **Tarr, P. I., C. A. Gordon, and W. L. Chandler.** 2005**.** Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet* **365:**1073–1086.
37. **Teel, D. L., J. A. Daly, R. C. Jerris, D. Maul, G. Svanas, A. D. O'Brien, and C. H. Park.** 2007. Rapid detection of Shiga toxin-producing *Escherichia coli* by optical immunoassay. *J. Clin. Microbiol.* **45:**3377–3380.

SUPPLEMENTAL READING

Bettelheim, K. A., and L. Beutin. 2003. Rapid laboratory identification and characterization of verocytotoxigenic (Shiga toxin producing) Escherichia coli (VTEC/STEC). *J. Appl. Microbiol.* **95:**205–217.

Ochoa, T. J., and T. G. Cleary. 2003. Epidemiology and spectrum of disease of *Escherichia coli* O157. *Curr. Opin. Infect. Dis.* **16:**259–263.

11.9 Serodiagnosis of *Helicobacter pylori*

In 1983, Marshall and Warren proposed the possible association of *Helicobacter pylori* with peptic ulcer disease and gastric cancer (17). In February 1994, the NIH Consensus Development Conference concluded that *H. pylori* infection is the major cause of peptic ulcer disease and that all patients with documented peptic ulcer disease associated with *H. pylori* infection should receive antimicrobial therapy (20).

The prevalence of peptic ulcer disease exceeds 6.5 million cases in the United States, approximating a rate of 2.5%, or 2,500 cases per 100,000 individuals (23). In developed countries, the overall prevalence of *H. pylori* infection ranges from 25 to 30% (5). Seroprevalence increases with age, ranging from 5 to 27% in early childhood to levels exceeding 50% in adults greater than 50 years of age (2, 12, 15, 17, 22). Seroprevalence studies demonstrate an acquisition rate in adults of 3 to 4% per decade. *H. pylori* infection is a likely contributing factor in the development of gastric neoplastic diseases, such as gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (5). *H. pylori* infection is considered to increase the risk of gastric adenocarcinoma, with pooled odds ratios of 1.9 to 2.5 (6, 15). *H. pylori* and its association with multiple gastroduodenal diseases have emphasized the importance of accurate and prompt diagnosis of symptomatic individuals.

Multiple methods for assessment of *H. pylori* infection are used in the clinical setting (Table 11.9–1). Invasive methods require biopsy by endoscopy and are recommended for patients with complicated infections who are suspected of having peptic ulcer disease. Serologic testing represents a primary screening method for the diagnosis of *H. pylori* infection in individuals with uncomplicated infections. Infection with *H. pylori* results in a vigorous local and systemic humoral response to multiple antigens (14, 24, 25). In contrast to serum immunoglobulin M (IgM), serum IgA and IgG levels persist for months and years and correlate with active infection in untreated individuals (1, 11, 19, 21).

Only a minority of individuals did not show evidence of systemic seroconversion following infection (21). Anti-*H. pylori* serum IgG levels are more consistently elevated than serum IgA levels. Consequently, serum IgG immunoassays yield superior sensitivities and specificities compared with those of serum IgA assays (16).

Commercial ELISAs detecting anti-*H. pylori* serum IgG are the serologic tests of choice for the primary screening of patients with uncomplicated infections. Assays to detect anti-*H. pylori* IgA in sera have reduced and variable sensitivities (39 to 82%) compared to those of serum IgG immunoassays. Serum IgA studies may be useful in testing of symptomatic individuals with equivocal or negative IgG findings. A key advantage of follow-up serum IgA testing is that the same serum specimen may be used. In one study, greater than 7% of patients with negative serum IgG results were found to have detectable anti-*H. pylori* serum IgA and symptoms consistent with *H. pylori* infection (13). As determined by using the IgA immunoassay from HYCOR Biomedical (Irvine, Calif.), five of six IgA-positive, IgG-negative patients had peptic ulcers by endoscopy. Salivary and urinary antibody assays have shown limited utility. Serum IgA assays lack the requisite sensitivity to serve as primary screening tests but may be useful in cases in which infection is strongly suspected and the serum IgG result is negative or equivocal.

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE (SERUM IgA OR IgG ELISA)

ELISAs depend on the adsorption of protein or carbohydrate antigens to plastic surfaces such as polystyrene (solid phase). When antigens bound to the solid phase make contact with a patient's serum, *H. pylori*-specific IgG (or IgA), if present, will bind to specific antigens on the solid phase, forming antigen-antibody complexes. Excess antibody is removed by washing. Washing is followed by the addition of goat anti-human IgG (or anti-human IgA) conjugated with horseradish peroxidase which then binds to the *H. pylori* antigen-antibody complexes. Excess conjugate is removed by washing and followed by the addition of the chromogenic substrate tetramethylbenzidine (TMB). If specific IgG (or IgA) antibodies to *H. py-*

lori antigens are present in the patient's serum, TMB is altered and a blue color develops. When the enzymatic reaction is stopped with 1 N sulfuric acid (H_2SO_4), the contents of the wells turn yellow. The color change is indicative of the presence of anti-*H. pylori* IgG in the patient's serum and can be detected by absorbance spectrophotometry in an ELISA microwell plate reader. ELISA-based detection of serum anti-*H. pylori* IgG is the preferred approach for antibody screening in assessment of chronic *H. pylori* infection.

II. SPECIMEN COLLECTION

Observe standard precautions.

Table 11.9–1 Diagnostic and follow-up testing approaches for assessment of *H. pylori* infection

Testing approach
Diagnostic testing
Noninvasive
IgG serology
IgA serology
Fecal antigen detection
Urea breath testing
Invasive[a]
Histopathology (special stains)
Rapid urease testing
Follow-up testing
Noninvasive
Fecal antigen detection
Urea breath testing

[a] Requires biopsy sampling by esophagogastroduodenal endoscopy.

Optimal detection of anti-*H. pylori* IgG antibodies depends upon the presence of fresh serum samples. Although point-of-care testing (POCT) kits for detection of anti-*H. pylori* IgG and total antibodies in whole blood are available, assay sensitivities are reduced compared to those of serum-based ELISAs (8). Recommended laboratory-based ELISAs include HM-CAP (Enteric Products, Westbury, N.Y.), *H. pylori* IgG ELISA (Wampole Laboratories, Cranbury, N.J.), and Premier IgG ELISA (Meridian Biosciences, Cincinnati, Ohio). A minimum volume of 50 μl is recommended for collection in case repeat testing is required to clarify equivocal findings. Ten microliters of serum is used for testing. Serum specimens should be stored at 2 to 8°C if testing will occur within 48 h of collection. If prolonged storage is required for batch testing, storage at −20°C or lower is recommended to minimize proteolysis of antibodies. Caution must be exercised to avoid hemolyzed, lipemic, or bacterially contaminated sera, as these samples may yield spurious results (e.g., false positive). Sera should not be heat inactivated prior to testing.

In order to facilitate in-office POCT for *H. pylori* infection, rapid serum and whole-blood IgG immunoassays have recently been developed by several manufacturers (e.g., Quickvue *H. pylori* test by Quidel, San Diego, Calif., and Flexsure HP by Beckman Coulter, Palo Alto, Calif.). Whole-blood assays may use small volumes (drops) of capillary blood for POCT. A rapid latex agglutination test format (Pyloriset, Orion Diagnostics, Espoo, Finland) is also available to detect serum anti-*H. pylori* IgG. Such immunoassays produce a qualitative result in 4 to 10 min using heparinized whole-blood or capillary blood specimens. Compared to those of serum EIAs, whole-blood serologic assays have reduced sensitivities (80 to 90% versus >90%), with comparable specificities (70 to 80%) and lower overall accuracy (5). Therefore, negative results must be confirmed by a laboratory-based serum IgG or IgA ELISA, fecal antigen testing, or urea breath testing.

III. MATERIALS

H. pylori antigens (derived from strain ATCC 43504 and others) are commonly used as reagents bound to solid phase for detection of *H. pylori*-specific antibodies. Pooled *H. pylori* antigens of high-molecular-weight surface-associated proteins, acid extracts, or whole-cell lysates (sonicates) are used in most serologic assays. Various cytosolic and cell surface-associated proteins (18) represent immunodominant antigens recognized by serum antibodies of infected individuals (14). Immunodominant antigens include housekeeping enzymes such as ATP synthase F1, RecA, and heat shock proteins HspA and HspB. Essential colonization factors such as urease subunit UreB and flagellar protein FlaA also comprised a group of greater than 20 different immunogenic proteins identified in *H. pylori* (14).

Goat anti-human IgG is commonly used for detection of *H. pylori*-specific antibodies in human serum. Goat IgG is conjugated with enzymes such as horseradish peroxidase to facilitate chromogen detection. Chromogenic substrates such as TMB are included for colorimetric detection by absorbance spectrophotometry. A single- or dual-wavelength microplate reader capable of absorbance spectrophotometry at the appropriate wavelength (e.g., 450 nm for TMB) is necessary for quantitative optical density determinations. If dual-wavelength spectrophotometry is performed, the reference filter is fixed at 600 to 650 nm.

ANALYTICAL CONSIDERATIONS

IV. QUALITY ASSURANCE AND QUALITY CONTROL

Include QC information on reagent container and in QC records.

Blank reagent, positive, and negative controls must be included with each assay. Commercial kits also provide calibrators and controls that should be included with each assay. All of the kits described in this procedure provide serum controls. Calibrators for verifying cutoff values should be provided in commercial serologic kits and are useful for maintaining reproducible assay performance. The reagent blank (usually the serum diluent provided in the kit) should be included with each assay to set the baseline for optical density readings on an ELISA plate reader. When read against an air blank, the absorbance should remain below 0.200 unit at the recommended wavelength. Negative controls (provided in kit) and patient serum negative controls should be included with each assay and yield readings below 0.200 absorbance unit. Positive controls should include low and high positive controls and may include prior reactive patient sera. Dilutions of known positive sera may provide useful low positive controls. Popular *H. pylori* serologic kits, such as the Wampole Laboratories *H. pylori* IgG ELISA kit, recommend verification of immune status ratios and stated ranges for high positive, low positive, and negative controls. Proficiency testing is available by subscribing to the VR3 (serology) survey of the CAP.

V. PROCEDURE (SERUM IgG ELISA)

Observe standard precautions.

Serologic testing for assessment of *H. pylori* infection depends upon the detection of serum antibodies (IgA, IgG, or total antibodies) by EIAs. The recommended approach for primary screening in the clinical laboratory is serum IgG detection by ELISA (16). Samples for which the results are negative or equivocal may be retested with serum IgA ELISA.

A. Serum preparation

Dilute the test sera from each individual patient in serum diluent provided by the manufacturer. Typically, 10 µl of patient or control sera will be diluted in 200 µl of diluent (1:21 dilution) prior to testing.

B. Primary *H. pylori* antigen-serum antibody binding

1. Add calibrators, controls, and patient sera (dilutions) to individual wells. Serum diluent must be added to reagent blank well for optical density calibration.
2. Incubate each well at room temperature for 20 to 30 min.
3. Wash strips of wells by light agitation or shaking liquid from wells. Alternatively, wells may be aspirated by vacuum-assisted suction using a Pasteur pipette tip at an angle. Repeat procedure a minimum of three times for sufficient removal of nonspecific antigen-antibody interactions.

C. Detection of anti-*H. pylori* antibodies

1. Add conjugate to each well. Conjugate or conjugated antibody usually is goat anti-human IgG linked covalently with horseradish peroxidase as reporter enzyme.
2. Incubate secondary antibodies in each well for 20 to 30 min.
3. Wash strips of wells by light agitation and aspiration as described above. Repeat procedure a minimum of three times for sufficient removal of nonspecific antigen-antibody interactions.
4. Add chromogenic substrate (e.g., TMB) solution to each well and incubate at room temperature for 10 to 15 min.
5. Stop reaction with 1 N H_2SO_4.

V. PROCEDURE (SERUM IgG ELISA) *(continued)*

6. Chromogenic reactions should be read on an ELISA plate reader equipped with an appropriate filter (e.g., for horseradish peroxidase-TMB, 450 nm). If dual-wavelength absorbance spectrophotometry will be performed, the reference filter should be used at 600 to 650 nm.

POSTANALYTICAL CONSIDERATIONS

VI. INTERPRETATION

Commercial EIAs detecting anti-*H. pylori* serum IgG are the tests of choice (16) for primary screening of patients with uncomplicated infections. Patients with mild dyspeptic symptoms or asymptomatic patients at risk may be screened by serologic testing. A positive serology should be confirmed with direct detection of *H. pylori* by fecal antigen testing or urea breath testing to verify the presence of active infection. Patients with severe symptoms or "alarm" features such as weight loss or bleeding should be diagnosed by endoscopy. Serologic results should be interpreted qualitatively as the presence or absence of antibodies. Overall, the medians of the sensitivity and specificity for *H. pylori* serology kits were 92 and 83%, respectively (16). Performance varied significantly among commercial serologic kits, with top performers exceeding 90% in sensitivity and specificity and bottom performers failing to reach 90% in sensitivity or 80% in specificity (16). Anti-*H. pylori* serologic assays from various commercial sources have variable sensitivities (57 to 100%) and specificities (31 to 100%) (16). Importantly, positive (95 to 100%) and negative (84 to 89%) predictive values for serology were comparable to those of histology, rapid urease testing, and urea breath testing (4). ELISA serologic testing had the lowest cost per correct diagnosis, but overall accuracy was reduced compared to that of stool antigen or urea breath testing (25). Patients infected with "*Helicobacter heilmannii*" were usually negative by anti-*H. pylori* IgG assays (9). Serum IgG immunoassays have markedly reduced sensitivities (50 to 60% versus >90%) with samples from human immunodeficiency virus type 1-infected patients (7). Therefore, a negative result in this setting must be confirmed by urea breath testing or gastric biopsy. In addition to qualitative serologic approaches, notable decreases in titer may be obtained within 6 to 12 months of completion of antimicrobial therapy with quantitative serologies (3). However, at least 50% of patients remain seropositive 6 months or longer following successful treatment (3, 10, 21).

REFERENCES

1. **Blecker, U., S. Lanciers, B. Hauser, D. I. Mehta, and Y. Vandenplas.** 1995. Serology as a valid screening test for *Helicobacter pylori* infection in asymptomatic subjects. *Arch. Pathol. Lab. Med.* **119:**30–32.
2. **Cullen, D. J., B. J. Collins, K. J. Christiansen, J. Epis, J. R. Warren, I. Surveyor, and K. J. Cullen.** 1993. When is *Helicobacter pylori* infection acquired? *Gut* **34:**1681–1682.
3. **Cutler, A., A. Schubert, and T. Schubert.** 1993. Role of *Helicobacter pylori* serology in evaluating treatment success. *Dig. Dis. Sci.* **38:**2262–2266.
4. **Cutler, A. F., S. Havstad, C. K. Ma, M. J. Blaser, G. I. Perez-Perez, and T. T. Schubert.** 1995. Accuracy of invasive and noninvasive tests to diagnose *Helicobacter pylori* infection. *Gastroenterology* **109:**136–141.
5. **Dunn, B. E., H. Cohen, and M. J. Blaser.** 1997. *Helicobacter pylori. Clin. Microbiol. Rev.* **10:**720–741.
6. **Eslick, G. D., L. L. Lim, J. E. Byles, H. H. Xia, and N. J. Talley.** 1999. Association of *Helicobacter pylori* infection with gastric carcinoma: a meta-analysis. *Am. J. Gastroenterol.* **94:**2373–2379.
7. **Fabris, P., L. Bozzola, P. Benedetti, M. Scagnelli, R. Nicolin, V. Manfrin, C. Scarparo, and F. De Lalla.** 1997. *H. pylori* infection in HIV-positive patients. A serohistological study. *Dig. Dis. Sci.* **42:**289–292.
8. **Faigel, D. O., N. Magaret, C. Corless, D. A. Lieberman, and M. B. Fennerty.** 2000. Evaluation of rapid antibody tests for the diagnosis of *Helicobacter pylori* infection. *Am. J. Gastroenterol.* **95:**72–77.
9. **Hilzenrat, N., E. Lamoureux, I. Weintrub, E. Alpert, M. Lichter, and L. Alpert.** 1995. *Helicobacter heilmannii*-like spiral bacteria in gastric mucosal biopsies. Prevalence and clinical significance. *Arch. Pathol. Lab. Med.* **119:**1149–1153.

REFERENCES *(continued)*

10. **Hirschl, A. M., and M. L. Rotter.** 1996. Serological tests for monitoring *Helicobacter pylori* eradication treatment. *J. Gastroenterol.* **31**(Suppl. 9)**:**33–36.
11. **Hunt, R. H.** 1997. Peptic ulcer disease: defining the treatment strategies in the era of *Helicobacter pylori. Am. J. Gastroenterol.* **92:**36S–40S.
12. **Isaacson, P. G.** 1994. Gastric lymphoma and *Helicobacter pylori. N. Engl. J. Med.* **330:**1310–1311.
13. **Jaskowski, T. D., T. B. Martins, H. R. Hill, and C. M. Litwin.** 1997. Immunoglobulin A antibodies to *Helicobacter pylori. J. Clin. Microbiol.* **35:**2999–3000.
14. **Kimmel, B., A. Bosserhoff, R. Frank, R. Gross, W. Goebel, and D. Beier.** 2000. Identification of immunodominant antigens from *Helicobacter pylori* and evaluation of their reactivities with sera from patients with different gastroduodenal pathologies. *Infect. Immun.* **68:**915–920.
15. **Kosunen, T. U., A. Aromaa, P. Knekt, A. Salomaa, H. Rautelin, P. Lohi, and O. P. Heinonen.** 1997. *Helicobacter* antibodies in 1973 and 1994 in the adult population of Vammala, Finland. *Epidemiol. Infect.* **119:**29–34.
16. **Laheij, R. J., H. Straatman, J. B. Jansen, and A. L. Verbeek.** 1998. Evaluation of commercially available *Helicobacter pylori* serology kits: a review. *J. Clin. Microbiol.* **36:**2803–2809.
17. **Marshall, B. J., and J. R. Warren.** 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **1:**1311–1315.
18. **Megraud, F.** 1996. *Helicobacter pylori:* bacterial factors and interaction with the epithelial cells. *Yale J. Biol. Med.* **69:**35–37.
19. **Morris, A. J., M. R. Ali, G. I. Nicholson, G. I. Perez-Perez, and M. J. Blaser.** 1991. Long-term follow-up of voluntary ingestion of *Helicobacter pylori. Ann. Intern. Med.* **114:**662–663.
20. **NIH Consensus Conference.** 1994. *Helicobacter pylori* in peptic ulcer disease. NIH Consensus Development Panel on *Helicobacter pylori* in Peptic Ulcer Disease. *JAMA* **272:**65–69.
21. **Perez-Perez, G. I., B. M. Dworkin, J. E. Chodos, and M. J. Blaser.** 1988. *Campylobacter pylori* antibodies in humans. *Ann. Intern. Med.* **109:**11–17.
22. **Sipponen, P., T. U. Kosunen, I. M. Samloff, O. P. Heinonen, and M. Siurala.** 1996. Rate of *Helicobacter pylori* acquisition among Finnish adults: a fifteen year follow-up. *Scand. J. Gastroenterol.* **31:**229–232.
23. **Sonnenberg, A.** 1996. Cost-benefit analysis of testing for *Helicobacter pylori* in dyspeptic subjects. *Am. J. Gastroenterol.* **91:**1773–1777.
24. **Tinnert, A., A. Mattsson, I. Bolin, J. Dalenback, A. Hamlet, and A. M. Svennerholm.** 1997. Local and systemic immune responses in humans against *Helicobacter pylori* antigens from homologous and heterologous strains. *Microb. Pathog.* **23:**285–296.
25. **Vakil, N., D. Rhew, A. Soll, and J. J. Ofman.** 2000. The cost-effectiveness of diagnostic testing strategies for *Helicobacter pylori. Am. J. Gastroenterol.* **95:**1691–1698.

11.10 Total Viable Cell Counting Procedure

I. PRINCIPLE

Trypan blue allows rapid observation of nonviable cells present in a peripheral blood mononuclear cell suspension (PBMCS). A count of viable cells present in a PBMCS allows the suspension to be adjusted accordingly for further use in assays.

II. SPECIMEN

A PBMCS in a known volume of cell culture medium

III. MATERIALS

A. Trypan blue (0.4%; Sigma catalog no. T81540, 100-ml bottle)
- **1.** Filter as needed.
- **2.** Store at room temperature.

B. Hemacytometer with appropriate coverslip

C. Pipettes

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Perform daily.

B. A drop of trypan blue is mixed with a drop of distilled water. The preparation is screened for contamination by bacteria.

V. PROCEDURE

Observe standard precautions.

A. In a 5-ml test tube, add 90 µl of the 0.4% trypan blue solution to 10 µl of the cell suspension.

B. Mix cells and trypan blue and let stand for 1 to 3 min.

C. Make sure hemacytometer is clean; place appropriate clean coverslip on empty unit.

D. Load one chamber of the double-sided hemacytometer with 10 to 12 µl of cell-trypan blue mixture. Avoid air bubbles.

E. Allow cells to settle; let stand for at least 1 min.

VI. READING AND REPORTING RESULTS

A. Screen chamber under low power.
B. Use 40× high-power magnification to count cells.
C. The blue-tinged cells are counted as dead, nonviable cells. The translucent cells are counted as live, viable cells.
D. Count all the cells present inside the large four corner squares of the hemacytometer. Record as viable or nonviable.
E. Calculate percentages of viable and nonviable cells.
F. Report total viable cell count.

VII. CALCULATIONS

A. Total viable cell count

$$\left(\frac{\text{Total no. of cells in 4 large squares}}{4\ \text{[number of squares counted]}}\right) \times 10^4 \times \text{volume of cell suspension} \times \text{dilution factor}$$

B. Dilution factor

$$\frac{90\ \mu\text{l of trypan blue} + 10\ \mu\text{l of cells}}{10\ \mu\text{l of cells}} = 10\ \text{(dilution factor)}$$

SUPPLEMENTAL READING

Kruse, P. F., and M. K. Patterson. 1973. *Tissue Culture—Methods and Applications*, p. 406. Academic Press, New York, NY.

11.11 Peripheral Blood Mononuclear Cell Cryopreservation Method

I. PRINCIPLE

To be able to cryopreserve peripheral blood mononuclear cells (PBMCs) for utilization in future testing

II. SPECIMEN

Blood collected in heparin or acid citrate dextrose

Observe standard precautions.

III. MATERIALS AND REAGENTS

A. Fetal calf serum (heat inactivated at 56°C for 30 min)

B. RPMI 1640 medium

C. Ficoll-Hypaque

D. Dimethyl sulfoxide (DMSO) (catalog no. 27,043-1; Aldrich, Milwaukee, Wis.)
DMSO must be fresh. Once the bottle is opened, the shelf life of DMSO should not exceed 6 months.

E. Cryogenic vials (cryovials): 2-ml polyethylene vial with screw cap (catalog no. 03-341-20; Fisher Scientific)
◩ **NOTE:** Polypropylene cryovials are not recommended for storage in liquid nitrogen unless sealed in Cryoflex tubing (catalog no. 12-565-177; Fisher) using a heat sealer (catalog no. 01-812-15; Fisher).

F. Nalgene "Mr. Frosty" (catalog no. 288-383; Curtis Matheson Scientific, Houston, Tex.)

G. Optional: cryotube holder that stabilizes tubes on ice (Corning workstation from Fisher Scientific; catalog no. 03-374-37A)

H. Plasticware, pipettes

I. Sterile phosphate-buffered saline, Ca^{2+} and Mg^{2+} free
Use for washing cells.

IV. PROCEDURE

Observe standard precautions.

A. Label cryovials and chill at −20°C for 30 min.

B. Prepare freezing solution (90% fetal calf serum plus 10% DMSO); chill on ice.

C. Prepare PBMCs by Ficoll-Hypaque density centrifugation; wash twice at 4°C. Adjust cells, and wash again at 4°C. (After Ficoll separation, keep cells chilled or on ice at all times.)

D. Resuspend cells in freezing solution at 10^7 cells/ml; aliquot 0.5 to 1.0 ml of the cell suspension per cryovial. Be sure cryovial caps are securely tightened.

E. Immediately place cryovials in "Mr. Frosty" and transfer to a −70°C freezer. Alternatively, place cryovials in a Cryomed freezing chamber, lowering the temperature at −1°C per minute to −70°C.

F. Transfer cryovials to liquid nitrogen within 24 h.

V. THAWING PROCEDURE

A. Transfer cryovial from liquid nitrogen to a 37°C water bath. If liquid nitrogen has seeped into the cryovial, loosen the cap slightly to allow the nitrogen to escape during thawing.

■ *Some cryovials have been reported to explode during the thawing process. To minimize this risk, use only unbreakable polyethylene vials for storage in liquid nitrogen (see item III above). If polypropylene tubes are used, they should be sealed in Cryoflex tubing.*

B. Hold the cryovial in the surface of the water bath with an occasional gentle flick during thawing. Do not leave cryovial unattended during the thawing process. (It is important for cell viability that the cells are thawed and processed quickly.) When a small bit of ice remains in the cryovial, transfer the cryovial to the biosafety hood. Dry off the outside of the cryovials before opening to prevent contamination.

C. Add RPMI 1640 plus 10% fetal calf serum dropwise into the cryovial containing the cell suspension, slowly (over 2 min), up to a volume which doubles the original volume (e.g., add 1 ml of medium to a vial containing 1 ml of cell suspension).

D. Transfer the cell suspension to a 15-cc conical-bottom centrifuge tube containing 8 ml of RPMI 1640 plus 10% fetal calf serum; wash twice by centrifugation, gently resuspending the cells between washes.

E. Determine cell number and viability.

REFERENCE

1. **Laboratory Technologists Committee of the Adult AIDS Clinical Trials Group.** 2002. Consensus protocol for PBMC cryopreservation and thawing, version 3.0. http://aactg.s.-3.com/pub/download/vir/freezingprotocol.doc.

11.12 Lymphocyte Proliferation Assay

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Measuring the proliferative capability of lymphocytes is important in the evaluation of an individual's immune system. This can be achieved in an assay which cultures lymphocytes with either a mitogen or antigen that can stimulate a proliferative response and then labels the cells with a radioactive marker to allow for measurement of response (1).

The lymphocyte proliferation assay (LPA) exposes lymphocytes to mitogens such as phytohemagglutinin or pokeweed mitogen, recall antigens such as *Candida albicans* and tetanus toxoid, and other antigens which may be of specific interest (such as human immunodeficiency virus proteins gp120 or p24). Separated peripheral blood mononuclear cells (PBMCs) are incubated at 37°C for 6 days in a 96-well plate with a mitogen or antigen in triplicate wells. [^{3}H]thymidine is then added to each well. The isotope is incorporated into the newly synthesized DNA of the proliferating cells, and after a 6-h incubation the cells are harvested. A scintillation counter is used to measure the activity of the tritiated thymidine, expressed as counts per minute. This measurement is directly proportional to the level of lymphocyte proliferation.

II. SPECIMEN

Observe standard precautions.

Whole blood collected in an acid-citrate-dextrose (ACD) or heparin tube. These samples must be processed within 30 h of draw time.

III. REAGENTS AND SUPPLIES

A. Reagents

1. LPA medium (*see* Appendix 11.12–1 for preparation)
2. Ficoll-Paque PLUS or equivalent
3. 1× phosphate buffer solution (PBS)
4. Crystal violet (*see* Appendix 11.12–1 for preparation)
5. Trypan blue
6. [^{3}H]thymidine, 6.7 Ci/mmol, 1 mCi/ml
7. Pooled heat-inactivated human AB serum (Lonza)
8. Mitogens (assay dependent)
9. Recall antigens (assay dependent)
10. Specific antigens of interest (assay dependent)

B. Supplies

1. Pipet-Aid
2. 10-ml sterile polystyrene pipettes
3. 5-ml sterile polystyrene pipettes
4. 2-ml sterile polystyrene aspirating pipettes
5. 1-ml sterile polystyrene aspirating pipettes
6. 20-, 100-, and 200-µl pipettors
7. Multichannel pipettor plus reservoirs, if desired
8. Pipette tips to deliver 5 to 200 µl
9. 15-ml sterile polystyrene or polypropylene centrifuge tubes with conical bottoms
10. 50-ml sterile polypropylene centrifuge tubes with conical bottoms
11. 12- by 75-mm sterile polystyrene culture tubes with closures (snap cap)
12. Glass microfiber filter paper
13. 6-ml glass scintillation vials with cap
14. Scintillation fluid—Betafluor. If you are using a TopCount NXT Microplate scintillation and luminescence counter, then you will need Microscint 0.

III. REAGENTS AND SUPPLIES *(continued)*

15. Unifilter plate, top seal, backing tape (if using a TopCount machine)
16. ^{3}H waste containers (follow institutional guidelines for appropriate waste disposal)
17. Hemacytometer with coverslip
18. Ziploc bags
19. 96-well round-bottom plates
20. Steriflips (0.2-µm pore size)
21. PES filter unit, 150 ml (0.2-µm pore size; 50-mm-diameter membrane)

C. Equipment

1. Variable-speed centrifuge
2. Cell harvester
3. Liquid scintillation counter or TopCount NXT Microplate scintillation and luminescence counter
4. Biosafety cabinet with vacuum trap system for aspiration
5. Incubator, 37°C, 5% CO_2, 96% relative humidity
6. Microscope

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

Calibration

A. Scintillation counter

1. Normalization of scintillation counter must be done once a week.
2. Background and ^{3}H standards must be assayed with each run.
3. Preventive maintenance should be performed twice per year.

B. TopCount machine

1. Calibration of TopCount instrument must be done once a week.
2. Preventive maintenance should be performed twice per year.

C. Pipettors

Pipettors should be calibrated twice per year.

D. Equipment

All laboratory equipment should have preventive maintenance performed according to the manufacturers' instructions.

V. PROCEDURE

It is imperative that these cultures be handled in a biological safety cabinet.

Observe standard precautions.

◩ **NOTE:** All of the pipetting and filtering should take place in a biological safety cabinet using sterile technique, and all reagents should be at room temperature when used. All radioactive materials must be used in designated areas and must be disposed of properly per the institution's radiation safety guidelines.

A. Plate preparation

LPA plates can be made in advance and stored at −70°C in Ziploc bags until they are needed. LPA plates consist of triplicate wells containing 100 µl of either medium (background), antigen, or mitogen prepared at 2× in medium. The medium used is LPA medium plus 20% pooled heat-inactivated human AB serum to yield a final concentration of 10% pooled human AB serum after the PBMCs are added. Some commonly used final concentrations are as follows.

1. Tetanus toxoid: 2.5 and 1.25 µg/ml
2. *C. albicans:* 50 and 10 µg/ml
3. Pokeweed mitogen: 10 µg/ml

A titration is recommended to determine the optimal concentration for all stimulants.

B. PBMC separation

1. Centrifuge ACD or heparin tube for 10 min at 350 × *g* at room temperature.
2. Gently invert the Ficoll-Paque PLUS bottle twice.
3. Pipette 5 ml of Ficoll-Paque PLUS into a 15-ml conical tube.
4. Using a pipette, remove the plasma from the ACD or heparin tube and place into a snap-cap tube. This can be aliquoted and frozen, or discarded if desired.

V. PROCEDURE *(continued)*

5. Dilute the blood twofold using 1× PBS. Mix gently by pipetting up and down.
6. Carefully layer the blood onto the 5 ml of Ficoll-Paque PLUS. Be sure not to let the diluted blood penetrate the Ficoll-Paque PLUS.
7. Centrifuge the conical tube containing the diluted blood on Ficoll-Paque PLUS according to the separation gradient manufacturer's instructions.
8. After centrifugation, four layers will be visible. Gently aspirate the top clear layer, which contains PBS and any remaining plasma. Be careful not to disturb the cloudy layer underneath, which contains the mononuclear cells. If you do not have an in-house vacuum system, use a 5-ml pipette to remove the top clear layer and dispose of it appropriately.
9. The cloudy mononuclear cell layer is removed next by placing the tip of a pipette on the layer and gently moving the pipette in a circular motion while applying suction with the pipette. The entire layer should be removed and transferred to a 15-ml conical tube containing 5 ml of 1× PBS. Care should be taken to remove as little as possible of the Ficoll-Paque PLUS layer below the mononuclear cells. Centrifuge the conical tube according to the separation gradient manufacturer's instructions.
10. After centrifugation, aspirate the supernatant, making sure not to disturb the cell pellet.
11. Pipette 10 ml of 1× PBS into the conical tube containing the pellet. Using the pipette, thoroughly mix to resuspend the pellet.
12. Centrifuge the conical tube according to the separation gradient manufacturer's instructions.
13. After centrifugation, aspirate the supernatant without disturbing the pellet.
14. Pipette 1 ml of LPA medium into the conical tube containing the pellet. Using the pipette, thoroughly mix to resuspend the pellet.
15. Count the PBMCs using the total viable cell counting procedure (*see* procedure 11.10). This procedure uses trypan blue dye to count the viability and number of PBMCs. To better distinguish mononuclear cells, it is recommended that the cell count be performed using crystal violet.
16. Adjust the concentration of the cells to 10^6/ml with LPA medium. The total number of cells needed for a plate can be found by multiplying the number of wells by 100,000. A minimum of 600,000 is usually required, which allows you to do one control triplicate and one mitogen triplicate.
17. Retrieve the appropriate premade LPA plate from the freezer. After removing the bag, put the plate in the incubator to defrost.
18. Once the plate is fully defrosted, label it appropriately with patient information, study information, and date.
19. Mix PBMCs well and add 100 µl of the PBMC suspension to each well of the plate that contains a stimulant, changing tips every triplicate. Also add 100 µl in triplicate to the medium control wells.
20. Tap plate gently to mix.
21. Place the plate in the incubator for 6 days.
22. On the sixth day, add 50 µl of [^{3}H]thymidine working solution per well to yield 1 µCi/well. The working solution should be made fresh monthly (order new stock vial of [^{3}H]thymidine every 6 months).
23. Tap plate gently to mix.
24. Return the plate to the incubator for 6 h.
25. After 6 h, harvest the plate using a cell harvester.

V. PROCEDURE *(continued)*

26. Leave the filters to dry overnight. The following day, put the filters in 6-ml glass scintillation vials and add 2 ml of a scintillation fluid such as Betafluor to each of these vials. Alternatively, you can use a Filtermate harvester to harvest the entire plate onto a Unifilter plate. Leave the Unifilter plate to dry overnight. The following day, put backing tape on the plate. Add 30 µl of Microscint 0 to each well, and put a top seal on the plate.

27. Count each vial or well for 1 min using a liquid scintillation counter or TopCount instrument.

POSTANALYTICAL CONSIDERATIONS

VI. CALCULATIONS

A. To determine net counts

Net counts = (experimental counts per minute − background unstimulated counts per minute)

B. To determine stimulation index (SI)

SI = (experimental counts per minute/background unstimulated counts per minute)

VII. INTERPRETATION

An SI greater than 3 (antigens) to 5 (mitogens) is generally considered to be a significant response to the specific antigenic or mitogenic stimulus.

REFERENCES

1. **Fletcher, M. A., N. Klimas, R. Morgan, and G. Gjerset.** 1992. Lymphocyte proliferation, p. 213–219. *In* N. R. Rose, E. Conway de Macario, J. L. Fahey, H. Friedman, and G. M. Penn (ed.), *Manual of Clinical Laboratory Immunology*, 4th ed. American Society for Microbiology, Washington, DC.

SUPPLEMENTAL READING

Froebel, K. S., N. G. Pakker, F. Aiuti, M. Bofill, H. Choremi-Papadopoulou, J. Economidou, C. Rabian, M. T. L. Roos, L. P. Ryder, and F. Miedema. 1999. Standardization and quality assurance of lymphocyte proliferation assays for use in the assessment of immune function. *J. Immunol. Methods* **227:**85–97.

APPENDIX 11.12–1

Include QC information on reagent container and in QC records.

Reagent Information and Preparation

A. LPA medium
   1. 48.5 ml of RPMI 1640
   2. 0.5 ml of 200 mM L-glutamine for a final concentration of 2 mM L-glutamine
   3. 0.5 ml of 10,000-U/ml penicillin and 100-µg/ml streptomycin solution for a final concentration of 100 U of penicillin per ml and 100 µg of streptomycin per ml in solution
   4. 0.5 ml of 1 M HEPES buffer for a final of 10 mM HEPES buffer

B. Crystal violet
   1. 98 ml of deionized H_2O
   2. 2 ml of glacial acetic acid
   3. 0.05 g of crystal violet dye (powder form)

C. [^{3}H]thymidine working solution (20 µCi/ml)
   1. 24.5 ml of 1× PBS
   2. 0.5 ml of [^{3}H]thymidine at 1 mCi/ml

D. Reagent and special supply information

See table on next page.

Reagent	Manufacturer	Catalog no.	Address and website	Phone no.
[^{3}H]thymidine (1 mCi/ml)	Perkin-Elmer	NET-027	940 Winter St. Waltham, MA 02451 http://www.perkinelmer.com	(800) 762-4000
Ficoll-Paque PLUS	GE Healthcare	17-1440-03	800 Centennial Ave. P.O. Box 1327 Piscataway, NJ 08855-1327 http://www.gehealthcare.com	(800) 526-3593
HEPES buffer	Invitrogen	15630-080	1600 Faraday Ave. P.O. Box 6482 Carlsbad, CA 92008 http://www.invitrogen.com	(800) 955-6288
L-Glutamine	Lonza	17-605E	8830 Biggs Ford Rd. Walkersville, MD 21793 http://www.lonza.com	(301) 898-7025
Penicillin-streptomycin	Invitrogen	15140-148	1600 Faraday Ave. P.O. Box 6482 Carlsbad, CA 92008 http://www.invitrogen.com	(800) 955-6288
RPMI 1640	Lonza	12-167F (500 ml) 12-167Q (1,000 ml)	8830 Biggs Ford Rd. Walkersville, MD 21793 http://www.lonza.com	(301) 898-7025
Glacial acetic acid	Sigma	A-6283	3050 Spruce St. St. Louis, MO 63103 http://www.sigma-aldrich.com/order	(800) 521-8956
1× PBS	HyClone	SH30256.01	1725 South HyClone Rd. Logan, UT 84321 http://www.hyclone.com	(800) hyclone
Crystal violet dye (powder)	Sigma	A-6158	3050 Spruce St. St. Louis, MO 63103 http://www.sigma-aldrich.com/order	(800) 521-8956
Human AB serum (heat inactivated)	Lonza	14-490E	8830 Biggs Ford Rd. Walkersville, MD 21793 http://www.lonza.com	(301) 898-7025
Trypan blue dye	Invitrogen	15250-061	1600 Faraday Ave. P.O. Box 6482 Carlsbad, CA 92008 http://www.invitrogen.com	(800) 828-6686

11.13.1 Introduction

Natural killer (NK) cells are another group of cytolytic lymphocytes, distinct from B lymphocytes and T lymphocytes, that participate in both innate immunity and adaptive immunity. NK cells are lymphocytes that lack B-cell receptors and T-cell receptors. They are designed to kill certain mutant cells and virus-infected cells. Normally present as a small subpopulation of circulating lymphocytes, NK cells morphologically appear as so-called "large granular lymphocytes." Like cytotoxic T cells, they attack and kill tumor cells and protect against a wide variety of infectious microbes. They are "natural" killers because they do not need additional stimulation or to recognize a specific antigen in order to attack and kill. NK cells appear to play a role in a variety of human diseases. Compromised or absent natural immunity, as measured in vitro by decreased NK activity and/or depressed absolute numbers of circulating NK cells, has been linked to the development and progression of cancer, chronic and acute viral infections (including AIDS), various immunodeficiencies, and certain autoimmune diseases.

These cells characteristically lack CD3 but express CD16, CD56, and CD57. NK cell proliferation occurs in a number of reactive and autoimmune conditions. The transient, reactive proliferations include associations with viral infections, hepatitis, nephrotic syndrome, and lymphoma. Chronic, reactive proliferations can be seen in a number of autoimmune diseases. In addition, a chronic, clonal proliferative disorder of NK cells (large granular lymphoproliferative disorder) has been described.

The role of NK cells in viral disease has been known for a long time. The correlation between low NK activity and serious viral infections in immunocompromised hosts after transplantation and in certain congenital immunodeficiencies has been well documented.

The Practice Parameter for the Diagnosis and management of Primary Immunodeficiency recommends testing NK cells, quantitative and qualitative function, in patients with severe disease caused by herpesviruses or papillomaviruses who do not have another defined immunodeficiency. The assays described in procedures 11.13.2 and 11.13.3 quantify NK cells by flow cytometry as well as evaluate NK cell function.

11.13.2 Natural Killer Cell Flow Cytometry Assay

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The Practice Parameter for the Diagnosis and Management of Primary Immunodeficiency (3) states that patients with severe disease caused by herpesviruses or papillomaviruses who do not have another defined immunodeficiency should have phenotypic and functional assessments of natural killer (NK) cells performed. These patients need to be assessed for complete absence of all NK cells (all $CD56^+$ cells), classical NK cells (absence of $CD56^+$ $CD3^-$ cells but presence of $CD56^+$ $CD3^+$ cells), and defective NK cell function (presence of $CD56^+$ $CD3^-$ cells but absence of NK cell cytotoxicity). In addition, the Practice Parameter for Immunology (3) states that a mutation in the gene (*FCGR3A*) coding for CD16 that causes an amino acid substitution results in impaired NK cell cytotoxicity and susceptibility to recurrent or severe herpesvirus infections. Patients with isolated defects of cellular immunity who do not have defects of gamma interferon/interleukin 12 axis should be screened for this particular mutation in FCGR3A by flow cytometry using anti-CD16 clone B73.1. Patients homozygous for this mutation have NK cells that fail to react with a commonly used anti-CD16 monoclonal antibody (clone B73.1) and thus will appear to be $CD56^+$ $CD16^-$ $CD3^-$. Thus, when severe or recurrent infection due to herpesvirus is encountered in patients with decreased NK cell function in the absence of other defined immunodeficiency, NK cells should be specifically assessed by flow cytometry using anti-CD16 clone B73.1. However, it is important to note that many clinical immunology laboratories use anti-CD16 clone B73.1 and anti-CD56 conjugated with the same fluorophore in the same tube (identifies cells $CD16^+$ *or* $CD56^+$). In this type of assay, patients with the L48H substitution will not be identified. Therefore, it is important to use anti-CD16 clone B73.1 alone and not in conjunction with anti-CD56 in the same tube. In our laboratory, we use two different clones of anti-CD16: anti-CD16(B73.1) and anti-CD16(3G8). If the patient is homozygous for the FCGR3A mutation, the NK cells will be positive for expression of anti-CD16(3G8) but not with anti-CD16(B73.1). Below is an example of the assay showing normal NK cells (Fig. 11.13.2–1). The cells are gated on the $CD3^-$ population and analyzed for expression of anti-CD16(3G8) and anti-CD16(B73.1) and CD56. The percent gated and absolute values are reported, in addition to an interpretation.

Flow cytometric, multiparametric analysis of subpopulations of cells from various biological tissues had its genesis in the early studies of Loken et al. (2) in 1976 and Shapiro et al. (4) in 1977. These studies developed the principles of using forward and angular light scatter measurements from individual cells passing by fluid laminar flow through various light sources to characterize subpopulations of cells from heterogeneous populations. Shapiro et al. expanded upon these studies to incorporate histochemical fluorochromes to identify subpopulations of peripheral blood leukocytes. These studies established the principles to be used by second- and third-generation instruments, which, with improved data acquisition and analysis capability, have produced extraordinarily sophisticated fluorescence-activated flow cytometers for both biomedical research and routine clinical diagnostics. Further, the development of monoclonal antibody technology provided the necessary reagents for the immunophenotyping of a broad range of hematopoietic cells, which has led to important diagnostic procedures such as the more detailed classification of leukemias and lymphomas.

Whole blood can be stained with antibodies against markers found on the cell surface. The antibodies used are directly conjugated with a fluorescent dye. The stained cells are read on a flow cytometer and the percentage of fluorescent cells is determined for each antibody.

II. SPECIMEN

Observe standard precautions.

A. Type

Peripheral blood; EDTA, acid-citrate-dextrose (ACD), or heparin can be used as an anticoagulant. Depending on the number of antibodies to be run, 1 to 3 ml of peripheral blood should be collected. One hundred microliters of whole blood is needed for each antibody.

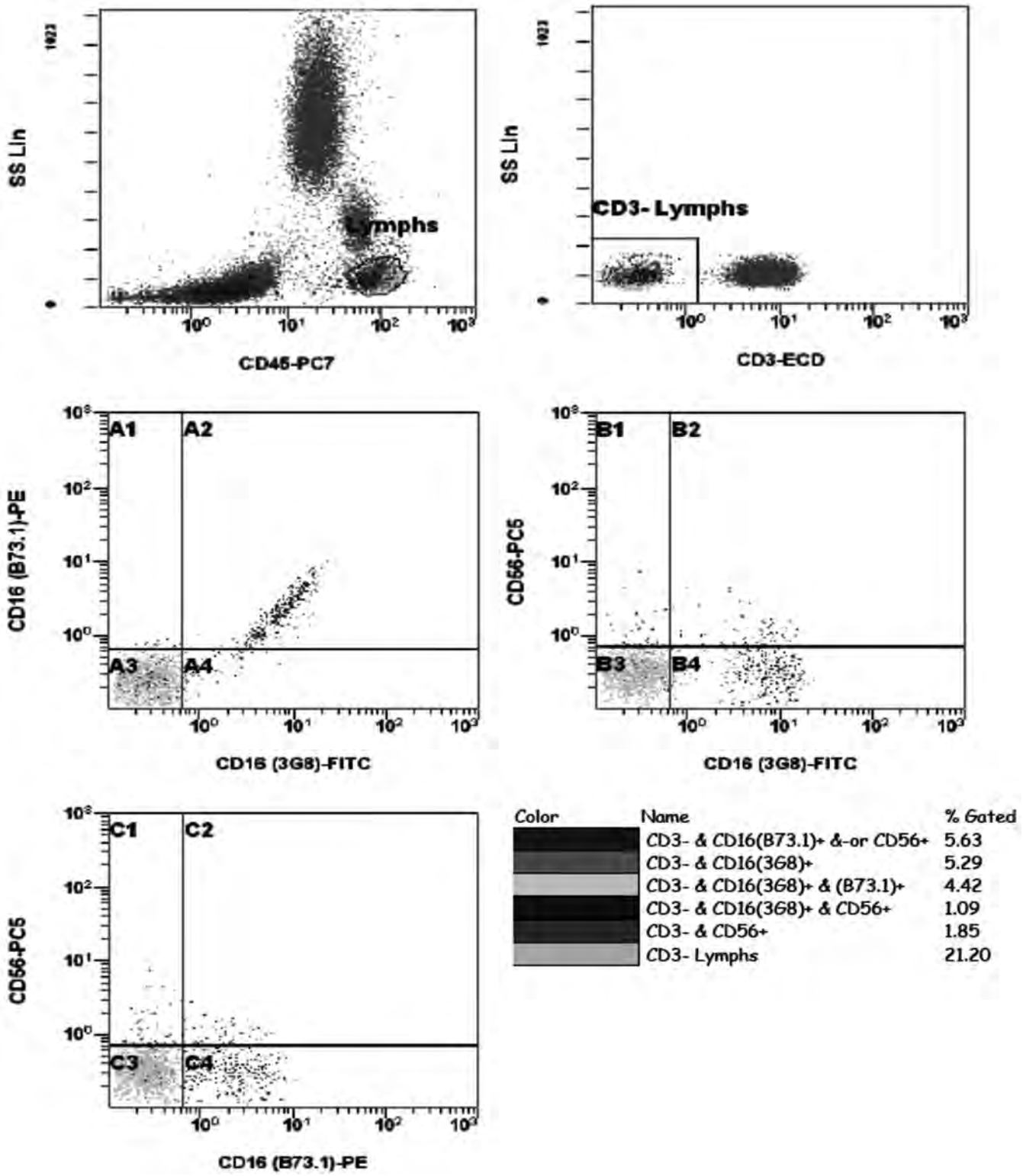


Figure 11.13.2–1 NK cell panel interpretation. As per the 2005 Practice Parameter for the Diagnosis and Management of Primary Immunodeficiency, a mutation in the Fc gamma R3III gene affecting NK cell function can be screened for by demonstrating decreased detection of CD16 using the B73.1 clone. If the CD16-positive population is detectable using both the anti-CD16 clones (B73.1 and 3G8) it is unlikely that the individual is homozygous for this mutation.

II. SPECIMEN *(continued)*

B. Handling

1. Specimens should be collected and brought immediately to the lab at room temperature. Specimens can be held for no more than 24 h at room temperature. This must also be kept in mind for specimens sent overnight.

2. Severely hemolyzed specimens or those with large clots should be rejected.

III. REAGENTS AND SUPPLIES

A. Adjustable-volume pipettor and tips
B. 12- by 75-mm polypropylene tubes
C. Beckman-Coulter Immunoprep reagent kits and Q-Prep instrumentation
D. Vortex
E. Centrifuge or serofuge
F. Vacuum source with pipette attached
G. Phosphate-buffered saline (PBS)
H. Transfer pipettes
I. Antibodies against cell surface markers—stored according to manufacturer's instructions
J. Healthy-donor control peripheral blood
K. Fetal calf serum
L. Plain red-top Vacutainer tubes
M. Flow cytometer

IV. CALIBRATION

A. Standards

The flow cytometer must be optically aligned and standardized for fluorescence intensity.

B. Procedure

1. Refer to flow cytometer instrument manual and startup, QC, and shutdown procedures.

2. New antibody lots (with the exception of isotype controls and CD45) must be tested in parallel with the current lot to ensure the sensitivity and specificity of the new lot.

ANALYTICAL CONSIDERATIONS

V. QUALITY CONTROL

Controls
Whole blood from a healthy donor

VI. PROCEDURE

Observe standard precautions.

A. Label one set of 12- by 75-mm polypropylene tubes for each patient and control. Label the tubes based on the antibodies or panel ordered. Refer to Table 11.13.2–1.

B. Pipette the appropriate volume of antibodies into all other corresponding tubes. Change pipette tips between each antibody vial to prevent contamination.

C. Add 100 μl of the control or patient blood to each of the appropriate tubes and vortex to mix.

D. Vortex all tubes and incubate for at least 15 min at 2 to 8°C.

E. Process all tubes containing peripheral blood after incubation. Select the necessary protocols/panels on the flow cytometer and load the sample tubes into a carousel in the appropriate order.

F. Gate on the lymphocyte population based on bright CD45 expression and low side scatter. Adjustments to this region should be made as needed. Refer to Fig. 11.13.2–2 for an example of a normal CD45/side scatter histogram.

G. If populations span quadrants, the positive and negative populations can be used as a guide to set cursors.

H. Percent positive results are obtained from the flow cytometer printouts.

Table 11.13.2–1 Example of tube labeling for NK cell flow cytometry assay

Tube	Panel[a]				
1	MsIgG1-FITC	MsIgG1-PE	CD3-ECD	MsIgG1-PC5	CD45-PC7
2	CD16-FITC (3G8 clone)	CD16-PE (B73.1 clone)	CD3-ECD	CD56-PC5	CD45-PC7

[a] MsIgG1, mouse immunoglobulin G1; FITC, fluorescein isothiocyanate; PE, phycoerythrin; ECD, phycoerythrin with Texas Red.

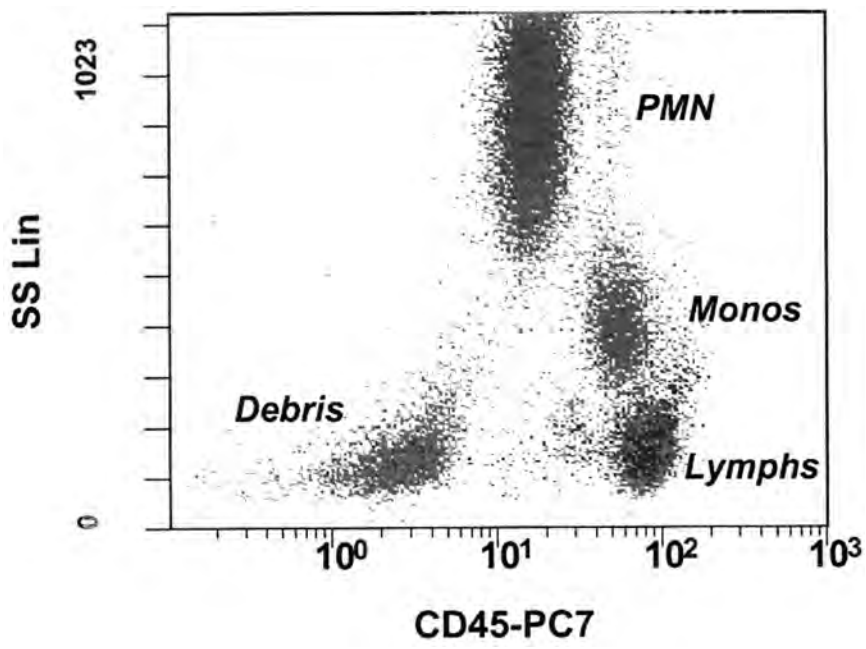


Figure 11.13.2–2 Example of a normal CD45/side scatter histogram.

VI. PROCEDURE *(continued)*

I. Surface marker-specific absolute counts are automatically calculated where appropriate.
   1. The absolute counts are calculated using the percent positive result along with the WBC and percent lymphocytes from a tandem complete blood count.
   2. If it is necessary to manually calculate a marker-specific absolute count, refer to item VII below.

POSTANALYTICAL CONSIDERATIONS

VII. CALCULATIONS

To manually calculate a marker-specific absolute count:

$$\text{Marker-specific absolute count} = \frac{(\text{WBC} - \text{cells}/\mu\text{l}) \times (\%\ \text{lymphocytes} + \%\ \text{atypical lymphocytes}) \times (\%\ \text{flow marker positive})}{10{,}000}$$

Example: Absolute $CD3^+\ CD4^+$ =

$$\frac{(6{,}800\ \text{cells}/\mu\text{l}) \times (37 + 0) \times (45.2)}{10{,}000} = 1{,}137\ CD3^+\ CD4^+\ \text{cells}/\mu\text{l}$$

VIII. INTERPRETATION

Results format

A. Results are reported as percent positive for each antibody. Histograms for NK panel specimens must be given to the medical director for an interpretation of the results (see previous example).

B. Normal range: age-matched reference ranges for NK lymphocyte subsets $CD3^-$ $CD16^+$ $CD56^+$ are from reference 1. All other reference ranges are derived from healthy-adult peripheral blood samples with a region set on lymphocytes.

REFERENCES

1. **Comans-Bitter, W. M., et al.** 1997. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. *J. Pediatr*. **130:**388–393.
2. **Loken, M. R., R. G. Sweet, and L. A. Herzenberg.** 1976. Cell discrimination by multiangle light scattering. *J. Histochem. Cytochem.* **24:**284.
3. **Parks, D. R., L. A. Herzenberg, and L. Herzenberg.** 1989. Flow cytometry and fluorescence-activated cell sorting, p. 781–802. *In* W. E. Paul (ed.), *Fundamental Immunology*, 2nd ed. Raven Press, New York, NY.
4. **Shapiro, H. M., E. R. Schildkraut, R. Curbelo, R. B. Turner, et al.** 1977. Cytomat-R: a computer-controlled multiple laser source multiparameter flow cytophotometer system. *J. Histochem. Cytochem.* **25:**836–844.

11.13.3 Natural Killer Cell Assay

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Natural killer (NK) cells mediate the spontaneous lysis of virus-infected or tumor cells. The functional capability of NK cells can be determined by exposing them to a known NK-specific target that is radioactively labeled. NK cell activity is quantified in this assay by using the K-562 cell line (*see* Appendix 11.13.3–1), which is known to be NK sensitive. K-562 target cells are incubated for 1 h with ^{51}Cr. The targets are then incubated with several different serial dilutions of peripheral blood mononuclear cells (PBMCs) (effectors) for 4 h at 37°C in a 96-well plate. The effectors lyse the target cells, releasing ^{51}Cr into the medium. At the end of the incubation, the plate is centrifuged and the supernatants are collected. The amount of ^{51}Cr released into the supernatants, which is measured with a gamma counter or TopCount, can be used to determine the lytic capability of the NK cells at each effector/target (E/T) ratio.

II. SPECIMEN

Observe standard precautions.

Whole blood drawn into a heparin (green top) tube; 5 to 10 ml is usually sufficient to perform this assay. These samples must be processed within 30 h of draw time.

III. REAGENTS AND SUPPLIES

A. Reagents (*see* Appendix 11.13.3–1 for preparation)

1. ^{51}Cr
2. K-562 cell line
3. RPMI 1640 (stored at 4°C)
4. Filtered RPMI 1640 complete with 10% fetal bovine serum (FBS) (R10 FBS) (stored at 4°C)
5. Ficoll-Paque PLUS or equivalent
6. 1% Triton X-100
7. Crystal violet
8. Trypan blue

B. Supplies

1. Sterile pipette tips to deliver 5 to 200 µl
2. 10-ml sterile polystyrene pipettes
3. 5-ml sterile polystyrene pipettes
4. 1-ml sterile polystyrene pipettes
5. 2-ml sterile polystyrene pipettes
6. 2-ml sterile polystyrene aspirating pipettes
7. 15-ml sterile polystyrene or polypropylene conical tubes
8. 12- by 75-mm sterile polystyrene culture tubes with closures (snap caps)
9. 12- by 75-mm sterile polystyrene culture tubes without closures (if using gamma counter)
10. Hemacytometer with coverslip
11. PES filter unit, 150 ml (0.2-µm pore size; 50-mm-diameter membrane)
12. 96-well round-bottom plate
13. LumaPlate and top seal (if using TopCount NXT Microplate scintillation and luminescence counter)

C. Equipment

1. 20-, 100-, and 200-µl pipettors
2. Pipet-Aid
3. Vacuum setup in biosafety cabinet
4. Centrifuge
5. Serofuge
6. ^{51}Cr radiation safety waste containers
7. Gamma counter (or TopCount machine)
8. 37°C, 5% CO_2, 96% relative humidity incubator
9. Microscope
10. Water bath

IV. CALIBRATION

Include QC information on reagent container and in QC records.

A. Gamma counter
   1. Cesium standard should be included with each run.
   2. Preventive maintenance should be performed according to the manufacturer's recommendations.

B. TopCount NXT Microplate scintillation and luminescence counter
   1. Calibration of TopCount machine must be done once a week.
   2. Preventive maintenance should be performed twice per year.

C. Pipettors
   Pipettors should be calibrated twice per year.

D. All laboratory equipment should have preventive maintenance performed according to the manufacturers' instructions.

ANALYTICAL CONSIDERATIONS

V. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Medium
   When R10 FBS is first prepared, it should be tested prior to use for the presence of contamination by pipetting 2 ml into 5 ml of BHI broth and placing in a 37°C, 5% CO_2, 96% relative humidity incubator for 5 days.

B. Assay validity
   In order for the assay to be considered valid, the spontaneous release must be ≤15% of the maximum release.

C. K-562 viability: ≥85% viable before and after being labeled with ^{51}Cr

D. New lots of ^{51}Cr
   Each new lot of ^{51}Cr is tested in parallel with the lot currently in use to ensure that the newest produces valid results.

VI. PROCEDURE

It is imperative that these cultures be handled in a biological safety cabinet.

Observe standard precautions.

◩ **NOTE:** All of the pipetting and filtering should take place in a biological safety cabinet using sterile technique, and all reagents should be at room temperature when used. All radioactive materials must be used in designated areas and must be disposed of properly per the institution's radiation safety guidelines.

A. PBMC preparation
   1. Centrifuge the heparin tube for 10 min at 350 × *g* at room temperature.
   2. Gently invert the Ficoll-Paque PLUS bottle twice.
   3. Pipette 5 ml of Ficoll-Paque PLUS into a sterile 15-ml conical tube.
   4. Using a 5-ml pipette, remove the plasma from the heparin tube and place into a sterile snap-cap tube. This can be aliquoted and frozen, or discarded if desired.
   5. Dilute the blood twofold using RPMI 1640. Mix gently by pipetting up and down.
   6. Carefully layer the blood onto the 5 ml of Ficoll-Paque PLUS. Be sure not to let the diluted blood penetrate the Ficoll-Paque PLUS.
   7. Centrifuge the conical tube containing the diluted blood on Ficoll-Paque PLUS according to the separation gradient manufacturer's instructions.
   8. After centrifugation, four layers will be visible. Using a sterile aspirating pipette connected to a vacuum system, gently aspirate the top clear layer that contains RPMI 1640 and any remaining plasma. Be careful not to disturb the cloudy layer underneath, which contains the mononuclear cells. If you do not have a vacuum system, use a 5-ml pipette to remove the top clear layer and dispose of it appropriately. Eject fluid into a waste container, such as an empty 15-ml conical tube, and treat as biohazardous waste.

VI. PROCEDURE *(continued)*

9. The cloudy mononuclear cell layer is removed next by placing the tip of a pipette on the layer and gently moving the pipette in a circular motion while applying suction with the Pipet-Aid. The entire layer should be removed and transferred to a sterile 15-ml conical tube containing 5 ml of RPMI 1640. Care should be taken to remove as little as possible of the Ficoll-Paque PLUS layer below the mononuclear cells. Add RPMI 1640 to bring the total volume to 14 ml. Centrifuge the conical tube according to the separation gradient manufacturer's instructions.
10. After centrifugation, aspirate the supernatant, making sure not to disturb the pellet.
11. Pipette 10 ml of RPMI 1640 into the conical tube. Thoroughly mix to resuspend the pellet.
12. Centrifuge the conical tube according to the separation gradient manufacturer's instructions.
13. After centrifugation, aspirate the supernatant, making sure not to disturb the pellet.
14. Pipette 1 ml of R10 FBS into the conical tube. Using the pipette, thoroughly mix to resuspend the pellet, being careful not to make bubbles.
15. Count the PBMCs using the total viable cell counting procedure (*see* procedure 11.10). This procedure uses trypan blue dye to count the viability and number of PBMCs. To better distinguish mononuclear cells, it is recommended to perform the cell count using crystal violet.
16. If lytic units are to be determined, the NK cells should be enumerated by flow cytometry. A total of 0.3×10^6 to 1.0×10^6 PBMCs is sufficient for this analysis.
17. Determine the amount of R10 FBS and cell suspension required to have 1 ml of the first E:T ratio. The E:T ratio is how many PBMCs there are for each K-562 cell. Typically, 2.5×10^6 PBMCs/ml are used for the 50:1 E:T ratio, so adjust the number you need for the first desired E:T ratio accordingly. If you have 12.5×10^6 PBMCs, the calculation is 2.5/12.5 = 0.20 ml of cell suspension (from step 14) + 0.80 ml of R10 FBS.
18. Label four sterile snap caps for the serial dilutions of PBMCs (effector cells). Commonly used E:T ratios are 50:1, 25:1, 12.5:1, and 6.25:1.
19. Pipette the appropriate amount of R10 FBS (calculated in step 17) into the starting E:T ratio snap cap.
20. Pipette 0.5 ml of R10 FBS into the next three snap caps.
21. Mix the cell suspension thoroughly and pipette the appropriate amount (calculated in step 17) into the starting E:T ratio snap cap.
22. With a new pipette, mix well, and pipette 0.5 ml from the first snap cap into the second one. Discard pipette.
23. Repeat this procedure until you come to the last snap cap, which will have a final volume of 1 ml after the addition of cells.
24. Using a 96-well plate, label the first 6 wells in the first row as "S." These wells are used to identify any spontaneous lysis of the target cells. (This creates two sets of triplicate wells.)
25. Label the next 6 wells as "M." These wells are used to demonstrate the maximum chromium released by the target cells. (This creates two sets of triplicate wells.)
26. Skip one row and mark off the patient row(s) in triplicate.
27. Pipette 100 µl of R10 FBS into each of the "S" wells.
28. Pipette 100 µl of 1% Triton X-100 into each of the "M" wells.
29. Pipette 100 µl of the first E:T ratio into each of the first three wells for that patient.
30. Changing tips between triplicates, pipette the remaining E:T ratios into the appropriate triplicate wells.

VI. PROCEDURE *(continued)*

B. K-562 preparation

1. Thoroughly mix the contents of the K-562 flask and pipette 2 ml into a sterile snap cap.
2. Count the K-562 cells using the total viable cell counting procedure (*see* procedure 11.10). Live K-562 cells appear translucent, while dead K-562 cells stain blue with trypan blue dye.
3. Add or remove K-562 suspension from the original snap cap as needed to obtain 2×10^6 to 3×10^6 K-562 cells to label. This is the recommended number of cells to label with 100 µl of 1-mCi/ml ^{51}Cr.
4. Serofuge the snap cap at 3,500 rpm for 3 min to pellet.
5. Decant the supernatant pellet side up so as not to disturb the pellet.
6. Resuspend the K-562 pellet in 100 µl of R10 FBS.
7. Add 100 µl of ^{51}Cr to the resuspended K-562 cells. It is recommended that this be timed so that the K-562 cells will be ready close to when the PBMCs will be ready, due to the highly cytotoxic nature of ^{51}Cr.
8. Put the snap cap into the bottom of a lead container containing warm water and place in a 37°C CO_2 incubator for 1 h. The ^{51}Cr will arrive in a lead container from the manufacturer. After you have either consumed or disposed of the contents, you will have an empty lead container that you can use for this incubation.
9. Gently shake the snap cap after 30 min to resuspend the K-562 cells.
10. After the 1-h incubation, add 2 ml of R10 FBS to the snap cap containing the K-562 cells. Mix well.
11. Spin in a serofuge for 3 min at 3,500 rpm.
12. Decant the supernatant into an appropriate radioactive waste container, pellet side up so as not to disturb the pellet.
13. Add 2 ml of R10 FBS to the snap cap. Mix well.
14. Spin in the serofuge for 3 min at 3,500 rpm.
15. Decant the supernatant into an appropriate radioactive waste container.
16. Repeat steps 13 through 15 two more times.
17. After the suspension is decanted completely for the fourth time, pipette 1 ml of R10 FBS into the snap cap. Mix thoroughly.
18. Count the K-562 cells using the total viable cell counting procedure (*see* procedure 11.10).
19. An NK assay utilizing one plate requires 10 ml of 5×10^4/ml K-562 cells. Remove from the snap cap the necessary amount of K-562 cells and dilute to 10 ml with R10 FBS in a sterile 15-ml conical tube. Sample calculation: if you have 2.3×10^6 K-562 cells, then the calculations would be as follows: 5×10^5 K-562 cells/2.3×10^6 K-562 cells = 0.22 ml of resuspended K-562 (from step 17) + 9.78 ml of R10 FBS.
20. Invert the 15-ml conical tube several times to mix it.
21. Pipette 100 µl of K-562 final suspension to each of the "S" wells, changing tips between triplicates.
22. Pipette 100 µl of K-562 final suspension to each of the "M" wells, changing tips between triplicates.
23. Pipette 100 µl of K-562 final suspension to each of the patient E:T ratio wells, changing tips between triplicates.
24. Spin the plate in the centrifuge for 5 min at $100 \times g$.
25. After centrifugation, incubate the plate at 37°C for 4 h.
26. After the incubation, spin the plate in the centrifuge for 10 min at $300 \times g$.
27. Label one nonsterile 12- by 75-mm plastic culture tube without closure for each well of the plate containing PBMCs and K-562 cells and the spontaneous and maximum wells (if using a gamma counter).

VI. PROCEDURE *(continued)*

28. Tilt the plate slightly and pipette 100 µl of supernatant from each well and eject into the appropriate tube. Be careful not to disturb the pellet. Repeat this procedure for all wells, changing tips every triplicate. Alternatively, if using a TopCount NXT instrument, you can pipette 50 µl of supernatant from each well to the corresponding well on a LumaPlate. The scintillant is already in this plate. Let it dry overnight, and put the top seal on the plate in the morning.

29. Load tubes into gamma counter (or plate into TopCount NXT instrument) to count for 1 min per tube or well.

30. Discard waste into a ^{51}Cr radioactive waste container after counting.

VII. CALCULATIONS

The mean of the triplicate counts per minute (cpm) is determined for each E:T ratio and for the spontaneous- and maximum-release wells. Percent specific target cell lysis is calculated for each E:T ratio as follows.

$$\% \text{ Specific lysis} = \frac{(\text{mean cpm of experimental release} - \text{mean cpm of spontaneous release})}{(\text{mean cpm of maximum release} - \text{mean cpm of spontaneous release})} \times 100$$

Lytic units/10^7 PBMCs and lytic units/10^7 NK cells can be calculated using the method of Bryant et al. (1).

POSTANALYTICAL CONSIDERATIONS

VIII. INTERPRETATION

The NK cell percent lysis at each E:T ratio can be used to determine the effectiveness of the subject's NK cells via comparison with the results for a healthy control. It should be noted, however, that variation in ^{51}Cr absorption and K-562 cell percent spontaneous release is to be expected, as the health of the K-562 cell line does have some minor variability.

REFERENCE

1. **Bryant, J., R. Day, T. L. Whiteside, and R. B. Herbeman.** 1992. Calculation of lytic units for the expression of cell-mediated cytotoxicity. *J. Immunol. Methods* **146:**91–103.

SUPPLEMENTAL READING

Douglas, S. D., S. Durako, N. B. Tustin, J. Houser, L. Muenz, S. E. Starr, and C. Wilson for the Adolescent Medicine HIV/AIDS Research Network. 2001. Natural killer cell enumeration and function in HIV-infected and high-risk uninfected adolescents. *AIDS Res. Hum. Retrovir.* **17:**543–552.

Evans, D., K. Lynch, T. Benton, B. Dube, D. Gettes, N. Tustin, J. Lai, D. Metzger, and S. Douglas. 2008. Selective serotonin reuptake inhibitor and substance P antagonist enhancement of natural killer cell innate immunity in human immunodeficiency virus/acquired immunodeficienty syndrome. *Biol. Psychiatr.* **63:**899–905.

Hay, R., J. Caputo, T. R. Chen, M. Macy, P. McClintock, and Y. Reid (ed.). 1994. *American Type Culture Collection (ATCC) Catalogue of Cell Lines and Hybridomas*, 8th ed. 1994 reference guide. American Type Culture Collection, Manassas, VA.

Lozzio, C. B., and B. B. Lozzio. 1975. Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. *Blood* **45:**321–334.

Whiteside, T., C. R. Rindalo, and R. B. Herberman. 1992. Cytolytic cell functions, p. 220–230. *In* N. R. Rose, E. Conway de Macario, J. L. Fahey, H. Friedman, and G. M. Penn (ed.), *Manual of Clinical Laboratory Immunology*, 4th ed. American Society for Microbiology, Washington, DC.

Whiteside, T. L. 2006. Measurement of NK cell activity in humans, p. 296–300. *In* B. Detrick, R. G. Hamilton, and J. D. Folds (ed.), *Manual of Clinical Laboratory Immunology*, 7th ed. ASM Press, Washington, DC.

APPENDIX 11.13.3–1

Observe standard precautions.

Assay Materials

A. K-562 cell line
   1. According to the *ATCC Catalogue of Cell Lines and Hybridomas* (1), the K-562 cell line was established by Lozzio and Lozzio (2) from the pleural effusion of a 53-year-old female with chronic myelogenous leukemia in terminal blast crises.
   2. The K-562 cell line can be purchased from the *ATCC Catalogue of Cell Lines and Hybridomas*, as CCL 243.
   3. The culture medium is RPMI 1640, 90%; FBS, 10%; antimicrobial agent free.
   4. The cell line is maintained in T_{25} flasks. When a concentration of 750,000 to 10^6/ml is reached, the flask is split 1:10 with culture medium.
   5. Splits should be maintained so that a T_{25} flask containing K-562 cells at a concentration of 10^6/ml is available for use in the NK cell assay.

B. Reagent information and preparation
   1. R10 FBS (500 ml)
      a. 435 ml of RPMI 1640
      b. 5 ml of 200 mM L-glutamine for a final concentration of 2 mM L-glutamine
      c. 5 ml of 10,000-U/ml penicillin and 10,000-µg/ml streptomycin solution for a final concentration of 100 U of penicillin and 100 µg of streptomycin per ml in solution.
      d. 5 ml of 1 M HEPES buffer for a final of 10 mM HEPES buffer
      e. 50 ml of heat-inactivated FBS
   2. 1% Triton X
      a. 0.1 ml of Triton X-100
      b. 9.9 ml of 1× phosphate-buffered saline
   3. Crystal violet
      a. 98 ml of deionized H_2O
      b. 2 ml of glacial acetic acid
      c. 0.05 g of crystal violet dye (powder form)
   4. Reagent information
      See table on next page.

REFERENCES

1. **Hay, R., J. Caputo, T. R. Chen, M. Macy, P. McClintock, and Y. Reid.** 1994. *American Type Culture Collection (ATCC) Catalogue of Cell Lines and Hybridomas*, 8th ed. 1994 reference guide. American Type Culture Collection, Manassas, VA.

2. **Lozzio, C. B., and B. B. Lozzio.** 1975. Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. *Blood* **45:**321–334.

Reagent	Manufacturer	Catalog no.	Address and website	Phone no.
^{51}Chromium	Perkin-Elmer	NEZ-030S	940 Winter St. Waltham, MA 02451 http://www.perkinelmer.com	(800) 762-4000
Fetal bovine serum (heat inactivated)	Atlanta Biologicals	S11550H	2150 Cedars Road, Suite 200 Lawrenceville, GA 30043 http://www.atlantabio.com	(800) 780-7788
Ficoll-Paque PLUS	GE Healthcare	17-1440-03	800 Centennial Ave. P.O. Box 1327 Piscataway, NJ 08855-1327 http://www.gehealthcare.com	(800) 526-3593
HEPES buffer	Invitrogen	15630-080	1600 Faraday Ave. P.O. Box 6482 Carlsbad, CA 92008 http://www.invitrogen.com	(800) 955-6288
L-Glutamine	Lonza	17-605E	8830 Biggs Ford Rd. Walkersville, MD 21793 http://www.lonza.com	(301) 898-7025
Penicillin-streptomycin	Invitrogen	15140-148	1600 Faraday Ave. P.O. Box 6482 Carlsbad, CA 92008 http://www.invitrogen.com	(800) 955-6288
RPMI 1640	Lonza	12-167F (500 ml) 12-167Q (1,000 ml)	8830 Biggs Ford Rd. Walkersville, MD 21793 http://www.lonza.com	(301) 898-7025
Trypan blue dye	Invitrogen	15250-061	1600 Faraday Ave. P.O. Box 6482 Carlsbad, CA 92008 http://www.invitrogen.com	(800) 955-6288
Triton X-100	Sigma	X-100	3050 Spruce St. St. Louis, MO 63103 http://www.sigma-aldrich.com	(800) 521-8956
1× phosphate-buffered saline	Lonza	17-516F	8830 Biggs Ford Rd. Walkersville, MD 21793 http://www.lonza.com	(301) 898-7025
Crystal violet dye (powder)	Sigma	A-6158	3050 Spruce St. St. Louis, MO 63103 http://www.sigma-aldrich.com	(800) 521-8956
Glacial acetic acid	Sigma	A-6283	3050 Spruce St. St. Louis, MO 63103 http://www.sigma-aldrich.com	(800) 521-8956
LumaPlate-96	Perkin-Elmer	6006633	940 Winter St. Waltham, MA 02451 http://www.perkinelmer.com	(800) 762-4000

11.14 Quantitation of Human Interleukin 4, Interleukin 6, and Gamma Interferon

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Interleukin 4 (IL-4), IL-6, and gamma interferon (IFN-γ) are cytokines with many cellular effects, including mediation of the immune response to infection. Among other biological activities, IL-4 and IL-6 stimulate B cells and IFN-γ has antiviral activities (6). There is an increasing interest in using cytokine levels to monitor disease progression (2). There are several commercially prepared kits available to quantify cytokines by ELISA. This procedure is written for use with kits manufactured by Pierce Endogen. If IL-4, IL-6, or IFN-γ is present in the clinical specimen, it will bind to an ELISA plate that is precoated with antibody specific for that cytokine. After the patient specimen is incubated with a biotinylated detection antibody, the plate is washed and then streptavidin conjugated to horseradish peroxidase (HRP) is added. After another incubation, tetramethylbenzidine (TMB) substrate is added to the plate. The enzyme-substrate reaction produces a color change that can be measured spectrophotometrically. The unknown values can be determined from a standard curve in which the color intensity is directly proportional to the standard concentration.

II. SPECIMEN

Observe standard precautions.

Serum, EDTA plasma, heparin plasma, sodium citrate plasma, and urine are acceptable specimens for cytokine measurement. In addition, in vitro cytokine production can be measured in peripheral blood mononuclear cell culture supernatants or whole blood culture supernatants (1, 7). Specimens should be stored at −70°C. If more than one ELISA is to be performed, the specimen or supernatant should be split into an appropriate number of 150-µl aliquots prior to freezing. If the cytokine concentration exceeds the highest standard concentration, prepare one or more 10-fold dilutions and reassay.

III. REAGENTS AND SUPPLIES

A. Reagents

1. Human IL-4, IL-6, or IFN-γ kit (Pierce Endogen, Rockford, IL)
2. Distilled water

B. Supplies

1. 5- and 10-ml nonsterile plastic pipettes
2. 20-, 100-, 200-, and 1,000-µl pipettors
3. Multichannel pipettor
4. 2-liter nonsterile flask or bottle to dilute wash buffer
5. Nonsterile pipette tips to deliver 5 to 1,000 µl
6. 12- by 75-mm nonsterile polypropylene tubes
7. Disposable reagent reservoirs
8. 15-ml nonsterile plastic centrifuge tubes with conical bottoms
9. Squirt bottle
10. Empty box to fit a 96-well plate

C. Equipment

1. Vortex
2. ELx800 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) capable of reading at 450 nm
3. Automated 96-well plate washer (optional)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. All reagents and samples should be brought to room temperature before use. Do not place in a water bath.

B. Reagents

1. Do not use any reagent that appears cloudy. This may indicate microbial contamination.

2. Do not mix reagents from different kits.

3. Use a new reagent reservoir for each reagent.

4. When using the multichannel pipettor, use new tips for each row to avoid cross contamination and bubble formation.

5. TMB substrate solution should be colorless. If it appears blue, discard it.

6. The standard diluent and biotinylated antibody reagent contain sodium azide as a preservative. If leftover reagent is poured down the drain, it must be flushed with water for several minutes.

C. Specimens

If any specimen is cloudy, lipemic, hemolyzed, or otherwise unusual, a note should be made on the plate plan or worksheet and the result should be reported with this notation.

D. Calibration

1. Standard curve

Each ELISA plate is specific for only one cytokine. This is identified by the kit being used. A curve using the enclosed standard must be run on each plate.

2. Medium validation

a. Medium validation must be performed in order to run culture supernatants and patient specimens (serum, etc.) on the same plate.

b. A standard curve diluted in culture medium is run in parallel with a standard curve diluted in the Pierce Endogen standard diluent. If the optical density (OD) values of the two curves are within 10% of the mean for each standard point of both curves then the culture medium is validated. For example, the average OD values for a particular point of a standard curve are 0.605 for the medium-diluted curve and 0.685 for the curve diluted in standard diluent. The mean for these OD values would be 0.645. The difference between the mean and the OD values would be 0.040, or 5.8%. This means that culture supernatants can be run on a plate with a curve diluted in standard diluent. If the difference is greater than 10%, culture supernatants must be run on a separate plate with a curve diluted in the culture medium.

3. Plate reader

The plate reader must be calibrated in accordance with the specific manufacturer's instructions.

V. PROCEDURE

Observe standard precautions.

It is imperative that these cultures be handled in a biological safety cabinet.

Plate setup

A plate plan or worksheet should be filled out completely for each plate to be run. This can be a diagram of a 96-well plate or a listing of each well. The standards and specimens to be run should be written in the places where they will be on the plate. The standards should always be run at the beginning of the plate (Fig. 11.14–1). All standards and samples are to be run in duplicate.

A. Remove the plate from the foil envelope. If a partial plate is being run, the unused strips should be resealed in the envelope with the desiccant and refrigerated as soon as possible.

Figure 11.14–1 Plate plan for Endogen IL-6 ELISA with 10 samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 1 400 pg/ml	Standard 5 10.24 pg/ml	Sample 3	Sample 7								
B	Standard 1 400 pg/ml	Standard 5 10.24 pg/ml	Sample 3	Sample 7								
C	Standard 2 160 pg/ml	Standard 6 0 pg/ml	Sample 4	Sample 8								
D	Standard 2 160 pg/ml	Standard 6 0 pg/ml	Sample 4	Sample 8								
E	Standard 3 64 pg/ml	Sample 1	Sample 5	Sample 9								
F	Standard 3 64 pg/ml	Sample 1	Sample 5	Sample 9								
G	Standard 4 25.6 pg/ml	Sample 2	Sample 6	Sample 10								
H	Standard 4 25.6 pg/ml	Sample 2	Sample 6	Sample 10								

V. PROCEDURE *(continued)*

B. Add 50 µl of the biotinylated antibody reagent to each well using a multichannel pipettor. This reagent is slightly foamy, so it will be necessary to use new tips frequently to avoid air bubbles.

C. Mix all samples by gently inverting the tubes.

D. Make 1:2.5 serial dilutions of the appropriate standard. See Appendix 11.14–1 for details.

E. Add 50 µl of standards or samples to the appropriate wells in duplicate. Check that no air bubbles are in the pipette tip. Mix by gently tapping the plate several times.

F. Cover the plate with a new adhesive plate sealer and incubate at room temperature (20 to 25°C) for 2 h. Ensure that the plate sealer edges are secure.

G. At the end of 2 h, wash the plate with wash buffer. See Appendix 11.14–1 for instructions to dilute wash buffer.

1. Empty the plate into the sink and use a squirt bottle to add wash buffer vigorously. Be sure to gently squeeze the long sides of the plate to ensure that all strips remain in place during the decanting step.

2. Empty the plate and repeat two more times.

3. After the third wash, pat the plate on paper towels to absorb all excess liquid.

4. An automated plate washer can be used for this process.

H. Add 100 µl of streptavidin-HRP solution to each well. See Appendix 11.14–1 for preparation instructions.

I. Cover the plate with a new adhesive plate sealer and incubate at room temperature for 30 min. Ensure that the plate sealer edges are secure.

J. After 30 min, wash the plate (*see* steps V.G.1 to V.G.4).

K. After washing the plate, add 100 µl of TMB substrate solution to each well.

◩ This reagent is extremely sensitive to light. Do not pour into reagent reservoir until immediately before use. Do not pour out more reagent than necessary.

L. Allow the plate to develop in the dark at room temperature for 30 min (e.g., place plate in empty box on lab bench). Do not use a plate sealer for this step.

M. After 30 min, add 100 µl of stop reagent to each well.

N. Read the plate using an automated plate reader at 450 nm. The plate must be read within 30 min after the addition of the stop reagent.

V. PROCEDURE *(continued)*

O. Subtract 550-nm values from 450-nm values to correct for optical imperfections in the microplate. If a 550-nm reading is not available, the 450-nm reading will suffice; however, the absorbance values will be higher.

POSTANALYTICAL CONSIDERATIONS

VI. CALCULATIONS

A. The standard curve can be created manually or with curve-fitting software. The cytokine concentration is plotted on the *x* axis, and the average OD of the standards is plotted along the *y* axis. The average OD of each specimen is used to interpolate its concentration from the standard curve. If curve-fitting software is used, a linear or point-to-point curve can be chosen. All OD values must agree within 10% of the duplicate value for that patient, control, or standard.

Example: A patient whose samples have been set up in duplicate has OD values of 0.123 and 0.167. Average these numbers.

$$0.123 + 0.167 = 0.29 \quad 0.29/2 = 0.145$$

Calculate the difference between the mean and one of the values.

$$0.145 - 0.123 = 0.022$$

Divide the difference by the mean and multiply by 100 to calculate the percent difference.

$$0.022/0.145 = 0.152 \times 100 = 15.2\%$$

These OD values are greater than 10% from the mean, and this sample would have to be retested on another run.

B. The results for any diluted specimens must be multiplied by the dilution factor.

VII. INTERPRETATION

A. In the absence of disease, cytokines are nearly undetectable in body fluids (6). See Table 11.14–1.

B. Sensitivity

1. The IL-4 and IFN-γ assays are sensitive to <2 pg/ml.

2. The IL-6 assay is sensitive to <1 pg/ml.

VIII. PROCEDURE NOTES

A. If the reader does not give an OD value for a specimen or if the cytokine concentration is greater than the highest standard, that specimen must be diluted and retested on another run.

B. Culture supernatants are diluted in the culture medium. Serum, plasma, or urine is diluted in the provided sample diluent.

Table 11.14–1 Pierce Endogen observed cytokine values in body fluids[a]

Body fluid	Concn (pg/ml) of cytokine		
	IL-4	IL-6	IFN-γ
Serum	Not detected (8)	0–149 (14)	0–1.5 (35)
Plasma	Not detected (8)	0–5 (14)	0–2.6 (45)
Urine	Not detected (5)	0–0.6 (5)	0.5–1.2 (5)

[a] The number of subjects evaluated is in parentheses (3–5).

REFERENCES

1. **Katial, R. K., D. Sachanandani, C. Pinney, and M. M. Lieberman.** 1998. Cytokine production in cell culture by peripheral blood mononuclear cells from immunocompetent hosts. *Clin. Diagn. Lab. Immunol.* **5:**78–81.
2. **Lee, B. N., J. G. Lu, M. W. Kline, M. Paul, M. Doyle, C. Kozinetz, W. T. Shearer, and J. M. Reuben.** 1996. Type 1 and type 2 cytokine profiles in children exposed to or infected with vertically transmitted human immunodeficiency virus. *Clin. Diagn. Lab. Immunol.* **3:**493–499.
3. **Pierce Endogen.** 2006. Package insert for human interleukin 4 ELISA. Pierce Endogen, Rockford, IL.
4. **Pierce Endogen.** 2007. Package insert for human interleukin 6 ELISA. Pierce Endogen, Rockford, IL.
5. **Pierce Endogen.** 2006. Package insert for human interferon gamma ELISA. Pierce Endogen, Rockford, IL.
6. **Remick, D. G.** 2002. Protein analysis and bioassays of cytokines and cytokine receptors, p. 320–337. *In* N. R. Rose, R. G. Hamilton, and B. Detrick (ed.), *Manual of Clinical Laboratory Immunology*, 6th ed. ASM Press, Washington, DC.
7. **Wallis, R. S., H. M. Lederman, J. Spritzler, J. L. Devers, D. Georges, A. Weinberg, S. Stehn, M. M. Lederman, and the ACTG Inducible Cytokines Focus Group.** 1998. Measurement of induced cytokines in AIDS clinical trials using whole blood: a preliminary report. *Clin. Diagn. Lab. Immunol.* **5:**556–560.

SUPPLEMENTAL READING

Aziz, N., P. Nishanian, and J. L. Fahey. 1998. Levels of cytokines and immune activation markers in plasma in human immunodeficiency virus infection: quality control procedures. *Clin. Diagn. Lab. Immunol.* **5:**755–761.

APPENDIX 11.14–1

Observe standard precautions.

Include QC information on reagent container and in QC records.

Reagent Preparation

Instructions for reagent preparation and standard dilutions from Pierce Endogen (1–3)

A. Wash buffer
   In a clean flask or bottle, dilute 50 ml of the 30× wash buffer concentrate with 1.45 liters of distilled water. Label the buffer with the lot number, date made, expiration date, and initials of the person who made it. Store unused wash buffer in a bottle at 4°C.

B. Streptavidin-HRP solution
   1. Do not prepare more than 15 min before it will be used. Do not prepare more than necessary. There is no extra reagent in the kit.
   2. Gently vortex the streptavidin-HRP concentrate.
   3. In a plastic 15-ml tube, dilute the streptavidin-HRP concentrate 1:40 with streptavidin-HRP dilution buffer.
   4. If an entire plate is being assayed, add 30 µl of concentrate to 12 ml of diluent. If less than a whole plate is being assayed, add 2.5 µl of concentrate to 1 ml of diluent for each strip used.

C. Standards
   1. Reconstitute one vial of standard with the volume of distilled water listed on the standard vial. The standard will take about 1 min to dissolve. Use standard within 1 h of reconstitution. Do not store reconstituted standard.
   2. Gently invert standard to mix.
   3. Make a 1:2.5 serial dilution of the standard. Label six polypropylene tubes for the standard dilutions as follows.
      a. For IL-4 and IL-6, label the tubes 400 pg, 160 pg, 64 pg, 25.6 pg, 10.24 pg, and 0 pg.
      b. For IFN-γ, label the tubes 1,000 pg, 400 pg, 160 pg, 64 pg, 25.6 pg, and 0 pg.
   4. If the specimens to be run are plasma, serum, or urine, the standard is diluted with the provided standard diluent. If the specimens are culture supernatants, the standard is diluted with the culture medium unless validation has been performed (*see* item IV.D.2 in procedure 11.14). If validation has been done, all specimen types can be analyzed on the same plate.
      a. Add 240 µl of the appropriate diluent to each of the six tubes.
      b. Pipette 160 µl of the reconstituted standard to the first tube and gently vortex to mix.
      c. Using the same pipette tip, transfer 160 µl to the second tube and gently vortex to mix.
      d. Continue this for the next three tubes, being careful that there are no air bubbles during the transfers.

APPENDIX 11.14–1 *(continued)*

e. Do not transfer anything into the sixth tube. This is the 0 standard tube and will contain only diluent or culture medium.
f. If more than one plate is being run, the standard dilutions can be made by increasing all volumes two- or threefold.

References

1. **Pierce Endogen.** 2006. Package insert for human interleukin 4 ELISA. Pierce Endogen, Rockford, IL.
2. **Pierce Endogen.** 2007. Package insert for human interleukin 6 ELISA. Pierce Endogen, Rockford, IL.
3. **Pierce Endogen.** 2006. Package insert for human interferon gamma ELISA. Pierce Endogen, Rockford, IL.

APPENDIX 11.14–2

Suppliers

Pierce Endogen
3747 North Meridian Rd.
P.O. Box 117
Rockford, IL 61105

Bio-Tek Instruments, Inc.
Highland Park, Box 998
Winooski, VT 05404

Fisher Scientific
2000 Park Lane Dr.
Pittsburgh, PA 15275
http://www.fishersci.com

11.15

Flow Cytometry Whole-Blood Intracellular-Cytokine Assay Using Phorbol Myristate Acetate, Ionomycin, and Brefeldin A

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Cytokines are soluble proteins produced by T and B lymphocytes, natural killer cells, monocytes, macrophages, and granulocytes. Specifically, cytokines regulate growth, differentiation, and function of a wide variety of cells and mediate normal and pathological responses. Most cytokine bioassays examine cytokines at the cell population level, but they cannot provide information concerning the phenotype of cytokine-producing cells or mechanism of cytokine products. The flow cytometry method described here measures production of cytokines by T-cell subsets using whole blood. Heparinized blood is stimulated with the polyclonal activator phorbol myristate acetate plus ionomycin (induces intracelluar signal cascades for polyclonal leukocyte activation) and the protein transport inhibitor brefeldin A (BFA) (a fungal metabolite that interferes with vesicular transport from the rough endoplasmic reticulum to the Golgi complex) for 4 h at 37°C and 5% CO_2. Activated cells are stained with monoclonal antibodies for lymphocyte surface markers, followed by lysing of RBCs. WBCs are fixed and permeabilized simultaneously and stained with monoclonal antibodies to intracellular cytokines. Stained cells are analyzed by flow cytometry.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. Peripheral blood in sodium heparin anticoagulant (2 ml)
2. Vacutainers or other collection tubes should be filled properly to account for the amount of anticoagulant.
3. Since all specimens should be regarded as potentially infectious, standard precautions for blood collection and handling should be properly followed.

B. Specimen transport

1. Specimens should be placed in well-stoppered tubes and should be placed with adequately absorbent material, preferably in a closed leakproof container, for delivery.
2. If delivery to reference laboratories is possible within the first 24 h, follow federal guidelines (International Air Transport Association Dangerous Goods Regulations, January 2001 [*see* procedure 15.5]).
3. Specimens should be maintained at room temperature (18 to 22°C) during transportation and storage.
4. *Testing must be performed within 24 h from the time of collection.*

C. Specimen labeling and request submission

1. Each patient sample must be properly labeled.
2. Each sample should be accompanied by some type of procurement form which should contain appropriate identifiers, e.g., patient's name, date of birth, and gender; date and time of collection, clinician; and other relevant patient information.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

D. Rejection criteria

1. Inadequate sample volume
2. Clotted specimen
3. Hemolyzed blood specimen
4. Specimen more than 24 h old
5. Specimen exposed to cold temperatures

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents (*see* Appendix 11.15–1 for reagent concentrations and preparation)

1. Dulbecco's phosphate-buffered saline without calcium, magnesium, or phenol red (dPBS) (Invitrogen, Grand Island, NY)
2. Dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO)
3. RPMI 1640 with glutamine (Invitrogen)
4. Heat-inactivated fetal bovine serum (FBS) (Invitrogen)
5. Monoclonal antibodies including isotype markers against cell surface and cytokine markers—stored according to manufacturer's instructions
6. Wash buffer (0.5% FBS, 0.1% sodium azide)
7. Sodium azide (J.T. Baker, Phillipsburg, NJ)
8. Phorbol 12 myristate 13 acetate (PMA) (Sigma)
9. Ionomycin (Sigma)
10. BFA (Sigma)
11. Lysing solution
a. NH_4Cl (Sigma)
b. $NaHCO_3$ (Fisher Scientific, Fair Lawn, NJ)
c. EDTA (Fisher Scientific)
d. Sterile distilled water
12. Permeabilization buffer
a. 10% Methanol-free formaldehyde (Polysciences, Inc., Warrington, PA)
b. Hanks balanced salt solution without calcium, magnesium, or phenol red (HBSS) (Invitrogen)
c. Saponin (Sigma)
d. HEPES buffer (Invitrogen)
13. Flow cytometer calibration beads or other calibration reagents (follow manufacturer's directions)

B. Supplies

1. Adjustable-volume pipette and tips
2. 12- by 75-mm polypropylene tubes (BD Biosciences, Franklin Lakes, NJ)
3. 12- by 75-mm polystyrene tubes (BD Biosciences)
4. Liquid-waste container with bleach
5. Disposable gloves
6. Disposable lab coat
7. Protective eyewear

C. Equipment

1. Centrifuge with swinging bucket rotor, capable of centrifugation at 300 × *g* and equipped with aerosol containment canisters
2. Vacuum source with pipette attached
3. Flow cytometer
4. Class II biosafety cabinet
5. Timer/stop watch
6. CO_2 incubator (5% CO_2)
7. Vortex

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

Controls

A. Surface and intracellular markers (e.g., CD4 and interleukin-2 [IL-2]) from a healthy volunteer donor. These cells should be stained in the same manner and with the same monoclonal antibodies as the patient specimens. These cells should be run with every cytokine assay.
B. Various blocking reagents may be used to ensure positivity.
C. Unstimulated control sample containing BFA.
D. Commercially available QC substance that is positive for particular cytokine.

V. CALIBRATION

A. Optical alignment

The flow cytometer must be optically aligned. Follow manufacturer's directions. (Some cytometers require a company engineer.)

B. Calibration

Depending on the flow cytometer manufacturer, either standardized fluorescent beads or chicken RBCs may be used for calibration and to assess instrument function. Follow manufacturer's procedures.

C. Verification with whole-blood preparation

Electrical fluorescent compensation on single stained cells. Follow manufacturer's procedures.

VI. CYTOKINE ASSAY

It is imperative that these cultures be handled in a biological safety cabinet.

Observe standard precautions.

A. Procedure

1. Label each of two 12- by 75-mm polypropylene tubes "Unstimulated" and "Activated."
2. Add 500 μl of RPMI 1640 to each tube.
3. Add 10 μl of BFA to the unstimulated tube.
4. Add 20 μl of PMA, 10 μl of ionomycin, and 10 μl of BFA to the activated tube.
5. Add 500 μl of heparinized (sodium heparin) whole blood. Vortex to mix.
6. Incubate for 4 h at 37°C in the 5% CO_2 incubator. (*Note:* One milliliter of activated blood provides enough cells for staining 10 samples, based on 100 μl/test.)
7. Aliquot 100 μl of activated blood into 12- by 75-mm polystyrene tubes preloaded with monoclonal antibody for surface antigens. (Follow manufacturer's instructions for appropriate volume. Antibody may have to be titered.)
8. Incubate for 15 min at room temperature in the dark.
9. Add 2 ml of prewarmed (37°C) lysing solution per tube. Vortex and incubate for 5 min at room temperature.
10. Centrifuge at 300 × *g* for 5 min, and aspirate supernatant. Vortex lightly to loosen cell pellet. Wash twice with 2 ml of wash buffer and centrifuge at 300 × *g* for 5 min.
11. Aspirate supernatant. Vortex lightly to loosen pellet. Add 500 μl of permeabilization buffer. Vortex lightly to mix. Incubate for 15 min at room temperature.
12. Wash twice with 2 ml of wash buffer. Spin at 300 × *g* for 5 min. Aspirate supernatant. Vortex lightly to loosen pellet.
13. Add cytokine monoclonal antibody for intracellular staining. (Follow manufacturer's instructions for appropriate volume. Antibody may have to be titered.) Vortex lightly.
14. Incubate for 25 to 30 min at room temperature in the dark.
15. Wash once with wash buffer. Spin at 300 × *g* for 5 min.
16. Aspirate supernatant to loosen pellet, and fix with 300 μl of 1% formaldehyde.

B. Flow cytometry analysis

1. Collect 5,000 events in the gate (small nongranular cells) on the flow cytometer. Cells can be stored at 4°C and collected the following day.
2. Gate lymphocytes using forward versus side light scatter parameters. Analyze fluorescent parameters for appropriate surface and cytokine markers. Negative markers will be set on unstimulated stained cells for the same antibodies.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

Report percentage of surface marker that expresses a particular cytokine (e.g., if the total percentage of CD4 is 25% and the $CD4^+$ IL-2^+ is 10%, then 10% divided by 25% would equal 40%, which is the percentage of CD4 that expresses IL-2).

VIII. INTERPRETATION

Normal values

A. Values for at least 50 healthy donors should be ascertained by each laboratory performing this test.
B. Compare percentage to means and standard deviations of healthy donors.

IX. LIMITATIONS OF TESTING

Stimulation with PMA and ionomycin does not occur in vivo.

SUPPLEMENTAL READING

Centers for Disease Control and Prevention. 1997. Revised guidelines for performing $CD4^+$ T-cell determinations in persons infected with human immunodeficiency virus (HIV). *MMWR Morb. Mortal. Wkly. Rep.* **46**(RR-2)**:**1–29.

Jung, T., U. Schauer, C. Heusser, C. Nermann, and C. Rieger. 1993. Detection of intracellular cytokines by flow cytometry. *J. Immunol. Methods* **159:**197–207.

Maino, V., M. A. Suni, and J. J. Ruitenberg. 1995. Rapid flow cytometric method for measuring lymphocyte subset activation. *Cytometry* **20:**127–133.

Picker, L. J., M. K. Singh, Z. Zdraveski, J. R. Treer, S. L. Waldrop, P. R. Bergstresser, and V. C. Maino. 1995. Direct demonstration of cytokine heterogeneity among human memory/effector T cells by flow cytometry. *Blood* **86:**1408–1419.

APPENDIX 11.15–1

Observe standard precautions.

Include QC information on reagent container and in QC records.

Reagent Preparation

A. PMA
   1. Stock solution of 1 mg/ml is prepared in DMSO, with aliquots frozen at 70°C.
   2. Working solution is prepared from stock solution diluted in dPBS for a 1-μg/ml concentration. Final concentration in assay is 20 ng/ml.

B. Lysing solution
   1. Stock solution is prepared using 8.02 g of NH_4Cl (Sigma), 0.84 g of $NaHCO_3$ (Fisher), and 0.37 g of EDTA (Fisher) and bringing to a volume of 100 ml with sterile distilled water. Store at 4°C. Keeps for 6 months.
   2. Working solution is prepared by adding 10 ml of stock solution to 90 ml of sterile distilled water. Keeps for a week.

C. Ionomycin
   1. Stock solution of 1 mg/μl is prepared in DMSO, with aliquots frozen at −70°C.
   2. Working solution is diluted in PBS for a 100-μg/ml concentration. Final concentration in assay is 1 μg/ml.

D. BFA
   1. Stock solution of 5-mg/ml is prepared in DMSO, with aliquots frozen at −70°C.
   2. Working solution is diluted in PBS for a 1-mg/ml concentration. Final concentration of assay is 10 μg/ml.

E. Permeabilization buffer
   1. Dilute 10% methanol-free formaldehyde in HBSS to make a 4% solution.
   2. Dissolve 0.1 g of saponin in 100 ml of 4% formaldehyde.
   3. Filter and add 1 ml of 1 M HEPES buffer.
   4. Cover in foil and store at 4°C. Keeps for 2 weeks.

F. Buffer
   Add 2.5 ml of heat-inactivated FBS and 0.5 g of sodium azide to 500 ml of PBS for a 0.5% FBS–0.1% sodium azide solution.

11.16 Whole-Blood Lymphocyte Immunophenotyping Using Cell Surface Markers by Flow Cytometry

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Whole blood can be stained with fluorochrome-labeled monoclonal antibodies against antigen markers found on the surfaces of lymphocytes. The stained samples are treated with a lysing solution to destroy erythrocytes. The flow cytometer is an instrument capable of rapid, quantitative, multiparameter analysis of heterogeneous cell populations on a cell-by-cell basis (single-cell analysis). During acquisition, the cells travel past the laser beam and scatter the laser light. The stained cells fluoresce. These scatter, and fluorescence signals provide information about the cells' size, internal complexity, and relative fluorescence intensity. The percentage of fluorescent cells is determined for each antibody. In addition, the use of the light scatter measurements from the individual cells along with fluorescence allows identification of the lymphocyte population.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. Peripheral blood in EDTA (acid citrate dextrose or heparin may also be used) anticoagulant
2. Vacutainers or other collection tubes should be filled properly to account for the amount of anticoagulant.
3. Since all specimens should be regarded as potentially infectious, standard precautions for blood collection and handling should be properly followed.

B. Specimen transport

1. Specimens should be placed in well-stoppered tubes and should be placed with adequately absorbent material, preferably in a closed leakproof container, for delivery.
2. Specimens should be maintained at room temperature (18 to 22°C) during transportation and storage.

C. Specimen labeling and request submission

1. Each patient sample must be properly labeled.
2. Each sample should be accompanied by some type of procurement form which should contain appropriate identifiers, e.g., patient's name, date of birth, and gender; date and time of collection; clinician; and other relevant patient information.

D. Rejection criteria

1. Inadequate sample volume
2. Clotted specimen
3. Hemolyzed blood specimen
4. Specimen more than 24 h old
5. Specimen exposed to cold temperatures

III. MATERIALS

A. Reagents (*see* Appendix 11.16–1 for reagent concentrations and preparation)

1. Lysing reagent (depends on manufacturer of flow cytometer)
2. Dulbecco's phosphate-buffered saline (dPBS) without calcium, magnesium, or phenol red (Invitrogen, Grand Island, NY)
3. Monoclonal antibodies against cell surface markers—stored according to manufacturer's instructions
4. 2% Working solution of 10% Formalin neutral buffered solution (Sigma, St. Louis, MO) or 2% working solution of 10% methanol-free Formaldehyde (PolySciences, Inc., Warrington, PA)
5. Flow cytometer calibration beads or other calibration reagents (follow manufacturer's directions)

B. Supplies

1. Adjustable-volume pipette and tips
2. 12- by 75-mm polystyrene tubes (BD Biosciences, Franklin Lakes, NJ)
3. Liquid-waste container with bleach
4. Disposable gloves
5. Disposable lab coat
6. Protective eyewear

C. Equipment

1. Vortex
2. Centrifuge with swinging bucket rotor, capable of centrifugation at 300 × *g* and equipped with aerosol containment canisters
3. Vacuum source with pipette attached
4. Flow cytometer
5. Class II biosafety cabinet
6. Timer/stop watch

IV. CALIBRATION

A. Optical alignment

Flow cytometer must be optically aligned. Follow manufacturer's directions. (Some cytometers require a company engineer.)

B. Calibration

Depending on the flow cytometer manufacturer, either standardized fluorescent beads or chicken RBCs may be used for calibration and to assess instrument function. Follow manufacturer's procedures.

C. Verification with whole-blood preparation

Electrical fluorescent compensation on single stained cells (unless not required due to calibration software specifications in which beads mimic the actual specimens). Adjust FL1-FL2 and FL2-FL1, FL2-FL3 and FL3-FL2, and FL3-FL4 and FL4-FL3. Parameters depend on the number of lasers present and which panel is being used. Follow manufacturer's procedure.

ANALYTICAL CONSIDERATIONS

V. QUALITY CONTROL

Include QC information on reagent container and in QC records.

Controls

A. Surface-stained cells (e.g., $CD3^+$ $CD4^+$) from a healthy donor

These cells should be stained in the same manner and with the same monoclonal antibodies as the patient specimens.

B. Commercially available QC substance (usually stabilized blood cells) that mimics actual specimens

These cells are stained with the same monoclonal antibodies as patient specimens. Results can be compared to expected values.

C. Frequency

The healthy donor blood should be drawn and run daily. If there are several working shifts, the blood should be stained and run for each shift. The QC cells should be stained and run daily.

V. QUALITY CONTROL
(continued)

D. Expected results

1. Healthy donor results should be compared to normal values ascertained by each laboratory.
2. QC substances should be compared to the individual company's published ranges.

E. Corrective action

1. All control results falling outside of the expected ranges should be considered suspect and should be brought to the attention of the person in charge.
2. Results of cell surface markers performed on blood from apparently healthy donors may be dependent on the current health of the individual or due to individual variation. They do not necessarily signify a failure of the test system. These results should be reviewed to determine if patient results are reportable. There must be documentation of these reviews.
3. In the event that a serious problem is found in the test system, corrective action should be performed and documented. If patient's values are suspect, they should not be released and a request should be made for submission of a new specimen.

VI. FLOW CYTOMETRY TWO-COLOR STAINING

It is imperative that these cultures be handled in a biological safety cabinet.

Observe standard precautions.

A. Panel

Isotype, CD45/CD14, CD3/CD4, CD3/CD8, CD3/CD19, CD3/CD(16 + 56), or CD3/CD16

B. Procedure (done in a laminar hood with the lights out)

1. Label a set of six tubes for each patient and one set for the normal control.
2. Pipette 100 µl (or amount specified by the monoclonal antibody manufacturer's directions) of the control's or patient's blood into each tube.
3. Pipette the appropriate volume of antibodies into all the tubes, changing pipette tips between each antibody vial to prevent contamination. Gently vortex to mix.
4. Incubate at room temperature in the dark for the appropriate time (check manufacturer's recommendations).
5. During the incubation time, remove any blood adhering to the walls of the tubes with a cotton swab moistened with PBS.
6. After incubation, vortex each sample tube and add appropriate amount of lysing reagent (per manufacturer's instructions).
7. Incubate for appropriate time (per manufacturer's instructions).
8. After the incubation period, centrifuge tubes at 300 × *g* for 5 min at room temperature.
9. Using an aspirating unit, remove the supernatant from the tubes to within 1/8 in. of the bottom. There should be about 200 µl of liquid left in the tube.
10. Gently vortex to resuspend the pellet.
11. Add 3 ml of PBS to all tubes. Centrifuge tubes at 300 × *g* for 5 min at room temperature.
12. Using the aspirating unit, remove the supernatant from the tube to within 1/8 in. of the bottom. There should be about 200 µl of liquid left in the tube.
13. Gently vortex each tube to resuspend the cell pellet.
14. Add at least 0.5 ml of a 2% formaldehyde fixative solution to each sample tube and vortex gently. Cap tubes, place in rack, and cover with aluminum foil to protect from light.
15. Store at 2 to 8°C until tubes can be acquired. This should be no longer than 48 h after they are completed.

VII. FLOW CYTOMETY TWO-COLOR ANALYSIS

Observe standard precautions.

A. Purity and recovery

1. Specimen must be properly mixed before collection.
2. Draw an electronic gate using forward versus side light scatter around the small nongranular population (lymphocytes). Care should be taken to include large granular lymphocytes and at the same time to exclude monocytes and granulocytes.
3. Draw a second, relatively larger light scatter gate to also include debris and monocytes.
4. Collect 20,000 total events or sufficient events so that there are at least 2,500 events collected in the lymphocyte population for each tube.
5. Using the first gate (lymphocyte gate), analyze the CD45 CD14 tube on a dual-parameter histogram plot. The CD45 should be on the *x* axis, and the CD14 should be on the *y* axis. Set quadrants so that they separate the bright CD45-positive cells from CD45-dim and -negative cells and the CD14-positive cells from the CD14-negative cells on the *y* axis (Fig. 11.16–1A and B). The purity of the lymphocyte gate is the percentage of cells within the gate that express bright $CD45^+$ and $CD14^-$ cells. The lymphocyte purity should optimally be at least 90% but may be as low as 85%. If it is less than 85%, the initial gate should be checked and if possible redrawn.
6. Using a dual dot plot for the larger gate, set the same quadrants as used for the smaller lymphocyte gate.
7. Determine the number of cells that meet both criteria for both gates (bright $CD45^+$ $CD14^-$ = total number of lymphocytes).
8. To determine the lymphocyte recovery (Fig. 11.16–1C and D), divide the number of lymphocytes determined from the analysis regions of the smaller gate by the total number of lymphocytes in the analysis regions of the larger gate and multiply by 100.
9. The lymphocyte recovery should optimally be at least 95% but may be as low as 90%. Optimal gates include as many lymphocytes and as few contaminants as possible.

B. Isotype tube

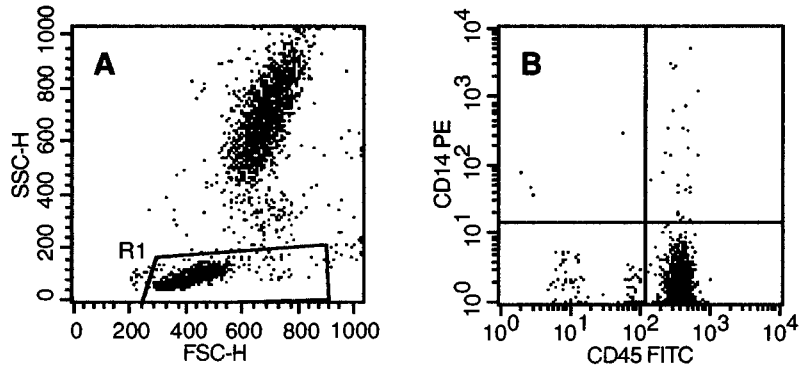
Using the smaller gate, analyze the isotype on a two-parameter dot plot. Set the quadrant markers to read between no more than 1 to 2% positive in quadrants 1, 2, and 4. If the background is above 2% and found to be present throughout the rest of the samples, it should be subtracted.

C. Remaining tubes

1. Read the remaining tubes for the same specimen using the isotype quadrant markers. If it is necessary, the quadrant markers may be optimized.
2. Correct all percentages of markers by dividing the percent positive of gated cells by the percent lymphocyte purity.

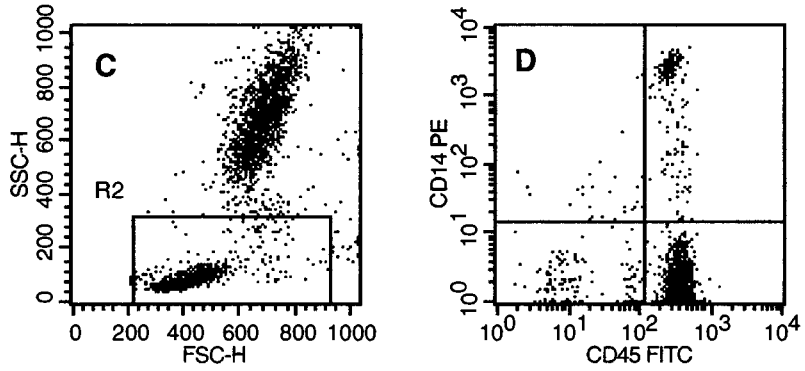
D. QA

1. Check lymphosum (total T cells plus B cells plus natural killer [NK] cells). Results should be between 95 and 100%.
2. Helper ($CD3^+$ $CD4^+$) plus suppressor ($CD3^+$ $CD8^+$) cells should equal total T ($CD3^+$) cells within $\pm$ 10%. (The presence of gamma delta cells could affect these results. In that case, the values could be less than -10%.)
3. The difference between $CD3^+$ cells in all four tubes should be within 0 to 3%.
4. If any of these values are out of range, try regating or restaining.



Gate: G1

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	4	0.10	0.04	16.46	5.45	115.40	80.77
UR	28	0.71	0.25	385.39	363.65	660.36	144.74
LL	104	2.63	0.94	47.96	29.74	1.84	1.59
LR	3812	96.56	34.43	374.14	366.28	1.42	1.26



Gate: G2

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	29	0.68	0.26	48.22	30.91	178.00	47.95
UR	206	4.86	1.86	297.35	285.51	1851.88	925.51
LL	157	3.70	1.42	37.22	19.26	2.18	1.76
LR	3847	90.75	34.75	373.93	365.85	1.43	1.27

Figure 11.16–1 (A) Forward versus side light scatter histogram showing the smaller light scatter gate (lymphocytes) which is used for analyzing the remaining tubes from that specimen. (B) CD45/CD14 histogram from gated R1. The lymphocyte purity is the percentage of positive cells within the gate that express bright CD45-positive cells that are negative for CD14 (quadrant LR). The purity in this sample is 97%. The number of cells in quadrant LR which are bright CD45 positive and negative for CD14 is the number of gated lymphocytes (3,812 cells). (C) The same light scatter gate as in panel A except that it has a rather large light scatter region drawn around the lymphocytes (R2). (D) The two-parameter histogram of CD45/CD14 fluorescence is gated on R2. The number of cells in quadrant LR which are bright CD45 positive and negative for CD14 is the total number of lymphocytes (3,847 cells). The lymphocyte recovery is 99%, obtained as follows: (3,812/3,847) × 100. FITC, fluorescein isothiocyanate; PE, phycoerythrin.

VIII. CD45 GATING AND THREE-COLOR ANALYSIS

It is easier to distinguish lymphocytes based on CD45 fluorescence and 90° (side) scatter. Many nonlymphocyte contaminants of a light scatter gate (e.g., unlysed RBCs) can be easily eliminated from a CD45 side scatter gate. The presence of nonlymphocyte elements within the boundaries of the lymphocyte gate is assumed to be negligible. Using a low side scatter and bright CD45 fluorescence for identification of lymphocytes, an assumption is made that the only cells meeting these criteria are lymphocytes and that therefore the lymphocyte purity of the gate is close to 100%. Therefore, it is not necessary to stain cells for determination of purity. An isotype control is not necessary, as the negatively labeled populations can serve this function.

A. Panel

CD45/CD3/CD4, CD45/CD3/CD8, CD45/CD3/CD19, CD45/CD3/CD(16 + 56), or CD16

B. Staining

Follow two-color procedure using four tubes instead of six.

C. Flow cytometry analysis

1. Set a lymphocyte gate using linear 90° side scatter and log of CD45 fluorescence. Lymphocytes are defined as $CD45^{bright}$ with low side scatter.
2. Set threshold to CD45 fluorescence and adjust so populations of interest are visible.
3. Collect a minimum of 2,500 events in the $CD45^{bright}$ low-scatter gate.
4. Analyze each tube using the negative and positive populations to determine cursor settings.
5. Ensure lymphocyte recovery by determining the lymphosum (total T cells plus B cells plus NK cells). Results should fall within 95 to 100%. If not, try regating.
6. The presence of nonlymphocyte elements within the boundaries of the lymphocyte gate is assumed to be negligible. Therefore, it is not necessary to report purity (percentage of lymphocytes in analysis gate).

D. QA

Follow two-color procedure.

IX. FLOW CYTOMETRY FOUR-COLOR ANALYSIS (Fig. 11.16–2)

A. Panel

CD45/CD3/CD4/CD8, CD45/CD3/CD19/CD(16 + 56), or CD16

B. Staining

Follow three-color procedure.

C. Flow cytometry analysis

Follow three-color procedure.

D. QA

Follow three-color procedure, except that the difference between the total T cells (CD3) in the two tubes should be 0 to 2%.

X. FLOW CYTOMETRY NO-WASH METHOD

Observe standard precautions.

By using fluorescence triggering on CD45 (setting a threshold on fluorescence instead of forward and side scatter), which stains all WBCs, it is possible to reduce contamination of unlysed or nucleated RBCs in the gate and analyze the lymphocyte population.

A. Staining using CD45 gating three- or four-color panel

1. Label a set of two or four tubes, depending on which panel you are using.
2. Pipette 100 µl (or monoclonal antibody manufacturer's directions) of the control's or patient's blood into each tube.

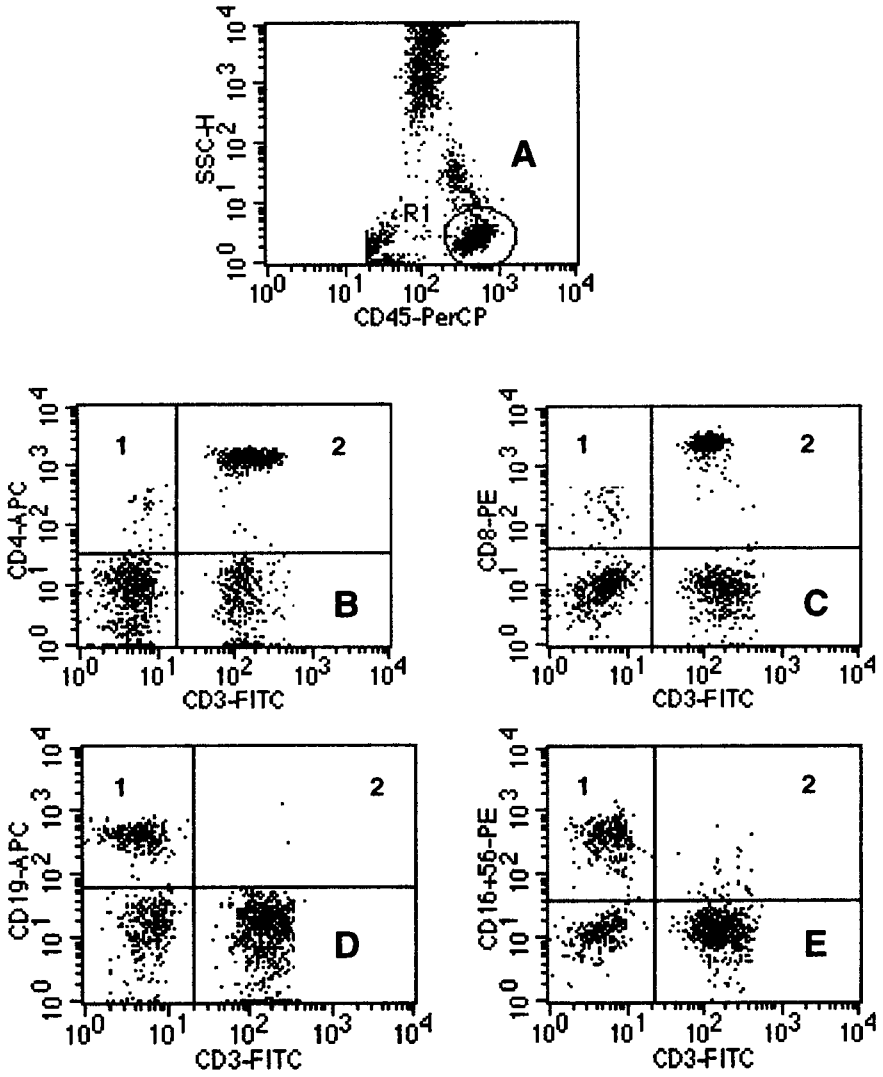


Figure 11.16–2 Four-color immunophenotyping using CD45 and side scatter gating. (A) Gate (R1) on the bright CD45 nongranular population (lymphocytes). This is the region used for analysis of both tubes 1 and 2. (B) $CD3^+$ $CD4^+$ (quadrant 2) from tube 1. (C) $CD3^+$ $CD8^+$ (quadrant 2) from tube 1. (D) CD^- $CD4^+$ (quadrant 1) from tube 2. (E) $CD3^-$ $CD(16^+56^+)$ (quadrant 1) from tube 2. FITC, fluorescein isothiocyanate; APC, allophycocyanin; PE, phycoerythrin.

X. FLOW CYTOMETRY NO-WASH METHOD *(continued)*

3. Pipette the appropriate volume of antibodies into all the tubes as in the three- and four-color procedures.
4. Incubate at room temperature in the dark for the appropriate time as in the three- and four-color procedures.
5. After incubation, add lysing reagent and incubate as in the three- and four-color procedures. Do not wash.

B. Flow cytometry analysis

Follow procedure for three- and four-color acquisition.

C. QA

Follow three- or four-color procedure.

POSTANALYTICAL CONSIDERATIONS

XI. REPORTING RESULTS

Reference range

A. Reference range values, including percent positive and absolute numbers, should be calculated from at least 50 healthy adult donors. Pediatric reference ranges should also be established.

B. Patient values should be compared to healthy donor ranges and reported as increased or decreased.

XII. INTERPRETATION

A. Patient values should be compared to healthy donor values. Increases or decreases in values may be related to a variety of diseases. A physician should be able to interpret these results based on patient symptoms and other clinical data.

B. New sample required if

1. Lymphosum (total T cells plus B cells plus NK cells) is out of range. Results should be between 95 and 100%.

2. The differences in CD3 values in tubes are not within the matching range (0 to 3% for two- or three-color staining and 0 to 2% for four-color staining).

3. Helper ($CD3^+$ $CD4^+$) plus suppressor ($CD3^+$ $CD8^+$) cells do not equal total T ($CD3^+$) cells within $\pm$ 10%. (The presence of gamma delta cells could affect these results. In that case the values could be less than $-$10%.)

SUPPLEMENTAL READING

Centers for Disease Control and Prevention. 1997. Revised guidelines for performing $CD4^+$ T-cell determinations in persons infected with human immunodeficiency virus (HIV). *MMWR Morb. Mortal. Wkly. Rep.* **46** (RR-2)**:**1–29.

NCCLS. 1998. *Clinical Applications of Flow Cytometry: Quality Assurance and Immunophenotyping of Lymphocytes.* Document H42-A, vol. 18, no. 21. NCCLS, Wayne, PA.

Nicholson, J., P. Kidd, F. Mandy, D. Livnat, and J. Kagan. 1996. Three-color supplement to the NIAID DAIDS guideline for flow cytometric immunophenotyping. *Cytometry* **26:**227–230.

APPENDIX 11.16–1

Observe standard precautions.

Reagent Preparation

Working solution of methanol-free 2% formaldehyde—stock solution from PolySciences or Sigma

A. 10% Formaldehyde solution (PolySciences no. 0418), which must be diluted 1:5 with dPBS to obtain a working concentration of 2% formaldehyde

B. 10% Formalin neutral buffered solution from Sigma (no. HT50-1-1) that is diluted 1:2 with dPBS to obtain a working concentration of 2% formaldehyde

C. Store diluted 2% formaldehyde in an amber glass container or wrap container in aluminum foil to avoid exposure to light. May be stored for up to 1 week at 2 to 8°C.

11.17 Neutrophil Function Whole-Blood Flow Cytometric Test for Leukocyte Adhesion Deficiency

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Normal neutrophil function is of great importance in the host defense against bacterial and fungal infections. As neutrophils recognize, adhere to, and phagocytose an invading microbe they generate an oxidative burst, which kills the pathogen. In the leukocyte adhesion deficiency (LAD) states, there are defects in the ability of neutrophils to adhere to both the vascular endothelium and opsonized microorganisms due to the absence or reduced expression of a group of cell surface leukocyte adhesion markers. This group of surface glycoproteins includes LFA-1 (CD11b/CD18), Cr3 (CD11b/CD18), and P150,95 (CD11c/CD18). Patients with this deficiency manifest recurrent infections involving the skin, subcutaneous tissues, middle ear, and oropharyngeal mucosa. In the assay described here, neutrophils are stimulated with phorbol 12 myristate 13 acetate (PMA) and stained with CD11b plus corresponding isotypic monoclonal antibody. Cellular fluorescence is measured on resting and stimulated neutrophils using flow cytometry.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. Peripheral blood in EDTA anticoagulant (2 ml)
2. Vacutainers or other collection tubes should be filled properly to account for the amount of anticoagulant.
3. Since all specimens should be regarded as potentially infectious, standard precautions for blood collection and handling should be properly followed.

B. Specimen transport

1. Specimens should be placed in well-stoppered tubes and should be placed with adequately absorbent material, preferably in a closed leakproof container, for delivery.
2. Specimens should be maintained at room temperature (18 to 22°C) during transportation and storage.
3. *Testing must be performed within 4 h from the time of collection.*

C. Specimen labeling and request submission

1. Each patient sample must be properly labeled.
2. Each sample should be accompanied by some type of procurement form which should contain appropriate identifiers, e.g., patient's name, date of birth, and gender; date and time of collection; clinician; and other relevant patient information.

D. Rejection criteria

1. Inadequate sample volume
2. Clotted specimen
3. Hemolyzed blood specimen
4. *Specimen more than 4 h old*
5. Specimen exposed to cold temperatures

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents (*see* Appendix 11.17–1 for reagent concentrations and preparation)

1. BD FACS Lysing Solution—working solution diluted 1:10 with distilled water (BD Biosciences, San Jose, CA). (If using different lysing reagent, follow manufacturer's instructions.)
2. Dulbecco's phosphate-buffered solution (dPBS) without calcium or magnesium (Invitrogen, Grand Island, NY)
3. Hanks balanced salt solution (HBSS) without calcium, magnesium, or phenol red (Invitrogen)
4. CD11b and isotype immunoglobulin G2a (IgG2a) phycoerythrin (PE)-labeled monoclonal antibodies, stored according to manufacturer's instructions
5. PMA (Sigma Chemical Co., St. Louis, MO)
6. 2% Working solution of 10% methanol-free formaldehyde
7. Flow cytometer calibration beads or other calibration reagents (follow manufacturer's directions)

B. Supplies

1. Adjustable-volume pipette and tips
2. 12- by 75-mm polystyrene tubes (BD Biosciences, Franklin Lakes, NJ)
3. Liquid-waste container with bleach
4. Disposable gloves
5. Disposable lab coat
6. Protective eyewear

C. Equipment

1. Vortex
2. Centrifuge
3. CO_2 incubator (5% CO_2)
4. Vacuum source with pipette attached
5. Centrifuge with swinging bucket rotor, capable of centrifugation at 300 $\times$ *g* and equipped with aerosol containment canisters
6. Class II biosafety cabinet
7. Timer/stop watch
8. Flow cytometer

IV. CALIBRATION

A. Optical alignment

Flow cytometer must be optically aligned. Follow manufacturer's directions. (Some cytometers require a company engineer.)

B. Calibration

Depending on the flow cytometer manufacturer, either standardized fluorescent beads or chicken RBCs may be used for calibration to assess instrument function. Follow manufacturer's procedures.

C. Verification with whole-blood preparation

Electrical fluorescent compensation on single stained cells. Follow manufacturer's procedures.

ANALYTICAL CONSIDERATIONS

V. QUALITY CONTROL

Controls

A. Surface-stained cells (e.g., CD11b) from a healthy donor. These cells should be stained and stimulated in the same manner and with the same monoclonal antibodies as the patient specimens. Values should fall within established normal ranges.

B. If for some reason the results for the healthy donor are abnormal (such as response to stimulants), then the patient's results should not be reported. Instruments and reagent integrity should be checked, and if necessary, service on the flow cytometer should be requested. A new patient sample should be submitted.

VI. LAD ASSAY

It is imperative that these cultures be handled in a biological safety cabinet.

Observe standard precautions.

A. Procedure

1. Set four tubes per run for both a healthy donor and the patient, labeled as follows.
   - **a.** IgG2a PE
   - **b.** CD11b PE
   - **c.** IgG2a PE + PMA
   - **d.** CD11b PE + PMA
2. Add 100 µl of whole blood to each tube.
3. Add 10 µl of HBSS to tubes a and b. Add 10 µl of PMA to tubes c and d. Vortex lightly.
4. Incubate for 15 min at 37°C in a CO_2 incubator.
5. Add 20 µl of monoclonal antibody (or follow amount suggested in monoclonal antibody manufacturer's directions) to appropriate tubes. Vortex lightly.
6. Incubate for 15 min in the dark at room temperature.
7. Add 2 ml of BD FACS Lysing Solution. Incubate for 10 min (follow appropriate amount and manufacturer's instructions if using a different lysing agent).
8. Centrifuge at 300 × *g* for 5 min. Aspirate supernatant.
9. Wash one time with 2 ml of HBSS. Aspirate supernatant.
10. Fix with 0.5 ml of 2% methanol-free formaldehyde.
11. Acquire as soon as possible on the flow cytometer.

B. Flow cytometry analysis

1. Using forward versus side light scatter parameters, set a region around the large granular cells (neutrophils).
2. Set the mean channel of the histogram from tube a (IgG2a) at around 2.0 on the log scale and collect specimen.
3. Collect 10,000 events for all four tubes.
4. Using the region around the neutrophils, print the histograms from all four tubes.
5. Record the mean fluorescence intensities (MFI) for CD11b, CD11b plus PMA, IgG2a, and IgG2a plus PMA and determine the upregulation index (UI).

$$UI = \frac{\text{MFI for CD11b stimulated with PMA/MFI for IgG2a stimulated with PMA}}{\text{MFI for unstimulated CD11b/MFI for unstimulated IgG2a}}$$

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

Report the MFI for CD11b stimulated with PMA divided by the MFI for IgG2a stimulated with PMA, the MFI for unstimulated CD11b divided by the MFI for unstimulated IgG2A, and the UI.

VIII. INTERPRETATION

Normal values

A. Values for at least 50 healthy donors should be ascertained by each laboratory performing this test.

B. Compare the following values.

1. The MFI for stimulated CD11b divided by the value for stimulated IgG2a
2. The MFI for unstimulated CD11b divided by the value for unstimulated IgG2a
3. UI

VIII. INTERPRETATION *(continued)*

C. Values for patients should fall within ranges for healthy donors. However, sometimes values may be slightly off for various reasons, e.g., the patient's MFI for unstimulated CD11b is increased. In cases such as these, it is important to ascertain that there was a sufficient increase in the MFI for stimulated CD11b. Patients with LAD will have very little increase in the MFI for CD11b when the cells are stimulated.

D. Increased values compared to the healthy donor ranges have no significance.

IX. LIMITATION OF TESTING

Stimulation with PMA does not occur in vivo.

SUPPLEMENTAL READING

Centers for Disease Control and Prevention. 1997. Revised guidelines for performing $CD4^+$ T-cell determinations in persons infected with human immunodeficiency virus (HIV). *MMWR Morb. Mortal. Wkly. Rep.* **46**(RR-2)**:**1–29.

Hassan, N. F., D. E. Campbell, and S. D. Douglas. 1988. Phorbol myristate acetate oxidation of 2′7′ dichlorofluorescein by neutrophils from patients with chronic granulatomatous disease. *J. Leukoc. Biol.* **43:**317–322.

Malech, H. L., and J. I. Gallin. 1987. Neutrophils in human diseases. *N. Engl. J. Med.* **317:**687–694.

O'Gorman, M. R. G., and A. C. Mcnally. 1993. A rapid whole blood lysis technique for diagnosis of moderate or severe leukocyte adhesion deficiency. *Ann. N. Y. Acad. Sci.* **677:**427–430.

Todd, R. F., and D. R. Freyer. 1988. The CD11/CD18 leukocyte glycoprotein deficiency. *Hematol. Oncol. Clin. N. Am.* **2:**13–31.

APPENDIX 11.17–1

Observe standard precautions.

Include QC information on reagent container and in QC records.

Reagent Preparation

A. PMA
   Stock solution of 1 mg/ml is prepared in dimethyl sulfoxide, with aliquots frozen at −70°C. Working solution is diluted in HBSS for a 1-μg/ml concentration. Final concentration in assay is 90 ng/ml.

B. Working solution of methanol-free 2% formaldehyde—stock solution from PolySciences or Sigma
   1. 10% Formaldehyde solution (PolySciences no. 0418), which must be diluted 1:5 with dPBS to obtain a working concentration of 2% formaldehyde
   2. 10% Formalin neutral buffered solution from Sigma (no. HT50-1-1) that is diluted 1:2 with dPBS to obtain a working concentration of 2% formaldehyde
   3. Store diluted 2% formaldehyde in an amber glass container or wrap container in aluminum foil to avoid exposure to light. May be stored for up to 1 week at 2 to 8°C.

11.18 Flow Cytometric Test for Chronic Granulomatous Disease

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Normal neutrophil function is of great importance in the host defense against bacterial and fungal infections. As neutrophils recognize, adhere to, and phagocytose an invading organism, they generate an oxidative burst which results in the reduction of molecular oxygen to superoxide. The superoxide produced is rapidly converted to hydrogen peroxide (H_2O_2, which kills the pathogen). In chronic granulomatous disease (CGD), microbial killing is defective because neutrophils from patients with CGD lack a respiratory burst. The assay described here uses dihydrorhodamine-123 (DHR-123) and phorbol 12 myristate 13 acetate (PMA) to measure oxidative burst activity, which is easily converted to hydrogen peroxide. DHR, a nonfluorescent compound, reacts with hydrogen peroxide and is oxidized to rhodamine-123, a green fluorescent compound. Cellular fluorescence is measured using flow cytometry.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. Peripheral blood in EDTA anticoagulant (2 ml)
2. Vacutainers or other collection tubes should be filled properly to account for the amount of anticoagulant.
3. Since all specimens should be regarded as potentially infectious, standard precautions for blood collection and handling should be properly followed.

B. Specimen transport

1. Specimens should be placed in well-stoppered tubes and should be placed with adequately absorbent material, preferably in a closed leakproof container, for delivery.
2. Specimens should be maintained at room temperature (18 to 22°C) during transportation and storage.
3. *Testing must be performed within 4 h from the time of collection.*

C. Specimen labeling and request submission

1. Each patient sample must be properly labeled.
2. Each sample should be accompanied by some type of procurement form which should contain appropriate identifiers, e.g., patient's name, date of birth, and gender; date and time of collection; clinician; and other relevant patient information.

D. Rejection criteria

1. Inadequate sample volume
2. Clotted specimen
3. Hemolyzed blood specimen
4. *Specimen more than 4 h old*
5. Specimen exposed to cold temperatures

III. MATERIALS

A. Reagents (*see* appendix 11.18–1 for reagent concentrations and preparation)

1. Ammonium chloride lysing solution
   - **a.** NH_4Cl (Sigma Chemical Co., St. Louis, MO)
   - **b.** $NaHCO_3$ (Fisher Scientific, Fair Lawn, NJ)
   - **c.** EDTA (Fisher Scientific)
2. Hanks balanced salt solution (HBSS) without calcium, magnesium, or phenol red (Invitrogen, Grand Island, NY)
3. DHR-123 (Molecular Probes, Eugene, OR)
4. Dimethyl sulfoxide (DMSO) (Sigma)
5. PMA (Sigma)
6. Flow cytometer calibration beads or other calibration reagents (follow manufacturer's directions)

B. Supplies

1. Adjustable-volume pipette and tips
2. 12- by 75-mm polystyrene tubes (BD Biosciences, Franklin Lakes, NJ)
3. Liquid-waste container with bleach
4. Disposable gloves
5. Protective eyewear

C. Equipment

1. Vortex
2. Timer/stop watch
3. Centrifuge with swinging bucket rotor, capable of centrifugation at 300 × *g* and equipped with aerosol containment canisters
4. CO_2 incubator (5% CO_2)
5. Vacuum source with pipette attached
6. Class II biosafety cabinet
7. Flow cytometer

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

Controls

A. Cells from a healthy donor. These cells should be treated in the same manner as the patient specimens. Values should fall within established normal ranges.

B. If for some reason the results for the healthy donor are abnormal (e.g., the neutrophil oxidative index [NOI]), then the patient's results should not be reported. Instruments and reagent integrity should be checked, and if necessary, service on the flow cytometer should be requested. A new patient sample should be submitted.

V. CALIBRATION

A. Optical alignment

Flow cytometer must be optically aligned. Follow manufacturer's directions. (Some cytometers require a company engineer.)

B. Calibration

Depending on the flow cytometer manufacturer, either standardized fluorescent beads or chicken RBCs may be used for calibration and to assess instrument function. Follow manufacturer's procedures.

VI. CGD ASSAY

It is imperative that these cultures be handled in a biological safety cabinet.

Observe standard precautions.

A. Procedure

1. Set two tubes per run for both a healthy donor and the patient, labeled as follows.
   - **a.** DHR-123
   - **b.** DHR-123 + PMA
2. Add 100 µl of whole blood to each tube.
3. Add 3 ml of lysing solution (prewarmed to 37°C) to each tube. Vortex and let stand at room temperature for 5 min.
4. Spin at 300 × *g* for 5 min. Aspirate supernatant.
5. Wash twice with 2 ml of HBSS. Aspirate supernatant.
6. Add 400 µl of HBSS. Vortex tubes lightly.

VI. CGD ASSAY *(continued)*

7. Add 10 µl DHR-123. Vortex lightly. Incubate in 5% CO_2 incubator at 37°C for 5 min.
8. Add 40 µl of HBSS to tube a. Add 40 µl of PMA to tube b. Vortex lightly and incubate in 5% CO_2 incubator at 37°C for 15 min.
9. Collect immediately on the flow cytometer.

B. Flow cytometry analysis

1. Using forward versus side light scatter parameter, set a region around the large granular cells (neutrophils).
2. Set the mean channel of the histogram from tube a (DHR) at around 10.0 on the log scale and collect specimen.
3. Collect 10,000 events for both tubes.
4. Using the region around the neutrophils, print the histograms from both tubes.

POSTANALYTICAL CONSIDERATIONS

VII. REPORTING RESULTS

Record the mean fluorescence intensity (MFI) from both the DHR tube and the tube with DHR plus PMA. Calculate the NOI. The NOI is the MFI of the cells with DHR plus PMA (stimulated) divided by the MFI of cells with DHR alone (unstimulated).

VIII. INTERPRETATION

Normal values

A. NOIs for at least 50 healthy donors should be ascertained by each laboratory performing this test.

B. Compare the NOIs to established normal ranges. Patients with CGD have a lack of oxidative burst function and will have a very low NOI (e.g., ~0 to 10). If the patient has an intermediate value, repeat studies should be done. A normal value indicates that there is no evidence of lack of oxidative burst function.

IX. LIMITATION OF TESTING

Stimulation with PMA does not occur in vivo.

SUPPLEMENTAL READING

Bass, D., W. Parce, L. Dechatelet, P. Szejda, M. Seeds, and M. Thomas. 1983. Flow cytometric studies of oxidative product formation by neutrophils: a grade response to membrane stimulation. *J. Immunol.* **136:**1910–1917.

Centers for Disease Control and Prevention. 1997. Revised guidelines for performing $CD4^+$ T-cell determinations in persons infected with human immunodeficiency virus (HIV). *MMWR Morb. Mortal. Wkly. Rep.* **46** (RR-2)**:**1–29.

Emmendorffer, A., M. Hecht, M. L. Lohmann-Matthes, and J. Roesler. 1990. A fast and easy method to determine the production of reactive oxygen intermediates by human phagocytes using dihydrorhodamine 123. *J. Immunol. Methods* **131:**269–275.

Hassan, N. F., D. E. Campbell, and S. D. Douglas. 1988. Phorbol myristate acetate oxidation of 2′7′ dichlorofluorescein by neutrophils from patients with chronic granulatomatous disease. *J. Leukoc. Biol.* **43:**317–322.

Malech, H. L., and J. I. Gallin. 1987. Neutrophils in human diseases. *N. Eng. J. Med.* **317:**687–693.

O'Gorman, M. R. G., and V. Corrochano. 1995. Rapid whole blood flow cytometry assay for the diagnosis of chronic granulomatous disease. *Clin. Diagn. Lab. Immunol.* **2:**227–232.

Vowells, S. J., S. Sekhsaria, H. L. Malech, M. Shalit, and T. A. Fleisher. 1995. Flow cytometric analysis of granulocyte respiratory burst: a comparison study of fluorescent probes. *J. Immunol. Methods* **178:**89–97.

APPENDIX 11.18–1

Observe standard precautions.

Include QC information on reagent container and in QC records.

Reagent Preparation

A. Ammonium chloride lysing solution
   1. Stock solution

NH_4Cl	8.02 g
$NaHCO_3$	0.84 g
EDTA	0.37 g

   Bring to a volume of 100 ml with distilled H_2O. Store at 4°C. Keeps for 6 months.
   2. Working solution diluted 1:10 in distilled H_2O. Keeps for 1 week.

B. DHR-123
   Stock solution of 5 mg/ml is prepared in DMSO, with aliquots frozen at −70°C. Working solution is diluted in HBSS for a 15-µg/ml concentration. Final concentration in assay is 1 µM.

C. PMA
   Stock solution of 1 mg/ml is prepared in DMSO, with aliquots frozen at −70°C. Working solution is diluted in HBSS for a 1-µg/ml concentration. Final concentration in assay is 90 ng/ml.

11.19 Gamma Interferon Enzyme-Linked Immunospot Assay

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The enzyme-linked immunospot (ELISPOT) assay was described more than 13 years ago for the detection of specific immune cells at the single-cell level. The utility of the gamma interferon (IFN-γ) ELISPOT assay in detecting antigen-specific T cells was initially demonstrated in models of autoimmune and infectious diseases. Optimization of the assay through the introduction of specifically designed antibodies, 96-well plates, substrate kits, and other modifications has broadened the potential uses for the IFN-γ ELISPOT assay. Today, it is being used for a wide range of applications, including monitoring responses in patients with cancer undergoing immunotherapeutic treatment and monitoring specific immune response patterns in patients with infectious, neoplastic, or autoimmune diseases. Additionally, it has been an important tool in the identification of immunodominant epitopes in human immunodeficiency virus and *Mycobacterium tuberculosis* infections, as well as in the development of specific vaccine strategies (1).

The presence of circulating IFN-γ-secreting T cells can be detected by IFN-γ ELISPOT assay using cryopreserved unfractionated peripheral blood mononuclear cells (PBMC) stimulated overnight with the appropriate antigen.

Cryopreserved PBMC are plated into 96-well plates and incubated overnight with the antigenic stimulus. After 6 h, the memory cells specifically primed for that antigen begin to secrete IFN-γ, which is, in turn, captured by the membrane-bound antibody. After approximately 20 h, the cells are washed off, and a biotinylated secondary antibody specific for IFN-γ is added. To make the antibody–IFN-γ–antibody sandwich visible, a streptavidin-horseradish peroxidase complex and an insoluble enzyme-specific substrate are added. Bound cytokine is visible through color development. Areas of color development form spots representing single cells that had secreted cytokine in response to recognition of the antigen added during the assay. The spot number per well denotes the estimated frequency of antigen-specific T cells among the cells plated.

II. SPECIMEN

Observe standard precautions.

All ELISPOT assays can be conducted on freshly isolated or cryopreserved PBMC using the established standard operating procedure (SOP) for collection processing and cryopreservation of PBMC. Acid-citrate-dextrose and heparin are the two anticoagulants of choice for the collection of whole blood samples.

III. REAGENTS AND SUPPLIES

Include QC information on reagent container and in QC records.

A. Reagents

1. L-Glutamine (200 mM L-glutamine, 100× concentration; store at −20°C)
2. Penicillin-streptomycin (10,000 U; store at −5 to −20°C)
3. Fetal bovine serum (FBS) (heat inactivate at 56°C for 30 min, aliquot, and store at −20°C)

   ◩ **NOTE:** FBS lots must be prescreened in ELISPOT assays for low background activity as well as the capacity to optimally support antigen-specific responses. A single lot of FBS must be used for all ELISPOT assays performed in conjunction with a given vaccine trial or study.
4.
   a. Benzonase (10,000 U/vial or 25 U/μl; store at −20°C)
   b. RPMI 1640 medium (RPMI 1640 with 25 ml of HEPES buffer and L-glutamine)

III. REAGENTS AND SUPPLIES *(continued)*

c. R10 (to make, add 55 ml of FBS and 5 ml of L-glutamine–penicillin-streptomycin into 500 ml of RPMI 1640 with HEPES buffer and L-glutamine; store at 4°C for 1 week)

d. R10/Benz thawing medium (to make, add 1.13 ml of benzonase [or 20 µl/10 ml if less medium is needed] into 565 ml of R10; do not store)

5. Phosphate-buffered saline solution without Ca^{2+} or Mg^{2+} (PBS)

6.

a. Tween 20

b. PBS-Tween (To prepare, add 0.05% Tween 20 in 1× PBS [to be prepared fresh]. Remove 5 ml of PBS from a full bottle and discard. Add 5 ml of FBS and 250 µl of Tween. Shake well. Incubate at 37°C for 15 min.)

7. ELISPOT assay-dedicated reagents (mandatory)

a. 96-well/plate Mabtech anti-human IFN-γ monoclonal antibody (MAb) 1-D1K precoated, opaque, and sterile

b. Secondary antibody: anti-human-IFN-γ MAb 7-B6-1, biotinylated, 0.25 mg

c. Streptavidin complex

d. Nova red substrate

8. Mitogen as polyclonal stimulation reagent (titration is recommended to avoid confluent wells)

9. Peptides or recall antigens as positive controls (assay dependent; final concentration of dimethyl sulfoxide [DMSO] <1% when used to resuspend peptides)

10. Study-specific peptides or recall antigens (assay dependent, with a final concentration of DMSO of <1% when used to resuspend peptides)

B. Supplies

1. Pipet-Aid or similar

2. 5- and 10-ml sterile polystyrene pipettes

3. 1- and 2-ml sterile polystyrene aspirating pipettes

4. 20-, 100-, 200-, and 1,000-µl pipettors

5. Multistep pipettors, 50 to 1,250 µl

6. Pipette tips to deliver 5 to 1,000 µl

7. 15- and 50-ml sterile polystyrene centrifuge tubes with conical bottoms

8. 12- by 75-mm sterile polystyrene culture tubes with closures (snap cap)

C. Instrumentation

1. Biological safety cabinet

2. Light microscope

3. Automated cell counter (e.g., Guava PCA [Guava Technologies] or similar)

4. Hemacytometer

5. Incubator (37°C, 5% CO_2; standard requirements)

6. Plate washer with adjustable flow rate and probe height (e.g., Elx405 plate washer [BIOTEK Instruments, Inc.])

7. Variable-speed centrifuge

8. ELISPOT reader (e.g., CTL Analyzers [LLC or Zeiss] or similar)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL (CALIBRATION)

Include QC information on reagent container and in QC records.

A. Plate washer

1. Rinsing of the probe and of the fluidic system must be done daily.

2. Decontamination of the fluidic system must be done weekly.

3. Preventive maintenance should be performed twice per year.

B. Automated cell counter

1. Checking for counting performance of microparticles must be done daily.

2. Preventive maintenance should be performed twice per year.

IV. QUALITY CONTROL (CALIBRATION) *(continued)*

C. ELISPOT reader

1. Calibration of the reading must be done daily with a calibration plate.
2. Preventive maintenance should be performed twice per year.

D. Pipettors

Pipettors should be calibrated twice per year.

E. Equipment

All laboratory equipment intended for clinical trials should be validated and have preventive maintenance performed according to the manufacturers' instructions. Include QC information on reagent container in QC records.

V. PROCEDURE

It is imperative that these cultures be handled in a biological safety cabinet.

Observe standard precautions.

NOTE: All of the pipetting should take place in a biological safety cabinet using sterile technique until the step of removal of the cells on day 3 by using the plate washer. It is imperative that these cultures be handled according to the institutional rules for working with biological material under universal precautions.

A. Day 1: cell thawing

NOTE: No more than two vials per thaw per operator and no more than three plates per operator should be used.

1. Transfer the cryovial from liquid nitrogen directly to a 37°C water bath.
2. Hold the cryovial on the surface of the water. Do not leave the cryovial unattended during the thawing process (it is important for cell viability that the cells are thawed and processed quickly; thawing takes only a few seconds). When a small bit of ice remains in the cryovial, transfer the cryovial to the laminar-flow hood. Dry off the outside of the cryovial and wipe with alcohol solution before opening to prevent contamination.
3. Add warmed R10/Benz dropwise slowly into the cryovial containing the cell suspension. The medium should be added to double the volume of the original cell suspension (e.g., add 1 ml of medium to a vial containing 1 ml of cell suspension). Be careful not to exceed the capacity of the cryovial.
4. Transfer the cell suspension to a 50-ml polypropylene conical-bottom centrifuge tube containing 8 ml of warm (37°C) R10/Benz per vial of cells thawed.
5. Centrifuge the cells at 400 × *g* for 10 min.
6. Decant the supernatant.
7. Gently resuspend the pellet with a 200-µl pipettor.
8. Resuspend in 10 ml of R10.
9. Determine cell number and viability according to the procedure for the automated cell counter of choice.
10. Centrifuge the cells at 400 × *g* for 10 min.
11. Decant the supernatant.
12. Gently resuspend the pellet with a 200-µl pipettor.
13. Resuspend in R10 at 2×10^6 cells/ml in a 50-ml polypropylene tube without exceeding 5 ml/tube. Incubate overnight at 37°C and 5% CO_2 with the cap of the tube loosened.

B. Day 2: cell preparation

1. Remove 50-ml tubes containing cells from the incubator. Combine cell samples of the same identifier.
2. Add R10 until the volume is equal to 20 ml.
3. Centrifuge the cells at 400 × *g* for 10 min.
4. Decant the supernatant.
5. Gently resuspend the pellet with a 200-µl pipettor.
6. Add 10 ml of R10.

V. PROCEDURE *(continued)*

7. Determine cell count and viability with the cell counter method of choice. Samples with less than 66% viability will not move forward in the assay and should be discarded, as they do not meet the required cutoff for reliable results. Cell counts and viability are reported on the daily worksheet. Refer to procedures listed in the Guava cell counter SOP.
8. Centrifuge the cells at 400 × *g* for 10 min.
9. Decant the supernatant.
10. Gently resuspend the pellet with a 200-µl pipettor.
11. Resuspend the cells in R10 at 4×10^6 cells/ml.

C. Day 2: control and test reagents

1. Negative control
   Each well contains 50 µl of cells and 50 µl of R10 medium without peptide. Use six wells per subject's PBMC on each plate and two wells for the control donor.
2. Phytohemagglutinin (PHA) control
   Each well contains 50 µl of cells and 50 µl of the mitogen. Use three wells per subject's PBMC on each plate and two for the control donor.
3. Recall antigen control (e.g., CEF pool)
   Each well contains 50 µl of cells and 50 µl of the 2-µg/ml positive control antigen. Use three wells per subject's PBMC on each plate and two for the control donor.
4. Study-specific antigen
   Each well contains 50 µl of cells and 50 µl of study-specific antigen solution. Use three wells per subject's PBMC on each plate.
5. Medium-only wells
   Each well contains 100 µl of R10. Use three wells containing 100 µl per plate.

D. Day 2: reagent preparation and plating

◩ **NOTE:** The plates should be brought to room temperature before starting the assay. Controls and peptide pools will be according to the plate layout designed prior to the assay.

1. Calculate the number of specific antigen vials needed according to the number of samples to be tested. During the preparation of the cells, thaw the necessary aliquots for each of the specific reagents and controls.
2. Dilute the study-specific antigen with R10 to reach a 2× working concentration.
3. Dilute the mitogen with R10 to reach a 2× working concentration.
4. Dilute the antigen used as the positive control with R10 to reach a 2× working concentration.
5. Add 50 µl of each antigen and controls into the appropriate wells.
6. Add 50 µl of cells (200,000 cells) to each well.
7. Incubate the plate at 37°C and 5% CO_2 for 16 to 24 h. Avoid moving the plates while they are in the incubator.

E. Day 3: develop spots

1. Prepare PBS-Tween and add this to the PBS-Tween bottle connected to the plate washer.
2. Add deionized water and PBS to the other bottles connected to the plate washer.
3. Prime the automatic plate washer according to the instrument SOP.
4. Prepare the secondary antibody by calculating how much secondary Ab is needed [(number of plates) (number of wells) (0.05 ml) = number of milliliters of PBS–10% FBS; (ml from above) (2 µg/ml) = number of microliters of secondary antibody]

V. PROCEDURE *(continued)*

5. Place the plate into the automatic plate washer and wash three times with PBS followed by three times with PBS-Tween.
6. Tap the excess moisture onto the absorbent pad.
7. Using the single-channel pipettor, add 50 µl of the vortexed secondary Ab to each well.
8. Incubate in the dark at room temperature for 3 h.
9. After the 3-h incubation, make a 1:500 dilution of streptavidin in PBS–10% FBS.
10. Place the plate into the automatic plate washer and wash six times with PBS-Tween.
11. Tap excess moisture onto the absorbent pad.
12. Using the single-channel pipettor, add 50 µl of streptavidin to each well.
13. Incubate in the dark at room temperature for 1 h.
14. After 1 h, wash plates six times with PBS-Tween without aspirating the final wash.
15. Incubate in the residual 200 µl of PBS-Tween at room temperature for 15 min.
16. Prepare the Nova red substrate.
    a. For one plate: pipette 15 ml of ultrapure water into a foil-wrapped tube. Scale up as needed.
    b. Add 3 drops of reagent 1 and invert.
    c. Add 2 drops of reagent 2 and invert.
    d. Add 2 drops of reagent 3 and invert.
    e. Add 2 drops of hydrogen peroxide and invert.
17. After 15 min, put the plate in the automatic plate washer and wash six times with PBS.
18. Tap out excess moisture onto the absorbent pad.
19. Using the single-channel pipettor, add 100 µl of Nova red substrate into each well and put in the dark at room temperature for 15 min to develop the spots.
20. After 15 min, take the plate out and rinse gently under tap water. Hold the plate at a 45° angle under slow-flowing water and rinse the plate from every side.
21. Take the bottom of the plate off and discard.
22. Rinse the back of the plate also.
23. Place the plates in the dark to dry overnight at room temperature.
24. Analyze the plate the next day using the ELISPOT reader of choice according to the instrument SOP.

    ◩ **NOTE:** Store dried plates, wrapped in foil, in the dark for up to 6 months to avoid color reduction.

POSTANALYTICAL CONSIDERATIONS

VI. PASS/FAIL CRITERIA

A. Background in the negative control wells

If test negative control wells (no antigen) result in 100 or more spots per million cells, when averaged across the wells, the samples should be retested. This threshold should be determined, as it could be assay specific and related to the population involved in the study as well as to the source of the responding cells.

B. Reactivity in the mitogen-stimulated wells

All assays should include three wells of samples treated with PHA. If the mean for the PHA control is below a predetermined number of spots per well according to the validation studies, that specimen must be retested.

VI. PASS/FAIL CRITERIA
(continued)

C. Reactivity in the recall antigen wells for the control donor

Additionally, antigen-stimulated samples from a known responder for a recall antigen may be used as a plate control. If the mean recall antigen responses in the control donor are outside the predetermined mean ± 3 standard deviations, all samples on the assay plate in question should be retested. These values should also be determined during the validation of the assay.

VII. INTERPRETATION

The algorithm to define positive results should be determined during the optimization of the assay based on the statistical analysis of the results, to include background among healthy donors and the patient population.

REFERENCE

1. **Dubey, S., J. Clair, T. M. Fu, L. Guan, R. Long, R. Mogg, K. Anderson, K. B. Collins, C. Gaunt, V. R. Fernandez, L. Zhu, L. Kierstead, S. Thaler, S. B. Gupta, W. Straus, D. Mehrotra, T. W. Tobery, D. R. Casimiro, and J. W. Shiver.** 2007. Detection of HIV vaccine-induced cell-mediated immunity in HIV-seronegative clinical trial participants using optimized and validated enzyme-linked immunospot assay. *J. Acquir. Immune Defic. Syndr*. **45:**20–27.

SUPPLEMENTAL READING

Janetzki, S., J. H. Cox, N. Oden, and G. Ferrari. 2005. Standardization and validation issues of the ELISPOT assay, p. 51–86. *In* A. E. Kalyuzhny (ed.), *Manual of ELISPOT*. Humana Press, Totowa, NJ.

SECTION 12 Molecular Diagnostics

SECTION EDITORS: *Amy L. Leber and Maurice Exner*
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12.1 Introduction

Molecular techniques have been used successfully to aid in the detection and identification of pathogens and for the treatment of many infectious diseases (1, 4, 7, 9, 10, 12). Some of the earliest diagnostic applications included direct nucleic acid probes for identification of bacterial or fungal isolates and for direct detection of microorganisms in patient samples. This was followed by the introduction of nucleic acid amplification techniques (NAATs) for which utility in clinical testing was originally complicated by the complexity of the testing processes. These processes included many manual steps, such as nucleic acid extraction, amplification, and detection, and methods were fraught with the potential for false-positive results due to a combination of contamination risks plus the exquisite sensitivity of the testing methods. With the acquisition of expertise and dissemination of information regarding appropriate molecular laboratory procedures, including the production of CLSI guidelines (8) and the recent inclusion of all molecular infectious disease testing into the CAP microbiology checklist (3), along with the advent of newer technologies, such as automated extraction and real-time detection, use of molecular techniques in the clinical setting has become more commonplace and is no longer considered to be an esoteric or specialized practice reserved for reference laboratories. This is best exemplified by the fact that molecular testing systems are now available for use in moderate-complexity laboratory settings.

The benefits and applications of molecular testing for infectious diseases are constantly expanding. Utilities pertaining to organism detection are particularly significant, especially in situations in which the organism is difficult, if not impossible, to detect using more conventional methods. Furthermore, in some instances, molecular techniques have become the standard of care, replacing less effective techniques (6). Beyond simple detection, monitoring viral loads with NAATs has become an integral part of patient management for human immunodeficiency virus (HIV), hepatitis, and various viral infections in the immunocompromised. Other applications, such as molecular fingerprinting and strain comparison methods, have been devised to characterize organisms and to serve in epidemiological investigations (5, 11). More recently, nucleic acid sequencing techniques have refined these methods to the level of identifying specific sequences that can be used to detect organisms, to monitor mutations leading to drug resistance, to identify virulence factors, and to determine epidemiological relatedness of isolated organisms (2, 7, 10).

Some of the commercially available tests that use nucleic acid probes and amplification-based techniques for direct detection of organisms in clinical material are listed in Tables 12.1–1 and 12.1–2. Table 12.1–3 lists available assays for identification of previously isolated or detected organisms. Despite proven utility of molecular methods, relatively few tests are commercially available, and most assays are still developed and run as laboratory-developed tests in individual laboratories. A scan of current literature shows a growing list of published molecular detection protocols, with assays available to detect nearly every significant infectious agent. A list of clinically important organisms for which laboratory-developed assays may be considered is found in Table 12.1–4. Noncommercial methods for typing and for characterizing infectious agents are found in Tables 12.1–5 and 12.1–6, respectively. Finally, a representative list of vendors which provide reagents that can be used for the production of laboratory-developed tests is provided in Table 12.1–7.

The following sections provide protocols that illustrate the application of molecular techniques used for the management of infectious diseases. This includes protocols for assays available from commercial manufacturers, along with representative protocols for laboratory-developed tests targeting organisms for which clinical importance is high and for which commercial tests are not readily available. Along with these protocols, readers are advised to consult package inserts for commercially available tests and to monitor the rapidly expanding literature available on new molecular tests and methods.

Table 12.1–1 Commercially available, FDA-cleared nucleic acid probe hybridization methods for direct pathogen detection in clinical specimens[a]

Type of probe	Organism(s)	Specimen type(s)	Method(s)	Manufacturer	Product name(s)	Intended use
Direct	*Chlamydia trachomatis*	Endocervical, male urethral, or conjunctival swabs	Chemiluminescent DNA probe	Gen-Probe Inc.[b]	PACE 2 CT PACE 2C CT/GC	Diagnosis of disease
	Neisseria gonorrhoeae	Endocervical or male urethral swabs	Chemiluminescent DNA probe	Gen-Probe Inc.	PACE 2 GC PACE 2C CT/GC	Diagnosis of disease
	Group A *Streptococcus*	Throat swab	Chemiluminescent DNA probe	Gen-Probe Inc.	Group A Streptococcus Direct Test	Diagnosis of disease
	Gardnerella vaginalis, *Trichomonas vaginalis*, and *Candida* spp.	Vaginal swab	Hybridization probes	Becton Dickinson[c]	BD Affirm VPIII	Diagnosis of disease
Signal amplification	Human papillomavirus	Cervical brush or biopsy, liquid Pap specimen (Cytyc PreserveCyt)	Hybrid capture	Qiagen[d]	HC 2 HPV DNA Test HC 2 High-Risk HPV DNA Test	Diagnosis of disease
	Chlamydia trachomatis	Cervical brush or swab	Hybrid capture	Qiagen	CT-ID HC2 DNA Test CT/GC HC2 DNA Test	Diagnosis of disease
	Neisseria gonorrhoeae	Cervical brush or swab	Hybrid capture	Qiagen	GC-ID HC2 DNA Test CT/GC HC2 DNA Test	Diagnosis of disease
	HCV	Plasma or serum	bDNA	Siemens Healthcare Diagnostics[e]	VERSANT HCV RNA 3.0 Assay	Quantitative detection of HCV RNA for prognosis and monitoring of therapeutic response
	HIV	Plasma	bDNA	Siemens Healthcare Diagnostics	VERSANT HIV RNA 3.0 Assay	Quantitative detection of HIV RNA for prognosis and monitoring of therapeutic response

[a] This table contains examples of FDA-cleared or -approved commercially available kits and is not intended to be all-inclusive. Abbreviations: HCV, hepatitis C virus; bDNA, branched-chain DNA.

[b] San Diego, CA.

[c] Sparks, MD.

[d] Gaithersburg, MD.

[e] Tarrytown, NY.

Table 12.1–2 Commercially available, FDA-cleared nucleic acid amplification tests[a]

Organism(s)	Specimen type	Method	Manufacturer	Test name(s)	Intended use
Chlamydia trachomatis	Urogenital[b]	SDA	Becton Dickinson[c]	BD ProbeTec ET *C. trachomatis* and *N. gonorrhoeae* Amplified DNA Assay	Diagnosis of disease
	Urogenital	PCR	Roche Molecular Systems, Inc.[d]	AMPLICOR CT/NG Test for *Chlamydia trachomatis*	Diagnosis of disease
	Urogenital	Real-time PCR	Abbott Molecular[e]	RealTime CT/NG	Diagnosis of disease
	Urogenital	TMA	Gen-Probe Inc.[f]	APTIMA Combo 2 Assay APTIMA CT Assay	Diagnosis of disease
Neisseria gonorrhoeae	Urogenital	SDA	Becton Dickinson	BD ProbeTec ET *C. trachomatis* and *N. gonorrhoeae* Amplified DNA Assay	Diagnosis of disease
	Urogenital	TMA	Gen-Probe Inc.	APTIMA Combo 2 Assay APTIMA GC Assay	Diagnosis of disease
	Urogenital	Real-time PCR	Abbott Molecular	RealTime CT/NG	Diagnosis of disease
	Urogenital	PCR	Roche Molecular Diagnostics	COBAS AMPLICOR CT/NG Test for *Neisseria gonorrhoeae*	Diagnosis of disease
Group B *Streptococcus*	Combined vaginal and rectal swab	Real-time PCR	Becton Dickinson	StrepB Assay	Detection of colonization
	Combined vaginal and rectal swab	Real-time PCR	Cepheid[g]	Smart GBS	Detection of colonization
	Combined vaginal and rectal swab	Real-time PCR	Cepheid	Xpert GBS	Detection of colonization
Staphylococcus aureus	Positive blood culture bottles	Real-time PCR	Becton Dickinson	BD GeneOhm StaphSR Assay	Diagnosis of methicillin-sensitive *S. aureus* and MRSA bloodstream infections
	Nasal swab	Real-time PCR	Becton Dickinson	BD GeneOhm MRSA Assay	Detection of colonization with MRSA
	Wound swab	Real-time PCR	Cepheid	Xpert MRSA/SA SSTI test	Diagnosis of methicillin-sensitive *S. aureus* and MRSA skin and soft tissue infections
	Positive blood culture bottles	Real-time PCR	Cepheid	Xpert MRSA/SA BC test	Diagnosis of methicillin-sensitive *S. aureus* and MRSA bloodstream infections
	Nasal swab	Real-time PCR	Cepheid	Xpert MRSA test	Detection of colonization with MRSA
Mycobacterium tuberculosis	Concentrated sediments: sputum, tracheal aspirates, bronchial specimens	TMA	Gen-Probe Inc.	AMPLIFIED MTD (Mycobacterium Tuberculosis Direct) Test	Diagnosis of disease

(continued)

Table 12.1–2 Commercially available, FDA-cleared nucleic acid amplification tests[a] *(continued)*

Organism(s)	Specimen type	Method	Manufacturer	Test name(s)	Intended use
Cytomegalovirus	Whole blood	NASBA	bioMérieux, Inc.[h]	NucliSENS CMV pp67	Diagnosis of disease
Enterovirus	CSF	Real-time RT-PCR	Cepheid	Xpert EV	Diagnosis of disease
	CSF	NASBA	bioMérieux, Inc.	NucliSENS EasyQ Enterovirus	Diagnosis of disease
HBV, quantitative	Serum, plasma	RT-PCR	Roche Molecular Diagnostics	COBAS TaqMan HBV Test	Quantitative detection of HBV RNA for prognosis and monitoring of therapeutic response
HCV	Serum, plasma	PCR	Roche Molecular Systems, Inc.	AMPLICOR HCV Test, v2.0 COBAS AMPLICOR HCV Test, v2.0 COBAS AmpliPrep/COBAS AMPLICOR HCV Test, v2.0	Quantitative detection of HCV RNA for prognosis and monitoring of therapeutic response
	Serum, plasma	TMA	Gen-Probe Inc., for Siemens Healthcare Diagnostics[i]	Versant HCV RNA	Diagnosis of disease
HCV, quantitative	Serum, plasma	RT-PCR	Roche Molecular Systems, Inc.	COBAS AmpliPrep/ COBAS TaqMan HCV Test	Quantitative detection of HCV RNA for prognosis and monitoring of therapeutic response
HIV, quantitative	Plasma	Real-time RT-PCR	Roche Molecular Systems, Inc.	COBAS AmpliPrep/ COBAS TaqMan HIV-1 Test	Quantitative detection of HIV RNA for prognosis and monitoring of therapeutic response
	Plasma	RT-PCR	Roche Molecular Systems, Inc.	AMPLICOR HIV-1 MONITOR Test, v1.5 COBAS AMPLICOR HIV-1 MONITOR Test, v1.5	Quantitative detection of HIV RNA for prognosis and monitoring of therapeutic response
	Plasma	NASBA	bioMérieux, Inc.	NucliSens HIV-1 QT	Quantitative detection of HIV RNA for prognosis and monitoring of therapeutic response
	Plasma	Real-time RT-PCR	Abbott Molecular	RealTi*m*e HIV-1	Quantitative detection of HIV RNA for prognosis and monitoring of therapeutic response

HIV, genotyping	Plasma	RT-PCR and cycle sequencing	Abbott Molecular	ViroSeq HIV-1 Genotyping System	Monitoring of HIV-1-infected individuals at initial presentation, before initial drug therapy, and at drug therapy failure (with increased viral load) before therapy switch
	Plasma	RT-PCR and cycle sequencing	Siemens Healthcare Diagnostics	TruGene HIV-1 Genotyping and Open Gene DNA Sequencing System	Monitoring of HIV-1-infected individuals at initial presentation, before initial drug therapy, and at drug therapy failure (with increased viral load) before therapy switch
Human metapneumovirus	Nasopharyngeal swabs	Real-time RT-PCR	Prodesse, Inc.[j]	Pro hMPV + Assay	Diagnosis of disease
Influenza A, influenza B, and respiratory syncytial viruses (3 targets)	Nasopharyngeal swabs	Real-time RT-PCR	Prodesse, Inc.	ProFlu + Assay (multiplex assay)	Diagnosis of disease
Influenza A and B viruses; respiratory syncytial virus; parainfluenza viruses 1, 2, and 3; adenovirus; human metapneumovirus; rhinovirus (12 targets)	Nasopharyngeal swabs	PCR, TSPE, Tag sorting	Luminex Molecular Diagnostics, Inc.[k]	xTAG Respiratory Viral Panel (multiplex assay)	Diagnosis of disease; epidemiology

[a] This table contains examples of FDA-cleared or -approved nucleic acid amplification tests that are commercially available in the United States. It is not intended to be all-inclusive and does not contain tests used for screening of blood products. Websites of the manufacturers are useful sources of the most up-to-date information. Abbreviations: RT-PCR, reverse transcriptase PCR; SDA, strand displacement amplification; TMA, transcription-mediated amplification; NASBA, nucleic acid sequence-based amplification; TSPE, target-specific primer extension; MRSA, methicillin-resistant *S. aureus;* HBV and HCV, hepatitis B and C viruses, respectively; HIV-1, HIV type 1.

[b] See Table 12.2.3–1 for additional details of collection devices for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*.

[c] Sparks, MD.

[d] Branchburg, NJ; distributed by Roche Diagnostics Corp., Indianapolis, IN.

[e] Des Plaines, IL.

[f] San Diego, CA.

[g] Sunnyvale, CA.

[h] Durham, NC.

[i] Tarrytown, NY.

[j] Waukesha, WI.

[k] Toronto, Canada.

Table 12.1–3 FDA-cleared nucleic acid probes for organism identification[a]

Method of identification	Organisms	Sample	Manufacturer
Chemiluminescent DNA probe	Bacteria: *Campylobacter, Enterococcus,* group A streptococcus *(Streptococcus pyogenes),* group B streptococcus *(Streptococcus agalactiae), Haemophilus influenzae, Neisseria gonorrhoeae, Staphylococcus aureus, Listeria monocytogenes,* and *Streptococcus pneumoniae*	Culture isolate	Gen-Probe Inc.[b]
	Fungi: *Blastomyces dermatitidis, Coccidioides immitis,* and *Histoplasma capsulatum*	Culture isolate	Gen-Probe Inc.
	Mycobacteria: *Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium avium* complex, *Mycobacterium gordonae, Mycobacterium kansasii,* and *Mycobacterium tuberculosis* complex	Culture isolate	Gen-Probe Inc.
PNA FISH	Bacteria: *Staphylococcus aureus, Staphylococcus aureus* and coagulase-negative staphylococci, *Enterococcus faecalis* and other enterococci, *Escherichia.coli,* and *Pseudomonas aeruginosa*	Positive blood culture from continuously monitored blood culture systems	AdvanDx, Inc.[c]
	Fungi: *Candida albicans; Candida albicans* and *Candida glabrata;* and Yeast Traffic Light—*Candida albicans* and/or *Candida parapsilosis, Candida tropicalis,* and *Candida glabrata* and/or *Candida krusei*	Positive blood culture from continuously monitored blood culture systems	AdvanDx, Inc.

[a] This table contains examples of FDA-cleared or -approved nucleic acid probe tests that are commercially available in the United States. It is not intended to be all-inclusive. Websites of the manufacturers are useful sources of the most up-to-date information. Abbreviation: PNA FISH, peptide nucleic acid fluorescence in situ hybridization.

[b] San Diego, CA.

[c] Woburn, MA.

Table 12.1–4 Clinically important pathogens tested for by noncommercial nucleic acid amplification-based tests[a]

Organism(s)	Specimen type(s)	Clinical indication(s)
Adenovirus	Urine, stool, plasma	Immunocompromised/transplant patients
Anaplasma phagocytophilum	Blood	Suspected granulocytic anaplasmosis
Aspergillus species	Blood, respiratory	Invasive aspergillosis
Babesia microti	Blood	Suspected babesiosis
BK virus	Plasma, urine	Immunocompromised/transplant patients
Bordetella pertussis	Nasopharyngeal swabs/aspirates	Whooping cough
Borrelia burgdorferi	Synovial fluid, CSF, blood	Suspected Lyme disease
Candida	Blood	Hematogenous candidiasis
Chlamydia pneumoniae	Respiratory	Atypical pneumonia
Clostridium difficile	Stool	Antimicrobial agent-associated diarrhea
Cytomegalovirus	Plasma, CSF, bronchial wash	Immunocompromised/transplant patients
Dengue virus	Serum	Dengue fever/hemorrhagic fever
Epstein-Barr virus	Blood, plasma, CSF	Lymphoproliferative disorders
Ehrlichia species	Blood	Suspected monocytic ehrlichiosis
Enterovirus	CSF, stool	Aseptic meningitis
Human herpesvirus 6	Plasma, CSF	Immunocompromised, encephalitis
Herpes simplex viruses 1 and 2	CSF, swabs	Encephalitis, genital and ocular herpes, congenital infections
Human metapneumovirus	Respiratory	Upper and lower respiratory symptoms
Influenza virus	Respiratory	Fever, myalgia, upper respiratory symptoms
JC virus	Plasma, CSF	Progressive multifocal leukoencephalopathy

(continued)

Table 12.1–4 *(continued)*

Organism(s)	Specimen type(s)	Clinical indication(s)
Legionella pneumophila	Respiratory	Atypical pneumonia
Mumps virus	Oral swab, urine, plasma	Parotitis, aseptic meningitis, encephalitis
Mycoplasma pneumoniae	Respiratory	Atypical pneumonia
Norovirus	Stool	Diarrhea or vomiting
Parainfluenza viruses 1, 2, and 3	Respiratory	Upper respiratory symptoms
Parechovirus	CSF	Aseptic meningitis, encephalitis
Parvovirus B19	Serum, amniotic fluid	Hydrops fetalis, immunocompromised
Rhinovirus	Respiratory	Upper respiratory symptoms
Respiratory syncytial virus	Respiratory	Upper and lower respiratory symptoms
Toxoplasma gondii	CSF, whole blood, amniotic fluid	Suspected toxoplasmosis, congenital infections
Treponema pallidum	Swabs of lesions, CSF	Suspected syphilis, congenital infections
Varicella-zoster virus	CSF, swabs of lesions	Fever and rash with meningitis or ataxia
West Nile virus	Plasma, CSF	Fever, encephalitis, meningitis, or acute flaccid paralysis

[a] All tests use PCR. The list is not intended to be all-inclusive.

Table 12.1–5 Molecular methods for epidemiological typing of microorganisms[a]

Method	Substrate	Principal characteristics	Examples
Plasmid analysis	Plasmid DNA	Potentially unstable owing to loss of plasmids. May be augmented by restriction endonuclease digestion.	*Staphylococcus aureus,* coagulase-negative staphylococci, *Klebsiella, Serratia, Enterobacteriaceae*
Restriction endonuclease analysis of chromosomal DNA with conventional electrophoresis	Chromosomal DNA	Broadly applicable. Large numbers of bands. Not amenable to computer analysis.	Enterococci, *S. aureus, Clostridium difficile, Candida* spp.
Pulsed-field gel electrophoresis	Chromosomal DNA	Broadly applicable. Uses infrequently cutting restriction enzymes to generate large DNA fragments (10–800 kb). Fewer bands. Excellent reproducibility and discriminatory power. Expensive equipment.	Staphylococci, enterococci, *Enterobacteriaceae, Pseudomonas, Candida*
Genome restriction fragment length polymorphism analysis with DNA probes	Chromosomal DNA	Broadly applicable. Multistep process. Amenable to computer analysis. Includes *IS*6110 analysis of *M. tuberculosis* and ribotyping.	*Mycobacterium tuberculosis, Candida* spp., *Enterobacteriaceae,* staphylococci, *Pseudomonas*
PCR-based methods: repetitive-element PCR spacer typing, selective amplification of genome restriction fragments, multilocus allelic sequence-based typing	Chromosomal DNA	Broadly applicable. Rapid and moderately easy to perform. Crude extracts and small amounts of DNA may suffice.	*Enterobacteriaceae, Acinetobacter,* staphylococci, *M. tuberculosis,* hepatitis C virus
Library probe genotypic hybridization schemes: multilocus probe dot blot patterns, high-density oligonucleotide patterns	Chromosomal DNA	Unambiguous yes-no result. Less discrimination than with other methods. Couple with DNA chip technology.	*Burkholderia cepacia, S. aureus, M. tuberculosis*

[a] This table contains examples of available methods and applications and is not intended to be all-inclusive. It has been adapted from reference 12.

Table 12.1–6 Application of molecular methods for detection of antimicrobial resistance[a]

Organism(s)	Antimicrobial agent(s)	Gene(s)	Detection method
Staphylococci	Methicillin or oxacillin	*mecA*[b]	PCR, probe, bDNA
	Aminoglycosides	*ant*	PCR, probe
	Macrolides	*erm, msr*	PCR, probe
	Fluoroquinolones	Point mutations in *gyrA* and *parC*	PCR and sequencing
Enterococci	Vancomycin	*vanA, -B, -C, -D*[c]	PCR, probe
	Aminoglycosides	*aac, aph, ant*	PCR, probe
Streptococcus pneumoniae	β-Lactams	*pbp1A, -2B, -2X*[d]	PCR
	Fluoroquinolones	Point mutations in *gyrA* and *-B* and *parC* and *-E*	PCR and sequencing
Enterobacteriaceae	β-Lactams	bla_{TEM}[e]	Probe
	Fluoroquinolones	bla_{SHV}[e]	PCR and RFLP
		Point mutations in *gyrA* and *-B* and *parC* and *-E*	PCR and sequencing PCR and sequencing
Mycobacterium tuberculosis[f]	Rifamycins	Point mutations in *rpoB*	PCR and SSCP; PCR and sequencing
	Isoniazid	Point mutations in *katG, inhA,* and *ahpC*	PCR and SSCP
	Ethambutol	Point mutations in *embB*	PCR and sequencing
	Streptomycin	Point mutations in *rpsL* and *rrs*	PCR and RFLP
Herpesviruses[g]	Acyclovir and related drugs	Mutations or deletions in the TK gene	PCR and sequencing
	Foscarnet	Point mutations in the DNA polymerase gene	PCR and sequencing
HIV	Nucleoside RT inhibitors	Point mutations in the RT gene	PCR and sequencing; PCR and LIPA
	Protease inhibitors	Point mutations in the PROT gene	PCR and sequencing

[a] Adapted from reference 12. Abbreviations: bDNA, branched-chain DNA; RFLP, restriction fragment length polymorphism; SSCP, single-stranded conformational polymorphism; LIPA, line probe assay; TK, thymidine kinase; RT, reverse transcriptase; PROT, protease.

[b] *mecA* encodes the altered penicillin binding protein PBP2a; phenotypic methods may require 48 h of incubation or more to detect resistance and are less than 100% sensitive. Detection of *mecA* has potential for clinical application in specific circumstances.

[c] Vancomycin resistance in enterococci may be related to one of four distinct resistance genotypes of which *vanA* and *vanB* are of the most importance. Genotypic detection of resistance is useful in validation of phenotypic methods.

[d] Penicillin and cephalosporin resistance in *S. pneumoniae* is due to the alteration of one or more penicillin binding proteins.

[e] The genetic basis of resistance to β-lactam antimicrobial agents among *Enterobacteriaceae* is extremely complex. The bla_{TEM} and bla_{SHV} genes are the two most common sets of plasmid-borne β-lactamase genes. The presence of either a bla_{TEM} or bla_{SHV} gene implies ampicillin resistance. Variants of the bla_{TEM} and bla_{SHV} genes (extended-spectrum β-lactamases) may also encode resistance to a range of broad-spectrum cephalosporins and to monobactams.

[f] *M. tuberculosis* is a very slow-growing organism. Four weeks or more may be required to obtain phenotypic susceptibility test results. Detection of resistance genes in *M. tuberculosis* has potential for clinical application in the short term.

[g] There are no phenotypic methods sufficiently practical for routine clinical detection of resistance to antiviral agents. Genotypic methods represent a practical approach to the routine detection of antiviral resistance.

REFERENCES

1. **Bergeron, M. G., and M. Ouellette.** 1998. Preventing antibiotic resistance using rapid DNA-based diagnostic tests. *Infect. Control Hosp. Epidemiol.* **19:**560–564.
2. **Clarridge, J. E.** 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clin. Microbiol. Rev.* **17**:840–862.
3. **College of American Pathologists.** 2009. Laboratory Accreditation Program, Microbiology Checklist, rev. 06/15/2009. College of American Pathologists, Northfield, IL.
4. **Cormican, M. G., and M. A. Pfaller.** 2001. Molecular pathology of infectious diseases, p. 1241–1253. *In* J. B. Henry (ed.), *Clinical Diagnosis and Management by Laboratory Methods*, 20th ed. W. B. Saunders Company, Philadelphia, PA.
5. **Hacek, D. M., T. Suriano, G. A. Noskin, J. Kruszynski, B. Reisberg, and L. R. Peterson.** 1999. Medical and economic benefit of a comprehensive infection control program that includes routine determination of microbial clonality. *Am. J. Clin. Pathol.* **111:**647–654.

Table 12.1–7 Suppliers of reagents used for laboratory-developed PCR tests[a]

Products	Vendor	Website
PCR mixes and components	Applied Biosystems	http://www.appliedbiosystems.com
	Bio-Rad	http://www.bio-rad.com
	Life Technologies	http://www.lifetechnologies.com
	Promega	http://www.promega.com
	Qiagen	http://www.qiagen.com
	Roche Applied Science	http://www.roche-applied-science.com
	Sigma Aldrich	http://www.sigmaaldrich.com
	Stratagene	http://www.stratagene.com
Oligonucleotide primer/probe synthesis	Biosearch Technologies	http://www.biosearchtech.com
	Eurogentec	http://www.eurogentec.com
	IDT	http://www.idtdna.com
	Life Technologies	http://www.lifetechnologies.com
	Operon	http://www.operon.com
	Sigma-Aldrich	http://www.sigmaaldrich.com
DNA and RNA calibrators, run controls, and validation panels	AcroMetrix	http://www.acrometrix.com
	Advanced Biotechnologies	http://www.abionline.com
	Asuragen	http://www.asuragen.com
	National Institute for Biological Standards and Control	http://www.nibsc.ac.uk
	Qnostics	http://www.qnostics.com
	SeraCare	http://www.seracare.com
	ZeptoMetrix	http://www.zeptometrix.com
Organism stocks	Advanced Biotechnologies	http://www.abionline.com
	American Type Culture Collection	http://www.atcc.org
	NIH Repositories	http://dtp.nci.nih.gov/repositories.html
	ZeptoMetrix	http://www.zeptometrix.com

[a] This table contains representative examples of vendors which supply reagents that can be used for the production of laboratory-developed PCR tests. It is not intended to be all-inclusive. In addition to the components listed above, analyte-specific reagents targeting specific organisms can be obtained from a variety of sources.

REFERENCES *(continued)*

6. **Lakeman, F. D., and R. J. Whitley.** 1995. Diagnosis of herpes simplex encephalitis: application of polymerase chain reaction to cerebrospinal fluid from brain-biopsied patients and correlation with disease. *J. Infect. Dis.* **171:**857–863.
7. **Nolte, F. S., and A. M. Caliendo.** 2007. Molecular detection and identification of microorganisms, p. 218–244. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.
8. **Nolte, F. S., F. R. Cockerill, P. J. Dailey, D. Hillyard, S. McDonough, R. F. Meyer, and R. G. Shively.** 2006. *Clinical and Laboratory Standards Institute (CLSI). Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline—Second Edition.* CLSI, Wayne, PA.
9. **Persing, D. H. (ed.).** 1996. *PCR Protocols for Emerging Infectious Diseases.* ASM Press, Washington, DC.
10. **Persing, D. H., F. C. Tenover, J. Versalovic, Y. Tang, E. R. Unger, D. A. Relman, and T. J. White (ed.).** 2004. *Molecular Microbiology: Diagnostic Principles and Practice.* ASM Press, Washington, DC.
11. **Pfaller, M. A.** 1999. Molecular epidemiology in the care of patients. *Arch. Pathol. Lab. Med.* **123:**1007–1010.
12. **Pfaller, M. A.** 2000. Diagnosis and management of infectious diseases: molecular methods for the new millennium. *Clin. Lab. Newsl.* **26:**10–13.

12.2.1 Introduction

The detection of pathogenic microorganisms directly in clinical specimens by molecular methods has been investigated extensively using a variety of nucleic acid probe hybridization, target amplification, and signal-generating formats. Commercial product development has focused on direct diagnosis of blood-borne and sexually transmitted diseases and respiratory pathogens using solid- and solution-phase hybridization with nonisotopic nucleic acid probes and several different target amplification methods, including conventional PCR, strand displacement amplification, transcription-mediated amplification, and, more recently, real-time PCR. Many laboratories are developing their own "home brew" assays for pathogen detection using real-time PCR for organisms not addressed by commercial kits (Table 12.1–4).

12.2.2 Nucleic Acid Probe-Based Methods

Direct diagnosis by nucleic acid probe hybridization is simple, rapid, and relatively free of the contamination and inhibition problems associated with target amplification methods. The sensitivity of probe hybridization methods is limited by the relatively large number of copies ($\sim 10^4$) of the target sequence required to generate a positive signal. Efforts to improve the sensitivity of the nucleic acid probe-based methods include the use of RNA targets, various signal amplification formats, and reduction of the background signal (1–2).

REFERENCES

1. **Murphy, D., P. Gorin, and M. Fauvel.** 1999. Reproducibility and performance of the second-generation branched-DNA assay in routine quantification of human immunodeficiency virus type 1 RNA in plasma. *J. Clin. Microbiol.* **37:**812–814.
2. **Wylie, J. L., S. Moses, R. Babcock, A. Jolly, S. Giercke, and G. Hammond.** 1998. Comparative evaluation of Chlamydiazyme, PACE 2, and AMP-CT assays for detection of *Chlamydia trachomatis* in endocervical specimens. *J. Clin. Microbiol.* **36:**3488–3491.

PART 1

Gen-Probe PACE 2C Nucleic Acid Hybridization Test for Detecting *Chlamydia trachomatis* and *Neisseria gonorrhoeae*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The Gen-Probe PACE 2C system for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* is a rapid DNA probe test that utilizes the technique of nucleic acid hybridization to screen for the presence of *C. trachomatis* and/or *N. gonorrhoeae* in endocervical and male urethral swab specimens. The Gen-Probe PACE 2C system uses a chemiluminescently labeled DNA probe that is complementary to the rRNA of the target organism. The labeled DNA-RNA hybrid is separated from nonhybridized probe, and the luminescence of the hybrid is measured. The Gen-Probe PACE 2C assay detects whether a patient is positive for *C. trachomatis* or *N. gonorrhoeae* but does not differentiate between the two organisms. If the assay is positive, it is advised to reflex to the individual Gen-Probe PACE 2 CT or Gen-Probe PACE 2 GC assay. These assays will give a definitive identification as to whether the screening assay is positive for *C. trachomatis*, *N. gonorrhoeae*, or both. The PACE 2 CT test can also be used to test conjunctival specimens for *C. trachomatis.* Due to the low prevalence of these organisms in some patient populations, screening with the screening assay might be more cost-effective. In high-prevalence populations, testing for the individual analytes directly using the PACE 2 CT or PACE 2 GC assay may be a better alternative. Each laboratory should determine the most effective testing algorithm.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

Specimens from the urogenital tract (endocervical and male urethral) must be obtained using the Gen-Probe specimen collection kit (containing a transport tube and swab).

B. Time of specimen collection

There are no particular timing issues to consider in collection of the specimens; however, the time of collection should be recorded.

C. Specimen transport

1. Collection tubes containing the appropriate swab specimens must be transported to the laboratory at 2 to 25°C and may be stored at 2 to 25°C for up to 7 days before testing.
2. If longer storage is necessary, process the specimen as described in section V.A below, and freeze at −20 to −70°C.

D. Specimen labeling and request submission

The laboratory should determine a mechanism to ensure that the specimen type and time of collection are recorded.

E. Rejection criteria

1. Test only the indicated specimen types (endocervical and urethral) in the indicated transport medium. Testing of other specimen types or use of other transport media has not been validated.
2. Specimens containing some blood will not interfere with the assay. Grossly bloody specimens (greater than 80 μl of whole blood in 1 ml of transport medium) could interfere with the assay. A repeat specimen should be requested if possible.
3. Repeat testing to evaluate efficacy of therapy is not indicated for non-culture-based tests.
4. Request a repeat specimen, or obtain the information, when the collection time or specimen type has not been provided.
5. Reject specimens that arrive in leaky containers.
6. Non-culture-based methods should not be used in cases of suspected sexual abuse.
7. If an improperly collected, transported, or handled specimen cannot be replaced, document in the final report that specimen quality may have been compromised.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Materials provided in the Gen-Probe PACE 2C System for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*

1. PACE 2 hybridization buffer
2. PACE 2C *Chlamydia trachomatis/Neisseria gonorrhoeae* probe reagent
3. PACE 2 selection reagent
4. PACE 2 STD separation reagent
5. PACE 2 STD negative reference
6. PACE 2 *Chlamydia trachomatis* positive control
7. PACE 2 *Neisseria gonorrhoeae* positive control
8. PACE 2 STD wash solution
9. Sealing cards
10. Gen-Probe disposable polystyrene reaction tubes (12 by 75 mm)

B. Materials required but not provided

1. Gen-Probe PACE specimen collection kits
2. Gen-Probe detection reagent kit
3. Magnetic separation unit
4. Vortex mixer
5. Covered water bath (60 ± 1°C)
6. Micropipettes (100 μl)
7. Pipettes capable of delivering 1 to 25 ml
8. Absorbent paper

C. Materials available from Gen-Probe

1. Gen-Probe detection reagent kit (catalog no. 1971)
2. Gen-Probe LEADER luminometer
3. Gen-Probe magnetic separation unit (catalog no. 1639)
4. Gen-Probe FAST Express reagent kit (catalog no. 2930)

III. MATERIALS *(continued)*

5. Wash bottle cap assembly (catalog no. 3919)
6. Gen-Probe bottle top dispenser adapter kit (catalog no. 4173)
7. Gen-Probe dispenser (5 ml) (catalog no. 3078)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that transport media and kits meet expiration date and QC parameters.

B. Negative reference—provides a measure of the assay background and is used to calculate run cutoff

1. The response of each negative reference should be ≤200 relative light units (RLU).
2. All negative reference values should fall within 30% of the mean response of the negative reference.
3. If one value falls outside these ranges, it may be deleted. Follow the instructions in the LEADER luminometer operator's manual.
4. If two values fall outside these ranges, the results should not be reported, and the assay must be repeated.

C. Positive control (provided)

1. The difference in the response of the positive control and the mean response of the negative reference should be >600 and <3,200 RLU or the run is invalid.
2. If the positive control value is not in the required range, the test results are not acceptable, and the assay must be repeated.
3. Each laboratory should establish its own mean and range for the negative reference and positive controls.
4. To test the sample processing effectiveness, positive and negative cell controls may be run with specimens. ATCC organisms can be used as positive and negative controls. Refer to package insert for details.

V. PROCEDURE

Observe standard precautions.

A. Sample preparation

1. Allow the transport tubes containing the swab specimens to come to room temperature, and then vortex for 5 to 10 s.
2. Express all liquid from the swab by pressing the swab against the wall of the tube. Discard the swab.
3. Vortex the tubes for 5 to 10 s to ensure homogeneity.

B. Reagent preparation

1. All reagents (except for the probe reagent, PACE 2 hybridization buffer, and separation reagent) must reach room temperature prior to use. The separation and probe reagents must be kept at 2 to 8°C until used.
2. Probe reagent
   - **a.** If the PACE 2 hybridization buffer has a gel formed or has been stored at 2 to 8°C, vortex for 10 s. Place the buffer in a water bath at 60 ± 1°C for 3 to 4 min. Vortex again for 10 s to ensure that the solution is homogeneous (this process might have to be repeated).
   - **b.** Pipette 6.0 ml of the PACE 2 hybridization buffer into the lyophilized PACE 2C *Chlamydia trachomatis/Neisseria gonorrhoeae* probe reagent (PACE 2C probe reagent). The reagent should stand at room temperature for 2 min and then be vortexed for 10 s prior to use. Visually inspect for reagent homogeneity, and record on the label the date the reagent was reconstituted.
   - **c.** Reconstituted probe is stable for 3 weeks stored at 2 to 8°C or until the date stamped on the reagent container, whichever comes first. After re-

V. PROCEDURE *(continued)*

constitution prior to use, allow the reagent to stand at room temperature as in the step immediately above.

3. Separation suspension
   - **a.** Determine the number of tests to be performed.
   - **b.** Calculate the volumes of selection reagent and separation reagent needed as follows.
     - (1) Selection reagent
       - (a) Volume (milliliters) of selection reagent = number of tests + 2 extra tests (when using an Eppendorf pipettor)
       - (b) Volume (milliliters) of selection reagent = number of tests + 10 extra tests (when using bottle top dispenser)
     - (2) Separation reagent: volume (milliliters) of separation reagent = volume of selection reagent divided by 20
   - **c.** Pour the required volume of selection reagent into a clean, dry container.
   - **d.** Add the required volume of separation reagent to the selection reagent, and mix well. The separation suspension is stable at room temperature for 6 h.

C. Hybridization

1. Label the tubes with sample identification numbers. Include three tubes for the negative reference and one for each positive control *(C. trachomatis* and *N. gonorrhoeae).* Label the tubes near the top. The reference and controls must be run with each batch of specimens.
2. Insert the tubes into the tube rack section of the Gen-Probe magnetic separation unit. The base portion of the unit will be used at a later time.
3. Vortex each specimen for 5 s.
4. Pipette 100 µl of each control and each specimen into the bottoms of the appropriate tubes.
5. Pipette 100 µl of the PACE 2C probe reagent to the bottom of each tube, taking care not to touch the top or sides of the tube.
6. Seal the tubes with sealing cards and shake the rack three to five times to mix the contents.
7. Incubate the rack with tubes in a water bath (60 ± 1°C) for 1 h. The magnetic separation unit base should not touch the water bath.
8. Prepare the Gen-Probe LEADER instrument for operation. The detection reagents, I and II, should be checked to make sure there is sufficient volume.

D. Separation

1. Remove the tube rack from the water bath, remove the sealing cards, and pipette 1 ml of the separation solution into each tube.
2. Cover the tubes with sealing cards, shake three to five times, and incubate in the water bath (60 ± 1°C) for 10 min.
3. Remove the rack from the water bath. Remove the sealing cards and place the tube rack on the base of the Gen-Probe magnetic separation unit for 5 min at room temperature.
4. Holding the tube rack and base of magnetic separation unit together, invert to decant the supernatants, shake the unit two or three times, and then blot the inverted tubes (three to five times) on absorbent paper.
5. Do not remove the tube rack from the magnetic separation base. Fill each tube to the rim with wash solution and let sit on the magnetic separation base for 20 min at room temperature.
6. Holding the tube rack and base together, decant supernatant material. Do not blot. Approximately 50 to 100 µl of wash solution should remain in each tube.
7. Separate the tube rack from the base, and shake to suspend the pellets.

V. PROCEDURE *(continued)*

E. Detection

1. Select the appropriate protocol assay from the LEADER luminometer software.
2. Wipe each tube to ensure that no residue is present on the outside of the tube.
3. Mix each tube to suspend the pellets, and insert into the instrument based on the prompts provided by the software.
4. Read the tubes in the following order.
   a. Negative reference: three tubes
   b. *C. trachomatis* positive control: one tube
   c. *N. gonorrhoeae* positive control: one tube
   d. Specimen tubes
5. When the analysis is completed, remove the last tube, replace it with an empty tube, and follow the instrument prompts.

F. Calculation of results

1. Results are calculated on the basis of the difference between the response in RLU of the specimen and the mean of the negative reference.
2. The mean of the negative reference equals the sum of the three negative reference replicates divided by 3.
3. The luminometer will print the specimen response and compare this to the calculated assay cutoff. A positive or negative interpretation compared to this cutoff will be printed for the operator. Refer to the operator's manual for more detailed information.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report results as positive or negative for the presence of *C. trachomatis* and/or *N. gonorrhoeae.*

B. Reporting should clearly state that a positive result does not differentiate between the presence of *C. trachomatis* or *N. gonorrhoeae.* If positives are reflexed to the individual assays (PACE 2 CT and PACE 2 GC assay), the presence of the individual organisms can be determined and should be reported.

C. Document all testing in hard copy or computerized work card.

VII. INTERPRETATION

A. The luminometer prints the specimen response (in RLU) and compares this response to an assigned assay cutoff. A positive or negative interpretation resulting from this comparison is printed.

B. A positive result indicates that the target organism is present in the specimen and strongly supports a diagnosis of chlamydial or gonococcal infection.

1. Positive: the difference is $\geq$300 RLU.
2. Negative: the difference is $<$300 RLU.

VIII. LIMITATIONS OF TESTING

A. This assay has only been tested with endocervical and male urethral specimens. Performance of other specimen types has not been validated.

B. Only standard culture methods for *N. gonorrhoeae* and *C. trachomatis* should be used for evaluation of suspected sexual abuse and for other medico-legal indications.

C. Additional testing is recommended in any circumstance when false-positive results could lead to adverse medical, social, or psychological consequences. Test results should be interpreted with consideration of clinical and laboratory findings.

VIII. LIMITATIONS OF TESTING *(continued)*

D. The presence of blood in cervical specimens may cause erroneous results (usually false positives). There may be other endogenous substances that could interfere with assay performance, but gynecological lubricants and spermicides have not been known to cause interference.

E. A negative result does not exclude the possibility of infection, because reliable results are dependent on adequate specimen collection.

F. Therapeutic success or failure cannot be determined, because target organism nucleic acids may persist after appropriate antimicrobial agent therapy.

G. Because the Gen-Probe PACE 2C System for *C. trachomatis* and *N. gonorrhoeae* is a screening assay designed to detect both organisms, follow-up testing of PACE 2C positives with individual *C. trachomatis* and *N. gonorrhoeae* assays is necessary.

SUPPLEMENTAL READING

Clarke, L. M., M. F. Sierra, B. J. Daidone, N. Lopez, J. M. Covino, and W. M. McCormack. 1993. Comparison of the Syva Micro Trak enzyme immunoassay and Gen-Probe PACE 2 with cell culture for diagnosis of cervical *Chlamydia trachomatis* infection in a high-prevalence female population. *J. Clin. Microbiol.* **31:**968–971.

Hale, Y. M., M. E. Melton, J. S. Lewis, and D. E. Willis. 1993. Evaluation of the PACE 2 *Neisseria gonorrhoeae* assay by three public health laboratories. *J. Clin. Microbiol.* **31:**451–453.

Hosein, I. K., A. M. Kaunitz, and S. J. Craft. 1992. Detection of cervical Chlamydia trachomatis and Neisseria gonorrhoeae with deoxyribonucleic acid probe assay in obstetric patients. *Am. J. Obstet. Gynecol.* **167:**588–591.

Iwen, P. C., R. A. Walker, K. L. Warren, D. M. Kelly, S. H. Hinrichs, and J. Linder. 1995. Evaluation of nucleic acid-based test (PACE 2C) for simultaneous detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in endocervical specimens. *J. Clin. Microbiol.* **33:**2587–2591.

Koumans, E. H., R. E. Johnson, J. S. Knapp, and M. E. St. Louis. 1998. Laboratory testing for Neisseria gonorrhoeae by recently introduced nonculture tests: a performance review with clinical and public health considerations. *Clin. Infect. Dis.* **27:**1171–1180.

Limberger, R. J., R. Biega, A. Evancoe, L. McCarthy, L. Slivienski, and M. Kirkwood. 1992. Evaluation of culture and the Gen-Probe PACE2 assay for detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in endocervical specimens transported to a state health laboratory. *J. Clin. Microbiol.* **30:**1162–1166.

Schachter, J. 1998. Two different worlds we live in. *Clin. Infect. Dis.* **27:**1181–1185.

Wylie, J. L., S. Moses, R. Babcock, A. Jolly, S. Giercke, and G. Hammond. 1998. Comparative evaluation of Chlamydiazyme, PACE 2, and AMP-CT assays for detection of *Chlamydia trachomatis* in endocervical specimens. *J. Clin. Microbiol.* **36:**3488–3491.

PART 2 Detection of Human Papillomavirus (HPV) DNA by Using Hybrid Capture 2 High-Risk HPV DNA Test

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

It is well established that human papillomavirus (HPV) causes a high percentage of cervical cancer in women. Since HPV is common in adults, the concern is that over time some women may not clear HPV infection acquired at a young age and go on to develop cervical cancer later in life. The American College of Obstetricians and Gynecologists has developed guidelines that include both Pap smear and HPV testing in the management of women's health as it relates to cervical cancer.

The Hybrid Capture 2 (hc2) HPV DNA Test is an in vitro nucleic hybridization assay using chemiluminescence to qualitatively detect the presence of 13 high-risk HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. The assay cannot determine which specific type is present. Specimen types include cervical specimens collected with a cervical brush and fresh cervical biopsy samples. Specimens collected using liquid-based cytology collection vials can also be tested. Indications for testing include screening women that are over 30 years of age in conjunction with collecting a Pap smear. Algorithms for testing women that are under 30 who present with an ASC-US (atypical squamous cells of undetermined significance) Pap smear are also used to determine the need for referral for colposcopy. The hc2 high-risk DNA test is not intended for use as a screening device for women with normal Pap smears.

Specimens containing the target DNA hybridize with a specific HPV RNA probe cocktail. The resultant RNA-DNA hybrids are captured onto the surface of a microplate well coated with antibodies specific for RNA-DNA hybrids. Immobilized hybrids are then reacted with alkaline phosphatase-conjugated antibodies specific for the hybrids and detected with a chemiluminescent substrate. Light emitted by the reaction is measured as relative light units (RLUs) using a luminometer. The inten-

sity of the light correlates with the presence or absence of HPV DNA in the patient specimen. The hc2 high-risk HPV DNA test can be performed manually or using the Rapid Capture system instrument for high-volume sample throughput testing.

The procedure in this section covers the hc2 HPV assay and is intended to provide the reader with a general outline of the methodology and work flow; it does not represent the full operational detail provided by the manufacturer. For the most up-to-date information, the reader is advised to consult the manufacturer and to obtain a package insert (1).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. Specimens collected for testing include cervical brush samples and cervical biopsy samples (at least 2 mm in diameter). Specimens collected with a brush or broom-type device can also be collected and placed in Cytyc ThinPrep PreservCyt solution.
2. Cervical brush specimens should be collected with the hc2 DNA collection device (cervical brush and specimen transport medium [STM]). The DNA collection device is not to be used for specimen collection from pregnant women.
3. Fresh biopsy specimens (up to 5 mm) should be collected using 1 ml of STM.

B. Time of specimen collection

There are no particular timing issues to consider in collecting these specimens; however, the time and date of collection and specimen type should be recorded.

C. Specimen transport

1. Specimens collected using the DNA collection device are stable for up to 2 weeks at room temperature. If specimens are to be tested after that time, they can be stored for an additional week at 2 to 8°C. Specimens can be stored for up to 3 months prior to testing but must be stored at −20°C.
2. Biopsy specimens can be shipped overnight at 2 to 30°C but stored at −20°C until testing.
3. PreservCyt solution specimens are stable for up to 3 months at 2 to 30°C.

D. Specimen labeling and request submission

The laboratory should determine a mechanism to ensure that the specimen type and time of collection are recorded.

E. Rejection criteria

1. Test only those specimens collected using the hc2 DNA collection device or biopsy samples collected in 1 ml of STM.
2. There must be at least 4 ml of PreservCyt remaining for HPV testing after the Pap smear has been completed.
3. Biopsy specimens less than 2 mm in diameter should not be tested.
4. Request a repeat specimen, or obtain the information, when the specimen type and the collection time have not been specified.
5. Reject specimens in leaky containers.
6. If an improperly collected, transported, or handled specimen cannot be replaced, document in the final report that specimen quality may have been compromised.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents and materials provided

1. Indicator dye
2. Denaturation reagent
3. Probe diluent
4. High-risk HPV probe
5. Low-risk HPV QC
6. High-risk HPV QC
7. Negative calibrator
8. High-risk HPV calibrator
9. Capture microplate
10. Detection reagent 1
11. Detection reagent 2
12. Wash buffer concentrate

B. Materials required but not supplied

1. hc2 DNA collection device
2. Hybridization microplate
3. Microplate lids

III. MATERIALS *(continued)*

4. Empty microplate strips (model no. 2581; Costar); optional for use with automated plate washer
5. Extralong pipette tips for removal of specimen
6. Specimen collection tubes
7. Specimen collection tube screw caps
8. Disposable reagent reservoirs
9. DuraSeal tube sealer film
10. Hybridization microtubes

C. Equipment (for manual hc2 testing)

1. Digene hc2 system, consisting of a Digene-approved luminometer, computer, and software
2. Hybrid Capture System rotary shaker I
3. Hybrid Capture System microplate heater I
4. Hybrid Capture System automated plate washer
5. Hybrid Capture System multispecimen tube (MST) vortexer 2
6. Conversion rack and lid
7. Tube sealer dispenser and cutting device (used with MST vortexer 2)
8. Wash apparatus
9. Automated plate washer
10. Specimen collection tube rack
11. Specimen tube rack
12. Plate sealer

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Low-risk and high-risk controls must be included with each run.

B. The RLU/cutoff of each control must fall into the acceptable range for the assay to be considered valid.

HPV type	Expected result (RLU/cutoff value) with high-risk HPV probe		
	Minimum	Maximum	Average
Low-risk HPV type 6	0.001	0.999	0.5
High-risk HPV type 16	2	8	5.0

C. The control material will not act as an appropriate processing control if PreservCyt specimens are being tested.

D. Controls supplied in the kit are for internal QC use. Additional external controls can be purchased separately and used for supplemental QC.

V. PROCEDURE

◩ **NOTE:** This procedure addresses only specimens collected using the hc2 DNA collection device using the microplate format.

Observe standard precautions.

A. Reagent preparation

1. Denaturation reagent (prepare first)
   Add 5 drops of indicator dye to the bottle of denaturation reagent and mix thoroughly. This reagent is corrosive, so handle with care. Denaturation reagent is stable for 3 months at 2 to 8°C. If the color does fade, 3 drops of indicator dye can be added. Mix well before use in testing.
2. High-risk HPV probe mix (prepare during specimen denaturation incubation)
   a. Care should be taken during this step to prevent RNase contamination of probe and probe mix. Use pipette tips that have an aerosol barrier. Incomplete mixing during this step can result in false negatives.
   b. Centrifuge the vial of high-risk HPV probe briefly to get liquid to the bottom of the tube.
   c. Determine the amount of probe mix required (25 µl per test). Make a small amount of extra probe mix to account for some loss in pipetting.

V. PROCEDURE *(continued)*

d. Transfer the required amount of probe diluent to a new disposable container. Make a 1:25 dilution of high-risk HPV probe in probe diluent. See package insert for guidance in making dilutions.

e. Placing the pipette tip against the inner wall of the tube just above the meniscus. Pipette the high-risk HPV probe into the probe diluent. *Do not immerse the pipette tip.*

f. Vortex for 5 s. Label as high-risk HPV probe cocktail.

3. Wash buffer (prepare during capture step). Refer to the package insert for how to prepare the wash buffer (for example, 33.3 ml of wash buffer concentrate should be added to 966.7 ml of distilled deionized water for a final volume of 1 liter).

4. Detection reagents 1 and 2

Refer to the package insert for how to mix detection reagents 1 and 2. Do not return the reagents to their original bottles. These reagents are ready to use and can be mixed just prior to use.

B. Setup

1. Allow the microplate heater I to equilibrate to 65 ± 2°C (allow 60 min).

2. The water bath should be at 65°C.

3. Specimens and all reagents should reach room temperature before use (allow 15 to 30 min).

4. Create a plate layout for the specimens.

5. Controls, calibrators, and specimens should be plated in a test tube rack in the order that they will be tested on the microplate.

6. The high-risk control and negative control must be tested in triplicate with the high-risk probe cocktail.

C. Denaturation *(not for PreservCyt specimens; refer to package insert for detailed instructions)*

1. Remove caps from the calibrator, controls, and specimens. Caps can be discarded.

2. Using a adjustable or repeating pipettor, pipette denaturation reagent with indicator dye into each calibrator, control, or specimen. See table below.

Calibrator, control, or specimen	Volume of denaturation reagent required
Negative control	1,000 µl
High-risk HPV calibrator	500 µl
Low-risk or high-risk QCs	500 µl
Cervical specimen	500 µl

If using an Eppendorf Repeater pipette, use a 12.5-ml tip and a pipettor setting of 2.

3. Mix the specimens.

◩ **NOTE:** There is more than one method for mixing the specimens. The MST vortexer 2 method is described below. Specimens then must be hybridized using the hybridization microplate and microplate heater I method.

a. Cover the tubes with DuraSeal tube sealer film.

b. Place the rack lid over the covered tubes. Lock the lid in place using clamps. Cut the film with the cutting device. Make sure lid is secure.

c. The red-handled lever should be in the horizontal position.

d. Place the MST or Digene specimen rack and lid on the MST vortexer 2. The largest notched corner should be located in the right front corner. The red lever should now be in the down position.

e. The speed should be at 100 (maximum speed), and the pulser button should be set to OFF.

V. PROCEDURE *(continued)*

f. Turn the vortexer to ON.
g. Vortex the tubes for 10 s.
h. Turn the vortexer to OFF.
i. Lifting the red-handled lever, remove the rack from the vortexer.
j. Incubate in a 65 ± 2°C water bath for 45 ± 5 min. Specimens can be tested or stored; refer to package insert.
k. Begin preparation of a combined probe cocktail (CPC) or low-risk and high-risk HPV probe cocktails during this incubation.
l. Remove the rack from the water bath. Dry the rack and place securely on the vortexer.
m. Turn the vortexer to ON.
n. Vortex the tubes for 5 s.
o. Turn the vortexer to OFF. Remove the rack.
p. Remove the rack lid and DuraSeal sealer film immediately from all the specimens.

D. Hybridization (microplate heater I method described below—used with hc2 DNA collection device specimens in STM; refer to package insert for other options for other specimen types)

1. Some cervical specimens may contain blood or other material. This could mask the color changes upon addition of the probe mix. Care should be taken when testing these specimens, since the appropriate color change might not be apparent.
2. If denatured specimen has been refrigerated or frozen, equilibrate to 20 to 25°C. Vortex for 5 s on maximum speed.
3. Preheat the microplate heater I to 65 ± 2°C for 60 min. See operator's manual for additional instructions.
4. Obtain a hybridization microplate. Label accordingly.
5. Into the bottom of an empty microplate well, pipette 75 µl of each control, calibrator, or specimen, following the plate map set up earlier. Avoid touching the sides and creating any air bubbles. It is important to not cross-contaminate specimens. It is advisable that a clean extralong pipette tip be used for each transfer. Denatured specimens can be capped with specimen collection caps and stored.
6. Cover the plate with Parafilm or a plastic lid after transferring samples.
7. Incubate the hybridization microplate for 10 min at 20 to 25°C.
8. Aliquot the high-risk HPV probe mix (thoroughly vortexed) into a disposable reagent reservoir.
9. Pipette 25 µl of high-risk probe mix into each well using an eight-channel pipettor and fresh tips for each row. It is important to prevent back splashing and touching the sides of the wells.
10. Cover the hybridization microplate using a plate lid. Place the plate on the Hybrid Capture System rotary shaker I. The shaker should be set at 1,100 ± 100 rpm for 3 ± 2 min.
11. Controls, calibrators, and specimens should all turn yellow after shaking.
12. Wells remaining purple might not have received the appropriate amount of probe mix. An additional 25 µl of probe mix can be added to the purple wells. Shake again. If they still remain purple, specimens must be retested.
13. Incubate in the microplate heater I for 60 ± 5 min.

E. Hybrid capture

1. Remove enough capture microplate wells for testing. Number each column of wells 1, 2, 3, and so on. Label the microplate with an appropriate identifier. The plate map will dictate where samples are placed.
2. Remove the hybridization microplate containing all the calibrators, controls, and samples from the microplate heater I. Remove the plate lid and place on a clean surface.

V. PROCEDURE *(continued)*

3. Using an eight-channel pipettor, transfer the entire contents from the hybridization microplate to the capture microplate, using new pipette tips for each column.
4. Cover the microplate with the plate lid. Shake using the rotary shaker I at 1,100 ± 100 rpm and 20 to 25°C for 60 ± 5 min.
5. Prepare wash buffer.
6. Check the rinse and waste reservoirs if using the Hybrid Capture System automated plate washer I.
7. Remove the plate from the shaker and remove the liquid by discarding in a sink. Invert the plate to fully empty the liquid from the plate. Blot the plate before reinverting by tapping on clean Kimtowels or lint-free paper towels.
8. All liquid should be removed from the well.

F. Hybrid detection

1. Detection reagent should be aliquoted into a reagent reservoir. Next, pipette 75 µl of detection reagent into each well of the capture microplate. An eight-channel pipettor can be used. Refer to the package insert for the reverse pipetting technique, which is recommended at this step.
2. Cover the plate using the plate lid or Parafilm.
3. Incubate at 20 to 25°C for 30 to 45 min.

G. Washing (automated plate washer)

1. Remove the plate lid. Place the plate on the automated plate washer I platform.
2. Turn power on. Display should read "Digene Wash Ready."
3. Press ROWS (and then + or −) to select the number of strips to be washed. Press ROWS key to return to "Digene Wash Ready."
4. The washer will perform six fill and aspirate cycles. This will take ~10 min. When this is completed, the display will read "Digene Wash Ready."
5. The microplate should be removed from the washer when the program has completed the cycle. The plate should appear white. There should be *no* residual pink color or liquid in the microwell.

H. Signal amplification

1. Use a new pair of clean gloves when handling detection reagent 2.
2. Aliquot only the amount of detection reagent 2 needed. Do not place aliquoted reagent back into the bottle.
3. This step is very critical, and the incubation times of all the wells should be as close as possible.
4. Do not touch the sides of the microwell or splash the reagent back onto the tips. Cross-contamination can occur if this happens.
5. Pipette 75 µl of detection reagent 2 into each well of the capture microplate. An eight-channel pipettor can be used, maintaining the pipetting technique as described above. The microwells should all turn yellow and have similar intensities.
6. Cover with a plate lid or Parafilm and incubate at 20 to 25°C for 15 to 30 min. Avoid placing the plate in any direct sunlight.
7. Read the plate using the DML 2000 instrument after at least 15 min of incubation (but no longer than 30 min).
8. If a full microplate was not used, unused microwells can be removed from the microplate holder, rinsed, and used at a later date.

I. Assay calibration

Assay calibration verification is performed to make sure all is functioning properly. This occurs with each assay. The Hybrid Capture System version 2 and Digene qualitative software with version 4.01 DML 2000 assay protocols (or higher) for HPV automatically verify the criterion that is detailed in the package insert. Please refer to the package insert for more information about assay calibration verification criteria and cutoff calculation information.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Negatives should be reported as negative for high/intermediate-risk group HPV (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68).
B. Positives should be reported as positive for high/intermediate-risk group HPV (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68).
C. Document all testing in hard copy or computerized work card.

VII. INTERPRETATION

A. Specimens with RLU/cutoff ratios of $\geq$1.0 are considered positive.
B. Specimens with RLU/cutoff ratios of $<$1.0 are considered negative or not detected. High-risk subtype HPV DNA is either absent from the specimen or below the analytical sensitivity of the assay.
C. If PreservCyt specimens are used and the RLU/cutoff ratio is *$\geq$1.0 and $<$2.5, the specimen should be retested.* If upon retesting the result is positive (RLU/cutoff ratio of $\geq$1.0), the specimen can be reported as positive. If the retest is negative (RLU/cutoff ratio of $<$1.0), then a second retest (third test) needs to be performed to call the specimen negative.
D. Results of the test should be interpreted in conjunction with clinical information about the patient, which would include the Pap smear result. Institutions may have algorithms that combine HPV test results with Pap smear results in determining the follow-up management of the patient.

VIII. LIMITATIONS OF TESTING

A. The Digene Hybrid Capture HPV DNA assay procedure, QC, and the interpretation of specimen results must be followed closely to obtain reliable results.
B. The hc2 high-risk HPV DNA test is not recommended in cases of sexual abuse or sexual assault, since patients can be positive for HPV prior to a sexual abuse event.
C. The positive predictive value of a test can decrease if the prevalence of infection in a specific population is low.
D. Test performance characteristics with specimens other than cervical swabs, biopsy samples, and PreservCyt have not been determined.
E. Biopsy specimens $<$2 mm in diameter should not be tested.
F. It is important to pipette the exact reagent volume indicated and to mix well after each reagent addition. Failure to do so could result in erroneous test results. Ensuring that the noted color changes occur will help to confirm that these conditions have been met.
G. A negative result does not exclude the possibility of HPV infection, since very low levels of infection or sampling error may cause a false-negative result.
H. The hc2 DNA collection device should not be used in women that are pregnant.
I. A positive hc2 test does not mean that the woman has cancer or will develop cancer. Most women infected with a high-risk type will not go on to develop cancer.
J. A negative hc2 test does not preclude future development of cancer.
K. There is some cross-reactivity with low-risk HPV types 6 and 42 and the high-risk HPV probe.
L. The presence of contraceptive jelly, antifungal cream, or douche at the time of specimen collection can interfere with the assay and can cause false negatives, especially if the specimen contains an amount of HPV that is right around the cutoff.
M. A visible pellet must be observed after centrifugation when processing PreservCyt specimens. If a pellet is not visible, false-negative results could occur.

VIII. LIMITATIONS OF TESTING *(continued)*

N. It is not advisable to process more than 36 PreservCyt specimens at once.

O. The denaturation step of the hc2 procedure is very important, and it is advised that the procedure not be deviated from. Failure to follow protocol during the denaturation step can lead to false-positive test results. It is very important for the micropipette tip to not touch the sides of the specimen denaturation tube during the transfer of denatured specimen to the microplate.

REFERENCE

1. **Qiagen/Digene Corporation.** Hybrid Capture® 2 (hc2) HPV DNA Test package insert, 5199-1220(L2290Rev2). Qiagen/Digene Corporation, Gaithersburg, MD.

SUPPLEMENTAL READING

Cope, J. U., A. Hildesheim, M. H. Schiffman, M. M. Manos, A. T. Lorincz, R. D. Burk, A. G. Glass, C. Greer, J. Buckland, K. Helgesen, D. R. Scott, M. E. Sherman, R. J. Kurman, and K. L. Liaw. 1997. Comparison of the Hybrid Capture tube test and PCR for detection of human papillomavirus DNA in cervical specimens. *J. Clin. Microbiol.* **35:**2262–2265.

Cox, J. T. 2009. History of the use of HPV testing in cervical screening and in the management of abnormal cervical screening results. *J. Clin. Virol.* **45**(Suppl. 1)**:**S3–S12.

Cox, J. T., A. T. Lorincz, M. H. Schiffman, M. E. Sherman, A. Cullen, and R. J. Kurman. 1995. Human papillomavirus testing by hybrid capture appears to be useful in triaging women with a cytologic diagnosis of atypical squamous cells of undetermined significance. *Am. J. Obstet. Gynecol.* **172:**946–954.

Moore, K. N., and J. L. Walker. 2004. High-risk human papillomavirus testing: guidelines for use in screening, triage, and follow-up for the prevention and early detection of cervical cancer. *J. Natl. Compr. Canc. Netw.* **2:**589–596.

Schiffman, M. H., N. B. Kiviat, R. D. Burk, K.V. Shah, R. W. Daniel, R. Lewis, J. Kuypers, M. M. Manos, D. R. Scott, M. E. Sherman, R. J. Kurman, M. H. Stoler, A. G. Glass, B. B. Rush, I. Mielzynska, and A. T. Lorincz. 1995. Accuracy and interlaboratory reliability of human papillomavirus DNA testing by hybrid capture. *J. Clin. Microbiol.* **33:**545–550.

Stillman, M. J., S. P. Day, and T. E. Schutzbank. 2009. A comparative review of laboratory-developed tests utilizing Invader HPV analyte-specific reagents for the detection of high-risk human papillomavirus. *J. Clin. Virol.* **45**(Suppl. 1)**:**S73–S77.

PART 3

BD Affirm VPIII Microbial Identification Test for Detecting *Gardnerella vaginalis, Candida* spp., and *Trichomonas vaginalis*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The Affirm VPIII assay is a moderately complex test to aid in the diagnosis of vaginitis in women, which is a significant health problem. The three main causes of vaginitis are bacterial vaginosis (BV), yeast vaginitis (candidiasis), and *Trichomonas vaginalis* (trichomoniasis) (2). BV alone can cause 15 to 50% of the vaginitis cases, depending on the patient populations being tested (3). BV and *Trichomonas* have been linked to both preterm labor and preterm birth in pregnant women. Even though BV is considered a more complex disease not just caused by *Gardnerella*, the principle of the Affirm test is that it is a nucleic acid (NA) hybridization test detecting the three following organisms: *Gardnerella vaginalis*, *Candida albicans*, and *Trichomonas vaginalis*.

The assay uses two single-stranded NA probes distinct for each of the three organisms. The two probes consist of a capture probe and a color development probe. These probes are complementary to genetic sequences that are unique to the target organism. A probe analysis card (PAC) and a multiwell reagent cassette (RC) are employed in the assay. The PAC contains the capture probes, which are immobilized on a bead. There is a separate bead for each organism. The RC contains the color development probes. Initially in the reaction, a sample lysis occurs and NA from the organism is released into solution. The NA is stabilized, and then the sample is added to the RC along with the PAC. After the addition of the NA, an automated process begins on the BD MicroProbe processor, where hybridization and color development occur. For expanded information, the reader is referred to the package insert (1).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

A vaginal specimen should be collected via the BD Affirm VPIII ambient-temperature transport system (ATTS). Specimen collection is outlined below.

1. The outer seal on the transport system should be opened to remove components.
2. Open up the pouch containing the ATTS reagent dropper.
3. Pressing firmly, break the ampoule in the ATTS reagent dropper. Break close to the center. Once it is broken, do not manipulate the ampoule; the plastic could cause injury.
4. Take the sample collection tube (SCT) and add the reagent from the ATTS reagent dropper.
5. Collect a patient specimen using the swab provided in the kit.
   a. Collect a vaginal specimen using a nonlubricated (without jelly or water) speculum.
   b. Obtain the specimen from the posterior vaginal fornix. The entire surface of the swab should touch the vaginal wall (twist or roll the swab two or three times against the wall). When removing the swab, swab the lateral vaginal wall.
6. The swab should be placed in the SCT containing the ATTS reagent as soon as possible.
7. Break the shaft at the prescored line. Discard any remaining portion of the shaft into a biohazard waste container.
8. Label the collection tube with patient information and the time and date the specimen was collected.

B. Time of specimen collection

There are no specified times for specimen collection.

C. Specimen transport

The transport system described above must be used if the specimen is going to be tested *past 1 h* for samples stored at 15 to 30°C or *past 4 h* for samples stored at 2 to 8°C. If the transport system is not used and specimens are held longer than 1 h at room temperature or longer than 4 h at 2 to 8°C, false-positive results could occur.

D. Specimen labeling and request submission

The laboratory should determine a mechanism to ensure that the specimen type and time of collection are recorded.

E. Rejection criteria

1. Sources other than vaginal
2. Specimens sent at 2 to 8°C or 15 to 30°C greater than 72 h old
3. Swabs other than those provided in the BD Affirm VPIII ATTS

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents (BD Affirm VPIII Microbial Identification Test)

1. PACs
   Each card will contain the following five beads.
   a. Negative control
   b. *Trichomonas*
   c. *Gardnerella*
   d. *Candida*
   e. Positive control
2. RC
   The RC wells contain, from front to back, the following.
   a. Well 1: patient sample reservoir (will be empty)
   b. Well 2: hybridization solution (350 µl), consisting of color development probe, formamide, and buffered chaotropic solution
   c. Well 3: wash solution (750 µl), consisting of detergent, buffer solution, and preservative (Proclin)
   d. Well 4: conjugate (500 µl), consisting of enzyme conjugate and preservative (Proclin)
   e. Well 5: wash solution (750 µl), consisting of detergent, buffer solution, and preservative (Proclin)

III. MATERIALS *(continued)*

f. Well 6: wash solution (750 µl), consisting of detergent, buffer solution, and preservative (Proclin)
g. Substrate buffer (500 µl), consisting of buffered peroxide solution
3. Substrate solution
4. Lysis solution
5. Buffer solution

B. Supplies
1. BD Affirm VPIII ATTS
a. Prescored, sterile swab
b. SCT
c. Sample collection cap
d. ATTS reagent dropper (in foil pouch)
2. Pipette (for dispensing 120 test kit lysis solution and buffer solution)
3. Filter tips (FTs)
4. Sample collection caps
5. Sterile swabs

C. Equipment
1. BD MicroProbe processor
2. BD MicroProbe lysis block
3. Thermometer

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. The assay includes a positive and negative internal control located on each PAC. Results can be reported only if the positive control is blue and the negative control is colorless.

B. Each reagent lot and shipment should be tested for adequate specimen lysis and release of target NA. Since *C. albicans* is more difficult to lyse than *Trichomonas* or *Gardnerella,* lysis control with *C. albicans* is the only test that needs to be performed to ensure adequate lysis. *C. albicans* ATCC strain 18804, 14053, 10231, or 60193 can be used for QC. Obtain 18- to 24-h growth of QC culture for testing.

C. QC should be recorded on a log sheet.

D. To further test the assay performance, additional QC can be conducted with commercially prepared swabs or fresh indicator cultures (*C. albicans* ATCC 10231, *T. vaginalis* ATCC 30001, or *G. vaginalis* ATCC 14018). If additional QC is used, then the following will apply.
1. If all three organism beads turn blue, patient results can be reported.
2. The entire QC is invalid if the *C. albicans* bead does not turn blue. This means that no patient results can be reported.
3. If the *Gardnerella* and *Candida* beads turn blue and the *Trichomonas* bead does not, this means that the *Trichomonas* QC is invalid. Patient results can be reported for *Gardnerella* and *Candida.* The run must be investigated and the result for *Trichomonas* cannot be reported.
4. If the *Trichomonas* and *Candida* beads turn blue and the *Gardnerella* bead does not, this means that that *Gardnerella* QC is invalid. Patient results can be reported for *Trichomonas* and *Candida.* The run must be investigated and the results for *Gardnerella* cannot be reported.

E. QC must be performed in accordance with applicable state and/or federal regulations or other laboratory accreditation requirements and individual laboratories' applicable QC requirements.

V. PROCEDURE

Observe standard precautions.

A. Sample preparation
1. The BD MicroProbe lysis block should be set at 85 ± 5°C. The reagents should be at 22 to 28°C and well mixed.
2. The SCT should be uncapped, with the shaft firmly embedded in the cap. Pipette 0.4 ml or add 12 drops of lysis solution to the SCT. If drops are being added, hold the dropper bottle vertical for best results.
3. Move the swab up and down against the side of the tube to mix. Do this for approximately 10 s.
4. Leave the swab in the tube and cap the tube.

V. PROCEDURE *(continued)*

5. Place the tube into the lysis block to heat.
6. Incubate for between 10 and 20 min in the lysis block. The time should not exceed 20 min.
7. Remove sample from the lysis block.
8. Pipette 0.6 ml or add 12 drops of well-mixed buffer solution to the tube with the swab. The tip of the bottle should *not* touch the patient sample tube.
9. Cap the tube and flick the tube 10 times to adequately mix the contents.
10. The swab should now be removed from the tube. As much liquid as possible should be expressed from the tube by pressing the swab against the wall of the tube. This should be done for at least 10 s. Dispose of the swab in a biohazard waste container. Press an FT firmly onto each SCT. This constitutes a prepared specimen.

    ◩ **NOTE:** Prepared specimens must be tested within 24 h. Specimens can remain at room temperature until being tested.

B. Automated processing

1. Reagents and the testing environment should be at 22 to 28°C.
2. The BD MicroProbe processor should be in the off position before proceeding. Next, the Affirm VPIII Microbial Identification Test program card is placed (printed side up/arrow pointing towards the instrument) into the slot on the front right side of the instrument.
3. The processor should be turned on. As the procedure is followed the operator should follow the prompts on the processor display. The HELP key on the processor can be pressed at any time for additional help if needed.
4. The cassette caddy should be removed from the processor for ease in loading the samples.
5. For each sample to be tested, select one RC. Label each RC with patient information using a permanent marker on the front end of the RC. The foil covering must be removed from each RC, lifting the foil from the end that does not have the upward-bent flap. When loading the cassette caddy, begin loading from the center and then balance each side with cassettes.
6. Remove the PAC from pouch and label with patient information on the space provided.
7. Press the RUN key on the instrument. The instrument will prompt the operator to “Add Substrate.” Four drops (or 0.1 ml) of substrate solution is added to well 7 of the RC.
8. The substrate bottle cap should be closed.
9. Press the RUN key on the instrument. The instrument will prompt the operator to “Add Sample.” Match up each RC with the previously prepared SCT/FT. To express the sample through the FT, invert the SCT and squeeze firmly. The entire contents of the sample should go through the FT into the reservoir of well 1 of the appropriate RC. Foam at the tip of the filter is a good sign that the sample has been added.
10. Discard the patient sample in a biohazard waste container.
11. Press the RUN key on the instrument. The instrument will prompt the operator to “Place PAC.” A labeled PAC should be added to well 1 of the corresponding labeled RC. The bead should not be touched by the operator.
12. Press the RUN key on the instrument. The instrument will prompt the operator to “Place Caddy.” The cassette caddy should be replaced on the processor, with care not to splash reagents. The caddy needs to be securely seated on all four locater pins.
13. Press the RUN key on the instrument. The processor arm will automatically move the PAC through the testing procedure. After the appropriate processing time, the instrument will present the PAC for removal.

V. PROCEDURE *(continued)*

14. After test run is completed, the PAC should be removed, with gentle blotting of the PAC with a paper towel. Interpret results as soon as possible after completion of the test.

15. View the PAC against a white background, under normal lighting intensity.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report results as detected or not detected for *G. vaginalis, Candida,* or *T. vaginalis* DNA.

B. A comment can be added explaining the clinical significance of the finding (e.g., "The presence of *G. vaginalis* DNA is indicative of bacterial vaginosis").

C. Document the testing according to institutional guidelines either by hard copy or by the laboratory information system.

VII. INTERPRETATION

A. A positive result in the test system is interpreted by the test bead showing any visible color blue. If the target organism bead is absent of any visible blue color (colorless), then the result for the target organism is negative.

B. A positive result for *Candida* means that nucleic acid for *Candida* species *(C. albicans, C. glabrata, C. kefyr, C. krusei, C. parapsilosis,* or *C. tropicalis)* is present, and therefore, the patient has candidiasis. Similarly, a positive result for *Gardnerella* or *Trichomonas* means that the patient has either BV or trichomoniasis, respectively. Mixed infections can occur.

C. A negative result suggests that the patient does not have candidiasis, BV, or trichomoniasis. A negative test does not rule out the presence of vaginosis/vaginitis in a sample in which clinical evaluation is warranted.

VIII. LIMITATIONS OF TESTING

A. The Affirm VPIII sample collection and processing procedure is to be used in conjunction with the Affirm VPIII Microbial Identification Test. Only swabs from the Affirm VPIII sample collection set can be used with the Affirm Microbial Identification Test.

B. Once the specimen is collected, it should be added to the SCT containing the ATTS reagent. The ATTS reagent should placed in the SCT immediately prior to specimen collection and not sooner.

C. Optimal results are seen when the specimens are held no longer than 72 h at ambient temperature (15 to 30°C) or refrigerated (2 to 8°C). If these conditions are not met, false-positive results can occur.

D. Since the analytical sensitivity of the assay varies per organism (1×10^4 *Candida* cells, 2×10^5 CFU of *G. vaginalis,* or 5×10^3 trichomonads), a negative result could mean that the organism is present but at a level below the analytical sensitivity of the assay.

E. The assay detects *G. vaginalis* at greater than 2×10^5 CFU per patient sample. The clinical significance of this has not been evaluated. *G. vaginalis* is a common organism that can be found in women that do not have BV/vaginitis. The presence of *G. vaginalis* can be suggestive of but not diagnostic for BV/vaginitis. As with other diagnosis, a single laboratory test should be used in conjunction with other diagnostic criteria.

F. The performance of this test is based on the use of vaginal fluid. Other specimens have not been evaluated.

G. *Cryptococcus neoformans* at concentrations greater than 10^8 yeasts/ml react with the Affirm VPIII *Candida* probe. *C. neoformans* is rarely encountered in the vagina.

VIII. LIMITATIONS OF TESTING *(continued)*

H. *Mobiluncus mulieris* ($<4 \times 10^6$ bacteria/ml) and *Bifidobacterium dentium* ($>8 \times 10^5$ bacteria/ml) may react nonspecifically with the Affirm *G. vaginalis* probe.

I. The Affirm VPIII Microbial Identification Test is not validated for use as test of cure following antimicrobial therapy.

REFERENCES

1. **Becton Dickinson and Co.** 2005. BD Affirm VPII Microbial Identification Test package insert, 670160JAA. Becton Dickinson and Co., Sparks, MD.
2. **Kent, H. L.** 1991. Epidemiology of vaginitis. *Am. J. Obstet. Gynecol.* **165**(4 Pt. 2)**:**1168–1176.
3. **Sobel, J. D.** 1990. Vaginal infections in adult women. *Med. Clin. N. Am.* **74:**1573–1602.

SUPPLEMENTAL READING

Briselden, A. M., and S. L. Hillier. 1994. Evaluation of Affirm VP microbial identification. *J. Med. Microbiol.* **32:**148–152.

Brown, H. L., D. D. Fuller, L. T. Jasper, T. E. Davis, and J. D. Wright. 2004. Clinical evaluation of Affirm VPIII in the detection and identification of Trichomonas vaginalis, Gardnerella vaginalis, and Candida species in vaginitis/vaginosis. *Infect. Dis. Gynecol.* **12:**12–17.

Witt, A., L. Petricevic, U. Kaufmann, H. Gregor, and H. Kiss. 2002. DNA hybridization test: rapid diagnostic tool for excluding bacterial vaginosis in pregnant women with symptoms suggestive of infection. *J. Clin. Microbiol.* **40:**3057–3059.

12.2.3 Amplification-Based Methods

Molecular methods based on nucleic acid amplification continue to assume greater importance in the clinical microbiology lab. PCR in particular allows the sensitive identification and accurate quantification of a wide variety of pathogens. The introduction of technologies that permit detection of amplicons upon production, that is, in "real time," has enhanced the utility of PCR. By eliminating the need to open reaction vessels for subsequent analysis, real-time PCR greatly reduces the risk of amplicon contamination. Other benefits include ease of quantification, reduced turnaround time, and amenability to automation.

Many real-time PCR assays employ fluorescently labeled oligonucleotide probes. When these probes hybridize to targets, fluorescence results from the transfer of energy between fluorophores (hybridization probes) or separation of reporter and quencher fluorophores (hydrolysis probes), among other variations. Special thermal cyclers allow the detection of the fluorescence emitted. By measuring differences in probe binding affinities (melting curve analysis), targets can be discriminated; by determining at which reaction cycle product becomes detectable, targets can be quantified.

Besides the refinements of amplification-based methods, advances such as nucleic acid extraction robots requiring little hands-on time have eased the implementation of molecular testing for infectious diseases.

SUPPLEMENTAL READING

Ferreira-Gonzalez, A., and A. M. Caliendo. 2006. Molecular methods in diagnosis and monitoring of infectious diseases, p. 1555–1587. *In* C. A. Burtis, E. R. Ashwood, and D. E. Bruns (ed.), *Tietz Textbook of Clinical Chemistry and Molecular Diagnostics*, 4th ed. Elsevier Saunders, St. Louis, MO.

Hayden, R. T. 2004. In vitro nucleic acid amplification techniques, p. 43–69. *In* D. H. Persing, F. C. Tenover, J. Versalovic, Y.-W. Tang, E. R. Unger, D. A. Relman, and T. J. White (ed.), *Molecular Microbiology: Diagnostic Principles and Practice*. ASM Press, Washington, DC.

Holland, C. A., and F. L. Kiechle. 2005. Point-of-care molecular diagnostic systems—past, present and future. *Curr. Opin. Microbiol.* **8:**504–509.

Mackay, I. M. 2004. Real-time PCR in the microbiology laboratory. *Clin. Microbiol. Infect.* **10:**190–212.

Mothershed, E. A., and A. M. Whitney. 2006. Nucleic acid-based methods for the detection of bacterial pathogens: present and future considerations for the clinical laboratory. *Clin. Chim. Acta* **363:**206–220.

PART 1

Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in Urogenital Specimens Using the Gen-Probe APTIMA Combo 2 Assay

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

There are currently five manufacturers in the United States of nucleic acid-based tests for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae:* hybrid capture, a signal amplification method, and five other target amplification assays (Tables 12.1–1 and 12.1–2). A more detailed analysis of the test systems available is presented in Table 12.2.3–1. The procedure described herein features the Gen-Probe APTIMA Combo 2 assay and is intended to provide the reader with a general outline of the methodology and work flow; it does not represent the full operational detail provided by the manufacturer. For the most up-to-date information, the reader is advised to consult the manufacturer and to obtain a package insert (6).

Table 12.2.3–1 Commercially available, FDA-cleared *Chlamydia trachomatis* and *Neisseria gonorrhoeae* tests[a]

Manufacturer	Method	Test name(s)	Specimen type(s)	Inhibition control	Automation
Abbott Molecular, Inc., Des Plaines, IL	Real-time PCR	RealTime CT/NG	Female Vaginal swab FCU[b] Male Urethral swab FCU	Yes	*m*2000sp—sample preparation *m*2000rt—amplification and detection
Becton, Dickinson and Co., Sparks, MD	SDA[c]	BD ProbeTec ET *C. trachomatis* and *N. gonorrhoeae* Amplified DNA Assay BD ProbeTec ET *C. trachomatis* Amplified DNA Assay	Female Cervical swab FCU Male Urethral swab FCU	Yes[d]	Manual operation *or* Viper—some offboard sample preparation, automation of amplification and detection
Qiagen, Gaithersburg, MD	Hybrid capture	CT/GC HC2 DNA Test CT-ID HC2 DNA Test	Female Cervical brush or swab	No	Manual operation *or* Rapid capture system—automates sample processing
Gen-Probe Inc., San Diego, CA	TMA	APTIMA Combo 2 Assay APTIMA GC Assay APTIMA CT Assay	Female Cervical swab Vaginal swab FCU ThinPrep[e] Male Urethral swab FCU	No	Manual operation *or* Direct tube sampling system (DTS 400, 800, 1600)—automates sample handling *or* Tigris—full automation of sample processing, amplification and detection
Roche Molecular Systems, Inc., Branchburg, NJ	PCR	AMPLICOR CT/NG Test for *Chlamydia trachomatis* COBAS AMPLICOR CT/NG Test for *Neisseria gonorrhoeae*	Female Cervical swab FCU[f] ThinPrep[g] Male Urethral swab FCU	Yes	Manual operation *or* COBAS AMPLICOR—some offboard sample preparation, automation of amplification and detection

[a] This table contains examples of FDA-cleared or -approved nucleic acid amplification tests that are commercially available in the United States. It is not intended to be all-inclusive. Websites of the manufacturers are useful sources of the most up-to-date information.

[b] FCU, first-catch urine.

[c] SDA, strand displacement amplification.

[d] Inhibition control is optional with manual assay performance but cannot be run on Viper platform.

[e] ThinPrep PreservCyt Liquid PAP medium for collection of cervical cells.

[f] Roche PCR is not cleared for *Neisseria gonorrhoeae* testing on female urine.

[g] Clearance granted to Cytyc Corporation, Boxborough, MA, not Roche Molecular Systems, Inc.

The Gen-Probe APTIMA Combo 2 assay is based on the amplification chemistry called transcription-mediated amplification (TMA). TMA is an isothermal RNA transcription amplification system using two enzymes, RNA polymerase and reverse transcriptase. TMA can amplify either DNA or RNA and produces RNA amplicons, in contrast to most other nucleic acid amplification methods, which produce only DNA. The APTIMA assay further combines the sample processing procedure of target capture with a product detection chemistry called dual kinetic assay (DKA).

In the APTIMA assay, swab or urine specimens are collected and transferred into their respective specimen transport devices. The transport solutions release the rRNA targets and protect them from degradation. Target rRNA molecules are isolated from the specimens using capture oligomers (target capture). The TMA reaction replicates a specific region of the 23S rRNA from *C. trachomatis* and a specific region of the 16S rRNA from *N. gonorrhoeae* via DNA intermediates. Detection of the rRNA amplicons is achieved using nucleic acid hybridization. Single-stranded chemiluminescent DNA probes, which are complementary to a region of each target amplicon, combine with amplicons to form stable RNA:DNA hybrids. The selection reagent differentiates hybridized from unhybridized probe, and then, during the detection step, light emitted from the labeled RNA:DNA hybrids is measured as photon signals in a luminometer and is reported as relative light units (RLU). In DKA, differences in the kinetic profiles of the *C. trachomatis* and *N. gonorrhoeae* labeled probes allow for the differentiation of signal; kinetic profiles are derived from measurements of photon output during the detection read time.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

Specimens must be obtained using APTIMA collection kits.

1. Endocervical swab specimens
   - **a.** Remove excess mucus from the cervical os and surrounding mucosa using the cleaning swab. Discard this swab.
   - **b.** Insert the specimen collection swab into the endocervical canal.
   - **c.** Gently rotate the swab clockwise for 10 to 30 s in the endocervical canal. Withdraw the swab carefully; avoid any contact with the vaginal mucosa.

2. Male urethral swab specimens
   - **a.** The patient should not have urinated for at least 1 h prior to sample collection.
   - **b.** Insert the specimen collection swab 2 to 4 cm into the urethra. Gently rotate the swab clockwise for 2 to 3 s in the urethra to ensure adequate sampling.

3. Vaginal swab specimens
   - **a.** Insert the swab into the vagina about 2 in. past the introitus and gently rotate the swab for 10 to 30 s. Make sure the swab touches the walls of the vagina so that moisture is absorbed by the swab. Withdraw the swab without touching the skin.
   - **b.** All swab specimens should be inserted into the respective transport device and broken at the score line. Recap the transport tube tightly.

4. First-catch urine
   - **a.** Approximately 20 to 30 ml of the initial urine stream should be collected into a clean collection cup. The patient should not have urinated for at least 1 h prior to specimen collection. Female patients should not cleanse the labial area prior to providing the specimen.
   - **b.** Remove the cap and transfer 2 ml of urine into the urine specimen transport tube using the disposable pipette provided. The correct volume of urine has been added when the fluid level is between the black fill lines on the urine specimen transport tube label.
   - **c.** Recap the urine specimen transport tube tightly. This is now known as the processed urine specimen.

5. PreserveCyt

◩ **NOTE:** The APTIMA specimen transfer kit allows for APTIMA assay testing of gynecological specimens collected and processed with the Cytyc ThinPrep 2000 System. To prevent potential cross-contamination during Pap

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

smear processing, a specific contamination mitigation procedure must be followed. Refer to the instructions provided with the APTIMA specimen transfer kit and the ThinPrep 2000 operator's manual (7, 8).

a. Vortex the PreservCyt vial for 3 to 10 s. Within 1 min of vortexing, transfer 1 ml of the processed PreservCyt specimen into the APTIMA specimen transfer tube.

b. Dispose of the pipette tip in a container of 0.5% sodium hypochlorite in distilled water.

c. Recap the APTIMA specimen transfer tube tightly, and gently invert the tube two or three times to ensure complete mixing. This specimen is referred to as a processed liquid Pap specimen.

B. Time of specimen collection

There are no particular issues related to timing of collection of female swab specimens. For male urethral swabs and urine samples, the patient should not have voided within 1 h of collection.

C. Specimen transport

1. Swabs

Transport and store the swab in the swab specimen transport tube at 2 to 30°C until testing. Specimens must be assayed with the APTIMA assay within 60 days of collection. If longer storage is needed, store at −20 to −70°C for up to 90 days after collection.

2. Urine

After collection, transfer the urine sample into the APTIMA urine specimen transport tube within 24 h of collection. Transport the processed urine specimens in the Gen-Probe APTIMA urine specimen transport tube at 2 to 30°C and store at 2 to 30°C until testing. Processed urine specimens should be assayed with the APTIMA assay within 30 days of collection. If longer storage is needed, freeze at −20 to −70°C for up to 90 days after collection.

3. Specimens can be stored in Cytyc PreservCyt vials for 30 days at 2 to 30°C prior to Pap processing and transfer to APTIMA specimen transfer tubes. Once transferred into the APTIMA specimen transfer tube, the specimen must be tested within 30 days when stored at 2 to 8°C or 14 days when stored at 15 to 30°C. If longer storage is needed, freeze at −20 to −70°C for up to 90 days after transfer.

D. Specimen labeling and request submission

The laboratory should determine a mechanism to ensure that the specimen type and time of collection are recorded.

E. Rejection criteria

1. Test only the indicated specimen types in the indicated transport medium. Testing of other specimen types or use of other transport media must be validated by the user prior to reporting results (4).

2. Swab specimen transport tubes containing no swab, two swabs, or a swab not supplied by the manufacturer may give inaccurate results and should be rejected.

3. Urine specimens that have been frozen or are greater than 24 h old are unacceptable for testing. Urine in a transport device that is not within the collection lines printed on the tube should be rejected.

4. Reject specimens that arrive in broken or leaky containers.

5. *In cases of suspected sexual abuse, both an amplification-based and a culture-based method should be considered.*

6. If an improperly collected, transported, or handled specimen cannot be replaced, testing may be considered after consultation with health care provider. Document in the final report that specimen quality may have been compromised.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Materials provided

1. APTIMA Combo 2 assay kit (three boxes)
- **a.** Refrigerated box (2 to 8°C)
  - (1) APTIMA Combo 2 amplification reagent
  - (2) APTIMA Combo 2 enzyme reagent
  - (3) APTIMA Combo 2 probe reagent
  - (4) APTIMA Combo 2 target capture reagent B (TCR-B)
- **b.** Nonrefrigerated box (15 to 30°C)
  - (1) APTIMA Combo 2 amplification reconstitution solution
  - (2) APTIMA Combo 2 enzyme reconstitution solution
  - (3) APTIMA Combo 2 probe reconstitution solution
  - (4) APTIMA Combo 2 selection reagent
  - (5) APTIMA Combo 2 target capture reagent (TCR)
  - (6) APTIMA wash solution
  - (7) APTIMA buffer for deactivation fluid
  - (8) APTIMA oil reagent
  - (9) Collars
  - (10) Sealing cards (one package)

2. Control kit (2 to 8°C)
- **a.** APTIMA positive control, CT/negative control, GC
- **b.** APTIMA positive control GC/negative control, CT

B. Materials required but not provided

1. APTIMA collection kits
- **a.** Unisex swab specimen collection kit for endocervical and urethral swab specimens
- **b.** Urine specimen collection kit for male and female urine specimens
- **c.** Vaginal swab specimen collection kit
- **d.** Specimen transfer kit (for PreservCyt)

2. Gen-Probe target capture system (TCS)

3. Gen-Probe LEADER HC+ luminometer

4. APTIMA autodetect kit

5. Two repeat pipettors

6. Repeat pipettor tips (2.5, 5.0, and 25.0 ml)

Either

7. Two multitube vortex mixers

8. Three circulating water baths (62 ± 1, 42 ± 1, and 62 ± 1°C)

9. Three water bath inserts

Or

10. Two SB100 dry heat bath/vortexers

11. Micropipettors (20 to 200 µl)

12. Tips, Pipetman P1000 style, APTIMA Combo 2

13. Tips, 1,000 µl conductive, liquid sensing, TECAN 10612513

14. Pipette tips (20 to 200 µl)

15. Bleach, 5 to 7% (0.7 to 1.0 M) sodium hypochlorite solution

16. Large capped plastic container

17. Standard urine collection containers, without preservatives

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

The reader is referred to Clinical and Laboratory Standards Institute document MM3-A2 for a useful and complete review of guidelines for QC in molecular infectious disease diagnostics (3).

A. The APTIMA positive control *C. trachomatis*/negative control *N. gonorrhoeae* and the APTIMA positive control *N. gonorrhoeae*/negative control *C. trachomatis* act as controls for the target capture, amplification, and detection steps of the assay and must be included with each test run. The *C. trachomatis* and *N. gonorrhoeae* positive control materials are noninfectious RNA containing sequences specific for each organism. The *C. trachomatis* positive control serves as the *N. gonorrhoeae* negative control; the *N. gonorrhoeae* positive control serves as the *C. trachomatis* negative control. No inhibition control is provided due to the use of target capture for sample processing.

IV. QUALITY CONTROL *(continued)*

B. The APTIMA assay software automatically evaluates the controls according to the criteria below and will report the run status.

1. The run status will be reported as PASS if the run control criteria are met and FAIL if the run control criteria are not met.

2. If the run status is FAIL, all test results in the same run are invalid and must not be reported.

3. The positive controls must produce the following test results.

Control	Total RLU (10^3)	*C. trachomatis* result	*N. gonorrhoeae* result
Positive control *C. trachomatis*/negative control *N. gonorrhoeae*	≥100 and <3,000	Positive	Negative
Positive control *N. gonorrhoeae*/negative control *C. trachomatis*	≥150 and <3,000	Negative	Positive

C. External controls

For all amplified molecular assays, it is advisable to test external control material to monitor assay performance. The controls provided in the APTIMA assay and most commercial kits are nucleic acid sequences and do not represent whole organisms. Therefore, they do not represent specimen matrices, such as urine or cervical cells, containing infectious organisms. To adequately control for all steps of the testing, including specimen processing and extraction, an additional whole-organism control in a suitable matrix is advisable. These controls should be taken through all steps of the procedure.

Positive patient specimens can be used for control material. There are also a number of companies that manufacture whole-organism controls for use as positive external controls. Such controls should be at concentrations in the low range of the assay to challenge assay sensitivity and so as not to serve as a source of contamination.

D. Contamination

Contamination may occur at any step in the testing process, so care must be taken to prevent such occurrences. Regular decontamination of work surfaces and instruments should be performed using a buffered bleach solution. Maintaining unidirectional work flow is also an important part of contamination control. Any unexpected positive results for control samples or environmental monitoring should be addressed by decontamination. Laboratory contamination monitoring should be performed as described in the package insert. If monitoring reveals positive results, further decontamination efforts should be undertaken.

E. Positivity rate

In order to avoid reporting false-positive results due to contamination, it is advisable to set up a system in the laboratory whereby data are collected to determine the prevalence of each pathogen and hence the number of positive results to be expected per day, per run, or by some other appropriate parameter.

V. PROCEDURE

A. Equipment preparation

◩ **NOTE:** The procedure below is for the direct tube sampling systems using water baths (as opposed to heat blocks). If the SB100 dry heat bath/vortexer is in use, refer to the SB100 application sheet. A separate procedure for the fully automated Tigris is also provided in the Gen-Probe APTIMA Combo 2 package insert.

1. Adjust one water bath to 62 ± 1°C (for target capture and primer annealing), a second water bath to 42 ± 1°C (for amplification), and a third water bath to 62 ± 1°C (for DKA).
2. Prior to starting the assay, wipe down work surfaces and pipettors with 2.5 to 3.5% (0.35 to 0.5 M) sodium hypochlorite. Allow the bleach to contact surfaces and pipettors for at least 1 min, and then follow with a water rinse. Do not allow the bleach to dry. Cover the bench surface on which the test will be performed with clean, plastic-backed absorbent laboratory bench covers.
3. Place a sufficient number of Ten Tip cassettes into the TCS. Ensure that the TCS wash bottle is filled with APTIMA wash solution and the aspirator is connected to the vacuum pump.

B. Reagent reconstitution

Reagent reconstitution should be performed prior to beginning specimen transfer.

1. Reconstitution of the APTIMA Combo 2 enzyme, amplification, and probe reagents
   - **a.** Pair the appropriate reconstitution solution with the dried reagent. The labels have been color coded so that they can be paired correctly.
   - **b.** Reconstitute according to the manufacturer's instructions in the package insert.
   - **c.** Discard reconstituted reagent after 30 days or by the expiration date, whichever comes first.
2. Prepare the TCR plus TCR-B as follows.
   - **a.** Determine the number of reactions to be performed (specimens plus controls).
   - **b.** Calculate the volumes of TCR and TCR-B as follows. See table below for examples.

$$\text{Volume of TCR (milliliters)} = (\text{number of reactions} + 5 \text{ extra reactions}) \times 0.1 \text{ ml}$$

$$\text{Volume of TCR-B (milliliters)} = \text{volume of TCR (milliliters)}/100$$

Number of reactions	Volume of TCR	Volume of TCR-B
25 + 5	3.0 ml	0.03 ml (30 µl)
75 + 5	8.0 ml	0.08 ml (80 µl)
100 + 5	10.5 ml	0.105 ml (105 µl)

   - **c.** Transfer the calculated volume of TCR to an appropriately sized, dedicated, clean, dry container and, using a micropipettor, add the calculated volume of TCR-B into the TCR. Thoroughly mix the solution by swirling.
   - **d.** The TCR plus TCR-B is stable for 30 days when stored at 15 to 30°C. Do not refrigerate.

V. PROCEDURE *(continued)*

C. Target capture

1. Allow the controls and specimens to reach room temperature prior to processing.
2. Do not vortex specimens.
3. Inspect transport tubes before piercing them.
4. In the Ten Tube unit (TTU) rack, place enough TTUs to accommodate the controls and specimens.
5. Thoroughly mix the TCR plus TCR-B. Using the repeat pipettor, add 100 µl into each reaction tube.
6. Hold the positive control *C. trachomatis*/negative control *N. gonorrhoeae* tube in one hand or keep it in a rack. To work properly with the APTIMA assay software, the positive control *C. trachomatis*/negative control *N. gonorrhoeae* must be in the first position of the first TTU. This label is pink. The label text is "CONTROL + CT PCT / CONTROL – GC NGC." Using a micropipettor, pierce the cap, taking care not to drive the tip into the bottom of the tube. Add 400 µl of the positive control *C. trachomatis*/ negative control *N. gonorrhoeae* to the first reaction tube. In the same manner, add 400 µl of the positive control *N. gonorrhoeae*/negative control *C. trachomatis* to the second reaction tube. The label for the second control is blue-green. The label text is "CONTROL + GC PGC / CONTROL − CT NCT."
7. Continue the rack setup procedure by adding 400 µl of each specimen to the remaining TTU tubes. Use a new pipette tip for each specimen and control. The acceptable volume of control or specimen added to the TTU is 400 ± 100 µl.
8. If specimens with standard (nonpenetrable) caps are to be tested, they must be centrifuged for 5 min at a relative centrifugal force of 420 to bring all of the liquid down to the bottom of the tube before uncapping. Avoid splashing and cross-contamination.

   ◩ **NOTE:** Use of the Gen-Probe TCS is described in the Target Capture System for APTIMA Combo 2 Assay operator's manual.
9. Cover the TTUs with sealing cards and shake the rack gently by hand. Do not vortex. Incubate the rack at 62 ± 1°C in a water bath for 30 ± 5 min.
10. Remove the rack from the water bath and blot the bottoms of the tubes dry on absorbent material.
11. Ensure that the sealing cards are firmly seated. If necessary, replace them with new sealing cards and seal the TTUs tightly.
12. Vortex the rack for 60 s on the multitube vortex mixer. Begin vortexing within 2 min of removal of the rack from the water bath.
13. Without removing the sealing cards, incubate the rack at room temperature for 30 ± 5 min.
14. Place the rack on the TCS magnetic base for 5 to 10 min.
15. Prime the dispense station pump lines by pumping APTIMA wash solution through the dispense manifold. Pump enough liquid through the system so that there are no air bubbles in the line and all 10 nozzles are delivering a steady stream of liquid.
16. Turn on the vacuum pump and disconnect the aspiration manifold at the first connector between the aspiration manifold and the trap bottle. Ensure that the vacuum gauge meets the leak test specification. It may take 15 s to achieve this reading. Reconnect the manifold, and ensure that the vacuum gauge meets the vacuum level specification. Leave the vacuum pump on until all target capture steps are completed.

V. PROCEDURE *(continued)*

17. Firmly attach the aspiration manifold to the first set of tips. Aspirate all liquid by lowering the tips into the first TTU until the tips come into brief contact with the bottoms of the tubes. Do not hold the tips in contact with the bottoms of the tubes.
18. After the aspiration is complete, eject the tips into their original tip cassette. Repeat the aspiration steps for the remaining TTUs, using a dedicated tip for each specimen.
19. Place the dispense manifold over each TTU and, using the dispense station pump, deliver 1.0 ml of APTIMA wash solution into each tube of the TTU.
20. Cover the tubes with a sealing card and remove the rack from the TCS. Vortex the rack once on the multitube vortex mixer.
21. Place the rack on the TCS magnetic base for 5 to 10 min.
22. Aspirate all liquid as described above.
23. After the final aspiration, remove the rack from the TCS base and visually inspect the tubes to ensure that all liquid has been aspirated and all tubes contain magnetic particle pellets. If any liquid is visible, place the rack back on the TCS base for 2 min, and repeat the aspiration for that TTU using the same tips as used previously for each specimen. If *any* magnetic particle pellet is visible after aspiration is completed, the tube may be accepted. If no pellet is visible, the specimen should be retested. If the same specimen does not contain a magnetic particle pellet at this step in a subsequent run, this may indicate a specimen-specific problem. Collection of another specimen is recommended in this situation.

D. Amplification

1. Using the repeat pipettor, add 75 µl of the reconstituted amplification reagent to each reaction tube. All reaction mixtures in the rack should now be red.
2. Using the repeat pipettor, add 200 µl of oil reagent.
3. Cover the tubes with a sealing card and vortex them on the multitube vortex mixer.
4. Incubate the rack in a water bath at 62 ± 1°C for 10 ± 5 min.
5. Transfer the rack into a water bath at 42 ± 1°C for 5 ± 2 min.
6. With the rack in the water bath, carefully remove the sealing card and, using the repeat pipettor, add 25 µl of the reconstituted enzyme reagent to each of the reaction mixtures. All reactions should now be orange.
7. Immediately cover the tubes with a fresh sealing card, remove the rack from the water bath, and mix the reactions by gently shaking the rack by hand.
8. Incubate the rack at 42 ± 1°C for 60 ± 15 min.

E. DKA

◩ **NOTE:** The repeat pipettor used in hybridization and selection should be dedicated for these steps only.

1. Hybridization
   - **a.** Remove the rack from the water bath and transfer to the DKA area. Add 100 µl of the reconstituted probe reagent, using the repeat pipettor. All reaction mixtures should now be yellow.
   - **b.** Cover the tubes with a sealing card and vortex the rack on the multitube vortex mixer.
   - **c.** Incubate the rack in a 62 ± 1°C water bath for 20 ± 5 min.
   - **d.** Remove the rack from the water bath and incubate at room temperature for 5 ± 1 min.
2. Selection
   - **a.** Using the repeat pipettor, add 250 µl of selection reagent to each tube. All reactions should now be red.
   - **b.** Cover the tubes with a sealing card, vortex the rack for 10 s or until the color is uniform, and incubate the rack in a water bath at 62 ± 1°C for 10 ± 1 min.

V. PROCEDURE *(continued)*

c. Remove the rack from the water bath.

3. Detection

Detection must be performed at 18 to 28°C.

a. Incubate the rack at 18 to 28°C for 15 ± 3 min.

◩ **NOTE:** This temperature range is critical for assay performance.

b. For use of the LEADER HC+ luminometer and the APTIMA assay software, refer to the LEADER HC+ luminometer for APTIMA assay operator's manual.

c. Prepare the LEADER HC+ luminometer by placing one empty TTU in cassette position 1 and performing the WASH protocol.

d. Ensure that there are sufficient volumes of autodetect 1 and 2 to complete the tests.

e. Load the TTUs into the luminometer.

f. Log on to the computer. Click on NEW RUN and enter the number of tubes (controls and specimens). Click NEXT to begin the run.

◩ **NOTE:** The run must be completed within 2 h of the end of the selection step incubation.

g. Prepare a buffered bleach deactivation solution by mixing equal volumes of 5 to 7% (0.7 to 1.0 M) sodium hypochlorite (bleach) and APTIMA buffer for deactivation fluid in a large capped plastic container. Label and write the expiration date on the plastic container. This buffered bleach solution is stable for 4 weeks at room temperature.

h. After removing the used TTUs from the luminometer, place the TTUs into the container with the buffered bleach solution. Allow the TTUs to sit in the container for 15 min before disposal.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report results as "detected" or "not detected" for the presence of *C. trachomatis* and *N. gonorrhoeae* nucleic acid (rRNA). Results of "equivocal" or "invalid" should be reported with instructions for collection of new specimens for testing, if clinically indicated.

B. Document all testing results in hard copy or computerized work card and notify appropriate health care personnel.

VII. INTERPRETATION

A. APTIMA Combo 2 test results are automatically interpreted by the manufacturer's software and are presented as individual *C. trachomatis* and *N. gonorrhoeae* results (*see* table below). The final patient results are calculated by utilizing an algorithm that incorporates several elements, including the kinetic output (measured in RLU) and kinetic type/curve shape of the light-off reaction.

B. A test result may be negative, equivocal, positive, or invalid for *C. trachomatis* and *N. gonorrhoeae.* Along with the qualitative results, the RLU for each sample are printed on the sample report.

C. A negative result does not preclude a possible infection, because results are dependent on adequate specimen collection. Test results may be affected by improper specimen collection, technical error, or specimen mix-up.

D. Positive results may be reported based on a single valid testing result per the package insert. In low-prevalence populations or in cases with serious psychosocial or legal consequences, repeat testing of the same specimen with the APTIMA Combo 2 or an alternate target may be considered. A second specimen may also be collected and tested to confirm positive results using the same or an alternate testing method (1, 9).

VII. INTERPRETATION *(continued)*

E. Individual testing for *C. trachomatis* or *N. gonorrhoeae* is also possible using the APTIMA Combo 2. The software suppresses the result for the analyte that is not ordered. With this mode of testing, the RLU for the reaction, which includes both analytes, are still reported and may indicate the presence of the unordered analyte. For example, high RLU in a *C. trachomatis*-negative specimen may indicate that *N. gonorrhoeae* is present in the sample. However, because the algorithm is based on RLU and curve kinetics, the presence of *N. gonorrhoeae* cannot be automatically inferred.

Kinetic type	Total RLU (10^3) to give CT result		
	Negative	Equivocal	Positive
C. trachomatis only	1 to <25	25 to <100	100 to <4,500
C. trachomatis and *N. gonorrhoeae*	1 to <85	85 to <250	250 to <4,500
C. trachomatis indeterminate	1 to <85	85 to <4,500	Not applicable
	Total RLU (10^3) to give GC result		
	Negative	Equivocal	Positive
N. gonorrhoeae only	1 to <60	60 to <150	150 to <4,500
C. trachomatis and *N. gonorrhoeae*	1 to <85	85 to <250	250 to <4,500
N. gonorrhoeae indeterminate	1 to <85	85 to <4,500	Not applicable

VIII. LIMITATIONS OF TESTING

A. For the vaginal swab, endocervical swab, male urethral swab, and urine specimen clinical studies, performance characteristics for detecting *C. trachomatis* and *N. gonorrhoeae* are derived from high-prevalence populations. Positive results in low-prevalence populations should be interpreted carefully, with the understanding that the likelihood of a false positive may be higher than that of a true positive.

B. No interference in the APTIMA Combo 2 by blood, gynecological lubricants, spermicides, or commonly used vitamins, minerals, and over-the-counter pain relievers assay is noted for swab and urine specimens. The effects of tampon use, douching, and specimen collection variables have not been assessed for their impact on the detection of *C. trachomatis* or *N. gonorrhoeae.*

C. The presence of mucus in endocervical samples does not interfere with the detection of *C. trachomatis* or *N. gonorrhoeae* by the APTIMA Combo 2 assay. However, to ensure collection of cells infected with *C. trachomatis,* columnar epithelial cells lining the endocervix should be sampled. If excess mucus is not removed, sampling of these cells is not ensured.

D. To mitigate the potential for cross-contamination during Pap processing, a specific contamination mitigation procedure was validated. Two important steps of the procedure include (i) bleaching of the PreservCyt filter cap (in 0.5% sodium hypochlorite in deionized water) for 1 min between samples and (ii) mandating that the operator change gloves between each sample. It is important to dilute bleach only with deionized water. The pH of tap water varies from laboratory to laboratory. Alkaline water can decrease the available chlorine, making the bleach less effective for decontaminating equipment.

E. The APTIMA Combo 2 assay is not intended for the evaluation of suspected sexual abuse or for other medico-legal indications. In cases of suspected sexual

VIII. LIMITATIONS OF TESTING *(continued)*

abuse, both an amplification-based *and* a culture-based method should be considered for optimal clinical care while covering concerns about legal interpretation (1, 2).

F. Test of cure is not recommended for most patients treated with recommended first-line antimicrobials. If nucleic acid amplification testing (NAAT) is used for test of cure for *C. trachomatis* infection, specimens should be collected >3 weeks following the end of treatment. NAAT done prior to 3 weeks may be falsely positive due to continued shedding of nonviable organisms (1, 2, 5).

G. Glove powder

As in any reagent system, excess powder on some gloves may cause contamination of opened tubes. Powderless gloves are recommended.

REFERENCES

1. **Centers for Disease Control and Prevention.** 2002. Screening tests to detect Chlamydia trachomatis and Neisseria gonorrhoeae infections—2002. *MMWR Morb. Mortal. Wkly. Rep.* **51**(RR-15)**:**1–39.
2. **Centers for Disease Control and Prevention.** 2002. Sexually transmitted diseases treatment guidelines. *MMWR Morb. Mortal. Wkly. Rep.* **51**(RR-6)**:**1–77.
3. **Clinical and Laboratory Standards Institute.** 2006. *Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline MM3-A2.* Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Federal Register.** 2008. Code of Federal Regulations. Medicare, Medicaid and CLIA Programs; laboratory requirements relating to certain personnel qualifications; final rule. 68 FR 50724, p. 1083–1084. http://edocket.access.gpo.gov/cfr_2008/octqtr/pdf/42cfr493.1253.pdf. Accessed 28 September 2009.
5. **Gaydos, C. A., K. A. Crotchfelt, M. R. Howell, S. Kralian, P. Hauptman, and T. C. Quinn.** 1998. Molecular amplification assays to detect chlamydial infections in urine specimens from high school female students and to monitor the persistence of chlamydial DNA after therapy. *J. Infect. Dis.* **177:**417–424.
6. **Gen-Probe Incorporated.** 2008. Gen-Probe APTIMA Combo 2 Assay package insert, 501011 Rev. C 2008-05. Gen-Probe Incorporated, San Diego, CA.
7. **Gen-Probe Incorporated.** 2008. Gen-Probe APTIMA Specimen Transfer Kit package insert, 501548 Rev. A 2008-04. Gen-Probe Incorporated, San Diego, CA.
8. **Hologic, Inc.** ThinPrep 2000 operator's manual, P/N 70354-001. Hologic, Inc., Bedford, MA.
9. **Leber, A. L., G. S. Hall, and W. D. LeBar.** 2006. *Cumitech 44, Nucleic Acid Amplification Tests for Detection of* Chlamydia trachomatis *and* Neisseria gonorrhoeae. Coordinating ed., S. E. Sharp. ASM Press, Washington, DC.

SUPPLEMENTAL READING

Chernesky, M. A., and D. E. Jang. 2006. APTIMA transcription-mediated amplification assays for Chlamydia trachomatis and Neisseria gonorrhoeae. *Expert Rev. Mol. Diagn.* **6:**519–525.

Cook, R. L., S. L. Hutchison, L. Østergaard, R. S. Braithwaite, and R. B. Ness. 2005. Systematic review: noninvasive testing for Chlamydia trachomatis and Neisseria gonorrhoeae. *Ann. Intern. Med.* **142:**914–925.

Gaydos, C. A., M. Theodore, N. Dalesio, B. J. Wood, and T. C. Quinn. 2004. Comparison of three nucleic acid amplification tests for detection of *Chlamydia trachomatis* in urine specimens. *J. Clin. Microbiol.* **42:**3041–3045.

Koumans, E. H., C. M. Black, L. E. Markowitz, E. Unger, A. Pierce, M. K. Sawyer, and J. R. Papp. 2003. Comparison of methods for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* using commercially available nucleic acid amplification tests and a liquid Pap smear medium. *J. Clin. Microbiol.* **41:**1507–1511.

Levett, P. N., K. Brandt, K. Olenius, C. Brown, K. Montgomery, and G. B. Horsman. 2008. Evaluation of three automated nucleic acid amplification systems for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in first-void urine specimens. *J. Clin. Microbiol.* **46:**2109–2111.

Moncada, J., E. Donegan, and J. Schachter. 2008. Evaluation of CDC-recommended approaches for confirmatory testing of positive *Neisseria gonorrhoeae* nucleic acid amplification test results. *J. Clin. Microbiol.* **46:**1614–1619.

Schachter, J., M. A. Chernesky, D. E. Willis, P. M. Fine, D. H. Martin, D. Fuller, J. A. Jordan, W. Janda, and E. W. Hook III. 2005. Vaginal swabs are the specimens of choice when screening for Chlamydia trachomatis and Neisseria gonorrhoeae: results from a multicenter evaluation of the APTIMA assays for both infections. *Sex. Transm. Dis.* **32:**725–728.

Schachter, J., J. M. Chow, H. Howard, G. Bolan, and J. Moncada. 2006. Detection of *Chlamydia trachomatis* by nucleic acid amplification testing: our evaluation suggests that CDC-recommended approaches for confirmatory testing are ill-advised. *J. Clin. Microbiol.* **44:**2512–2517.

Van Der Pol, B., J. A. Williams, N. J. Smith, B. E. Batteiger, A. P. Cullen, H. Erdman, T. Edens, K. Davis, H. Salim-Hammad, V. W. Chou, L. Scearce, J. Blutman, and W. J. Payne. 2002. Evaluation of the Digene Hybrid Capture II Assay with the Rapid Capture System for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae. J. Clin. Microbiol.* **40:**3558–3564.

PART 2

Detection of Colonization with Methicillin-Resistant *Staphylococcus aureus* (MRSA) in Nasal Swab Specimens Using the Cepheid Xpert MRSA Assay

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major pathogen in the hospital setting and is an increasing problem in the community. Isolates of MRSA contain a plasmid with a gene encoding *mecA*, which confers resistance to beta-lactam antimicrobial agents such as penicillin, methicillin, and the cephalosporins. A concerted effort has been made to prevent the spread of MRSA among hospitalized patients. Toward this end, active surveillance using culture has been adopted in many settings to identify colonized patients. Nucleic acid amplification testing has also been applied to the detection of MRSA in surveillance specimens (1, 2, 6, 7). Table 12.1–2 lists the currently available tests for amplified testing of MRSA for both surveillance and direct detection of organisms in clinical specimens.

In this procedure, the Cepheid Xpert MRSA assay is presented. The assay is a qualitative test designed for rapid detection of MRSA from nasal swabs from patients at risk for nasal colonization. The assay is performed using the GeneXpert Dx system, a platform that automates sample purification, nucleic acid amplification, and detection using real-time PCR. The system consists of an instrument, computer, and software for running of the test and interpretation of results. The test utilizes single-use disposable GeneXpert cartridges that contain the PCR reagents and host the PCR process. Because the cartridges are self-contained, cross-contamination between samples is minimized. Each cartridge includes reagents for MRSA detection as well as a sample processing control (SPC) to test for adequate processing of the target bacteria and to monitor for the presence of inhibitors. The probe check control (PCC) verifies reagent rehydration, PCR tube filling in the cartridge, probe integrity, and fluorophore stability. The primers and probes in the Xpert MRSA assay detect a proprietary sequence for the presence of a cassette inserted into the *S. aureus* chromosome. Due to its simplicity and process controls, the FDA has designated the Xpert MRSA assay to be of moderate complexity. For a full description of the system's complete instructions for use, the reader is advised to consult the GeneXpert Dx system operator's manual and the Xpert MRSA assay package insert (3, 4).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. The assay is performed on nasal swabs. Specimens must be collected with the Cepheid collection device.
2. Insert dry swabs approximately 1 to 2 cm into each nostril and rotate against the inside of the nostril for 3 s. Apply slight pressure with a finger on the outside of the nose to help ensure good contact between the swab and the inside of the nose.
3. Using the same swabs, repeat for the second nostril, trying not to touch anything but the inside of the nose.
4. Remove the plastic transport tube. Twist off the tube cap and discard it. Place the swabs into the plastic transport tube.

B. Time of specimen collection

There are no particular issues related to timing of collection of specimens; however, the time of collection should be recorded.

C. Specimen transport

1. The swab specimen may be stored at 15 to 30°C if it will be processed within 24 h; otherwise, store the swab at 2 to 8°C.
2. The swab specimen is stable for up to 5 days when stored at 2 to 8°C.

D. Specimen labeling and request submission

The laboratory should determine a mechanism to ensure that the specimen type and time of collection are recorded.

E. Rejection criteria

1. Test only nasal swabs collected with the Cepheid collection device.
2. Reject specimens that arrive in broken or leaky containers.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

3. If an improperly collected, transported, or handled specimen cannot be replaced, testing may be considered after consultation with the health care provider. Document in the final report that specimen quality may have been compromised.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Material provided

1. The Xpert MRSA assay (GXMRSA-100N-10) contains sufficient reagents to process 10 specimens.

a. Xpert MRSA assay cartridge

Each cartridge contains the following.

(1) Bead 1 (freeze-dried), one per cartridge
- (a) Polymerase
- (b) Deoxynucleoside triphosphates
- (c) Bovine serum albumin (BSA)

(2) Bead 2 (freeze-dried), one per cartridge
- (a) Primers
- (b) Probes
- (c) BSA

(3) Bead 3 (freeze-dried), one per cartridge
- (a) SPC
- (b) Noninfectious sample preparation control spores

2. Xpert MRSA reagent pouches
- **a.** Elution reagent (guanidinium thiocyanate and surfactants)
- **b.** Reagent 1 (sodium hydroxide)
- **c.** Reagent 2 (Tris buffer, EDTA, and surfactants)

B. Materials required but not provided

1. GeneXpert Dx system
2. Printer
3. Cepheid sample collection device
4. Vortex mixer
5. Disposable, sterile transfer pipettes
6. Sterile gauze

C. Materials available but not provided

1. External control organism
- **a.** MRSA (KWIK STIK catalog no. 0158; MicroBioLogics, Inc.)
- **b.** Methicillin-sensitive *S. aureus* (KWIK STIK catalog no. 0371; MicroBioLogics, Inc.)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Cartridge controls

1. Each test cartridge includes an SPC and PCC.

2. Specimen processing control (SPC)

The SPC contains dry spores of *Bacillus globigii.* The SPC verifies lysis of MRSA and verifies that specimen processing is adequate. Additionally, this control detects specimen-associated inhibition of the real-time PCR assay.
- **a.** The SPC should be positive in a negative sample and can be negative or positive in a positive sample.
- **b.** The SPC passes if it meets the validated acceptance criteria.

3. Probe check control (PCC)

Before the start of the PCR, the GeneXpert Dx system measures the fluorescence signal from the probes to monitor bead rehydration, reaction tube filling, probe integrity, and dye stability. The probe check passes if it meets the assigned acceptance criteria.

B. External controls

1. External controls should include an MRSA isolate and a methicillin-sensitive *S. aureus* isolate. The controls may be culture isolates processed as patient samples.

IV. QUALITY CONTROL *(continued)*

2. The external controls should be run with each new lot of Xpert MRSA reagents. Because the Xpert assay has built-in controls in each cartridge, running the external controls with greater frequency is not required (5). Other state, local, or federal regulations may apply to control frequency.
3. The KWIK-STIK organisms (catalog no. 0158 MRSA as a positive control and catalog no. 0371 methicillin-sensitive *S. aureus* as a negative control; MicroBioLogics, Inc.) may be used for training, proficiency testing, and external QC of the GeneXpert Dx system. Follow the MicroBioLogics external control procedure described in the package insert (3).

V. PROCEDURE

Observe standard precautions.

A. Preparing the cartridge

1. Remove the cartridge and reagents from the package. It is important to start the test within 15 min of adding the reagents to the cartridge.
2. Remove the swabs from the transport container and then remove one swab from the red cap. Use only one of the swabs. The second swab is required for repeat testing.
3. Insert the swab into the tube containing the elution reagent (black cap). Use sterile gauze to minimize the risk of contamination.
4. Hold the swab by the stem near the rim of the tube, lift the swab a few millimeters from the bottom of the tube, and push the stem against the edge of the tube to break it. Make sure the swab is short enough to allow the cap to close tightly.
5. Close the cap and vortex at high speed for 10 s.
6. Open the cartridge lid. Using a sterile transfer pipette, transfer the entire contents of the elution reagent (black cap) to the "S" chamber of the GeneXpert cartridge (Fig. 12.2.3–1).
7. Add reagent 1 into cartridge chamber 1. Squeeze the ampoule until the entire contents are added to the cartridge.
8. Add reagent 2 into cartridge chamber 2. Squeeze the ampoule until the entire contents are added to the cartridge.
9. Close the cartridge lid.

B. Running the test

1. Turn on the computer, and then turn on the GeneXpert Dx instrument.
2. On the Windows desktop, double-click the GeneXpert Dx shortcut icon.
3. Log on to the GeneXpert Dx system software using your user name and password.
4. In the GeneXpert Dx system window, click Create Test. The Scan Cartridge Barcode dialog box appears.

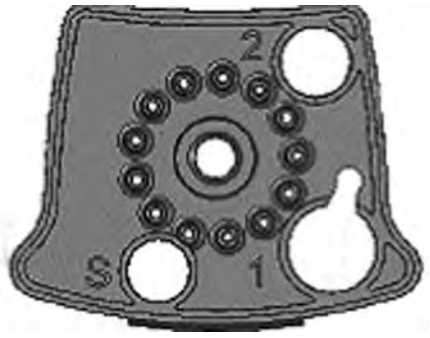

Figure 12.2.3–1 GeneXpert cartridge. S, sample; 1, reagent 1; 2, reagent 2.

V. PROCEDURE *(continued)*

5. Scan the barcode on the Xpert MRSA cartridge. The Create Test window appears. Using the barcode information, the software automatically fills the boxes for the following fields: Select Assay, Reagent Lot ID, Cartridge SN, and Expiration Date.
6. In the Sample ID box, scan or type the sample identification (ID). Make sure to type the correct sample ID. The sample ID is associated with the test results and is shown in the View Results window and all the reports.
7. Click Start Test. In the dialog box that appears, type in your password.
8. Open the instrument module door with the blinking green light and load the cartridge.
9. Close the door. The test starts and the green light stops blinking. When the test is finished, the light turns off.
10. Wait until the system releases the door lock before opening the module door and removing the cartridge.
11. Dispose of the used cartridges in the appropriate specimen waste containers according to the institution's standard practices.
12. Viewing and printing results
    For detailed instructions on how to view and print the results, see the GeneXpert Dx system operator's manual.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report results as "detected" or "not detected" for the presence of MRSA DNA.

B. For results of "invalid," "error," and "no result," repeat testing should be attempted to obtain a valid and reportable result. If repeat testing does not result in a positive or negative result, report as "unable to obtain valid result; please collect and submit additional specimen for testing."

C. Document all testing in hard copy or computerized work card and notify appropriate health care personnel.

VII. INTERPRETATION OF RESULTS

A. Results are interpolated by the GeneXpert Dx system from measured fluorescent signals and embedded calculation algorithms and will be shown in the View Results window. Possible results are as follows.

1. MRSA—POSITIVE: target DNA is detected (presumptive positive for MRSA colonization)
   - **a.** MRSA target has a crossing threshold (Ct) within the valid range and an endpoint above the minimum setting.
   - **b.** SPC—NA (not applicable): the SPC is ignored since MRSA amplification may compete with this control
   - **c.** Probe Check—PASS: all probe check results pass
2. MRSA target DNA is not detected (presumed not colonized with MRSA)
   - **a.** MRSA—NEGATIVE: MRSA target DNA is not detected
   - **b.** SPC—PASS: the SPC has a Ct within the valid range and an endpoint above the minimum setting
   - **c.** Probe Check—PASS: all probe check results pass
3. INVALID: the presence or absence of MRSA cannot be determined, repeat test with the extra swab. The SPC does not meet acceptance criteria, the sample was not properly processed, or PCR was inhibited.
   - **a.** MRSA—INVALID: the presence or absence of MRSA DNA cannot be determined
   - **b.** SPC—FAIL: MRSA target result is negative, and the SPC Ct is not within the valid range and the endpoint is below the minimum setting
   - **c.** Probe Check—PASS: all probe check results pass

VII. INTERPRETATION OF RESULTS *(continued)*

4. ERROR: the presence or absence of MRSA cannot be determined; repeat test with the extra swab. The PCC failed, probably because the reaction tube was filled improperly, because a probe integrity problem was detected, or because the maximum pressure limits were exceeded.
   a. MRSA—NO RESULT
   b. SPC—NO RESULT
   c. Probe Check—FAIL; all or one of the probe check results fail.
   If the Probe Check passed, the error is caused by a system component failure.
5. NO RESULT: the presence or absence of MRSA cannot be determined; repeat test with the extra swab. Insufficient data were collected to produce a test result (for example, the operator stopped a test that was in progress).
   a. MRSA—NO RESULT
   b. SPC—NO RESULT
   c. Probe Check—NA (not applicable)

VIII. LIMITATIONS OF TESTING

A. A positive test result does not necessarily indicate the presence of viable organisms. It is, however, presumptive for the presence of MRSA.

B. A negative result does not rule out colonization, because results are dependent on adequate specimen collection. Test results may be affected by improper specimen collection, technical error, or specimen mix-up.

C. Rerunning the Xpert MRSA test when results are INVALID, ERROR, and NO RESULT should depend on practices and policies within each facility. Alternate procedures (e.g., culture using selective agar plates with or without overnight incubation in a selective enrichment broth) should be available. For culturing, remaining swab specimens should be placed in appropriate transport systems and cultured within 4 days.

D. The Xpert MRSA assay is not intended to diagnose MRSA infection or to guide or monitor treatment for MRSA infections.

E. Test results might also be affected by concurrent antimicrobial therapy. Therefore, therapeutic success or failure cannot be assessed using this test because DNA might persist following antimicrobial therapy.

F. Mutations or polymorphisms in primer or probe binding regions may affect detection of new or unknown MRSA variants, resulting in a false-negative result.

G. Potentially interfering substances evaluated include blood, mucus, and nasal sprays used to relieve decongestion, nasal dryness, or irritation. The presence of these substances did not significantly inhibit PCR and did not give invalid or erroneous results.

REFERENCES

1. **Boyce, J. M., and N. L. Havill.** 2008. Comparison of BD GeneOhm methicillin-resistant *Staphylococcus aureus* (MRSA) PCR versus the CHROMagar MRSA assay for screening patients for the presence of MRSA strains. *J. Clin. Microbiol.* **46:**350–351.
2. **Carroll, K. C.** 2008. Rapid diagnostics for methicillin-resistant Staphylococcus aureus: current status. *Mol. Diagn. Ther.* **12:**15–24.
3. **Cepheid.** Cepheid Xpert MRSA Assay package insert 300-5188, Rev. C. Cepheid, Sunnyvale, CA.
4. **Cepheid.** Cepheid GeneXpert® Dx system operator manual. Cepheid, Sunnyvale, CA.
5. **College of American Pathologists.** 2009. Laboratory Accreditation Program, Microbiology Checklist, rev. 06/15/2009. College of American Pathologists, Northfield, IL.
6. **de San, N., O. Denis, M. F. Gasasira, R. De Mendonça, C. Nonhoff, and M. J. Struelens.** 2007. Controlled evaluation of the IDI-MRSA assay for detection of colonization by methicillin-resistant *Staphylococcus aureus* in diverse mucocutaneous specimens. *J. Clin. Microbiol.* **45:**1098–1101.
7. **Wolk, D. M., E. Picton, D. Johnson, T. Davis, P. Pancholi, C. C. Ginocchio, S. Finegold, D. F. Welch, M. de Boer, D. Fuller, M. C. Solomon, B. Rogers, M. S. Mehta, and L. R. Peterson.** 7 January 2009. Multicenter evaluation of the Cepheid XpertTM methicillin-resistant *Staphylococcus aureus* (MRSA) test as a rapid screening method for detection of MRSA in nares. *J. Clin. Microbiol.* doi:10.1128/JCM.01714-08.

SUPPLEMENTAL READING

Chaix, C., I. Durand-Zileski, C. Alberti, and B. Buisson. 1999. Control of endemic methicillin resistant Staphylococcus aureus. *JAMA* **282:**1745–1751.

Daum, R. S. 2007. Clinical practice. Skin and soft-tissue infections caused by methicillin-resistant Staphylococcus aureus. *N. Engl. J. Med.* **357:**380–390.

Shopsin, B., and B. N. Kreiswirth. 2001. Molecular epidemiology of methicillin-resistant Staphylococcus aureus. *Emerg. Infect. Dis.* **7:**323–326.

PART 3

Detection of *Mycobacterium tuberculosis* in Respiratory Specimens by Using the Gen-Probe Amplified *Mycobacterium tuberculosis* Direct Test

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The Gen-Probe Amplified *Mycobacterium tuberculosis* Direct (MTD) test is a target-amplified nucleic acid probe test for the in vitro diagnostic detection of *M. tuberculosis* complex rRNA in acid-fast bacillus (AFB) smear-positive and smear-negative concentrated sediments prepared from sputum, bronchial specimens (e.g., bronchoalveolar lavage specimens or bronchial aspirates) or tracheal aspirates.

The MTD test is a two-part test in which amplification and detection take place in a single tube. After nucleic acid extraction, the MTD test utilizes transcription-mediated amplification (TMA) and the hybridization protection assay to detect *M. tuberculosis* complex rRNA. In contrast to PCR, which requires thermal cycling and uses a thermostable polymerase to produce multiple copies (amplicons) of DNA, TMA uses a constant 42°C temperature and amplifies a specific rRNA target by using RNA polymerase and reverse transcriptase enzymes to produce multiple RNA amplicons. Detection of the amplicons is accomplished with a chemiluminescence-labeled DNA probe that is complementary to the RNA amplicons. When stable RNA-DNA hybrids are formed, separation of the hybridized from the unhybridized probe is accomplished by adding a selection reagent that hydrolyzes the chemiluminescent label on the unhybridized probe. The label on the hybridized probe is *protected* (hybridization protection) and may be detected in a luminometer. The entire assay is performed in a single tube.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. Acceptable specimens include sputum (induced or expectorated) or bronchial (e.g., bronchoalveolar lavage specimens or aspirates) or tracheal aspirates.
2. The efficacy of this test has not been demonstrated (and it is not Food and Drug Administration [FDA] approved) for the direct detection of *M. tuberculosis* rRNA with other clinical specimens (e.g., blood, urine, stool, or tissue).
3. The Gen-Probe MTD test (enhanced) has been FDA approved for testing AFB smear-positive and -negative respiratory specimens.

B. Timing of specimen collection

Generally, an early-a.m. expectorated sputum should be collected each day for 3 days. The time of collection and type of specimen (expectorated or induced sputum, bronchoalveolar lavage specimen, or trachael aspirate) should be recorded.

C. Specimen transport

1. Specimens must be collected in sterile, plastic containers and stored at 2 to 8°C until transported or processed.
2. Specimens should be processed (decontaminated and concentrated) within 24 h of collection (including transport time) in a proper biosafety hood (*see* section 7) as recommended by the CDC.
3. Specimens that are grossly bloody should not be tested with the MTD test.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

4. The MTD test is designed to detect rRNA from members of the *M. tuberculosis* complex by using sediments prepared from generally accepted current adaptations of the *N*-acetyl-L-cysteine-NaOH or NaOH decontamination protocols, described by the CDC, with 1 to 1.5% NaOH for 15 to 20 min and centrifugation at ≥3,000 × *g*.
   ◩ **NOTE:** See Section 7 for complete description of sputum concentration.
5. Processed sediments may be stored at 2 to 8°C for up to 3 days before testing.

D. Specimen labeling and submission

The laboratory should determine a mechanism to ensure that the specimen type and time of collection are recorded.

E. Rejection criteria

1. Test only the indicated specimen types (sputum, bronchial and tracheal aspirates) submitted within 24 h of collection in the proper container.
2. Reject specimens that arrive in leaky containers.
3. Request a repeat specimen, or obtain the information, when the collection time or specimen type has not been provided.
4. Repeat testing to evaluate efficacy of therapy is not indicated.
5. If an improperly collected, transported, or handled specimen cannot be replaced, document in the final report that specimen quality may have been compromised.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Materials provided (*see* package insert)

1. Specimen dilution buffer
2. Amplification reagent
3. Reconstitution buffer
4. Lysing tubes
5. Oil reagent
6. Enzyme reagent
   a. Reverse transcriptase
   b. RNA polymerase
7. Enzyme dilution buffer
8. Termination reagent
9. Hybridization positive control
10. Hybridization negative control
11. Probe reagent
12. Hybridization buffer
13. Selection reagent
14. Sealing cards

B. Materials required but not provided (*see* package insert)

1. Luminometer
2. Sonicator
3. Detection reagent kit
4. Dry-heat bath (95°C [±5°C])
5. Sonicator rack
6. Pipette tips with hydrophobic plugs
7. Polypropylene tubes (12 by 75 mm)
8. Micropipettes capable of dispensing 20, 25, 50, 100, 200, and 300 μl
9. Snap-top polypropylene caps for 12- by 75-mm tubes
10. Water bath and/or dry heat bath (42°C [±1°C] and 60°C [±1°C])
11. Vortex mixer
12. Sterile water
13. Culture tubes
14. Glass beads (3 mm)
15. Screw-cap microcentrifuge tubes
16. Positive cell controls (e.g., *M. tuberculosis* ATCC 25177 or ATCC 27294)
17. Negative cell controls (e.g., *Mycobacterium gordonae* ATCC 14470 or *Mycobacterium terrae* ATCC 15755).
18. Household bleach (5.25% hypochlorite solution)
19. Plastic backed bench covers

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. The amplification cell negative control and amplification cell positive control should produce the following values.

1. Amplification cell negative control, <20,000 relative light units (RLU)
2. Amplification cell positive control, ≥500,000 RLU

IV. QUALITY CONTROL *(continued)*

B. The hybridization negative control and hybridization positive control should produce the following values.
  1. Hybridization negative control, $<$5,000 RLU
  2. Hybridization positive control, $\geq$15,000 RLU

C. Patient test results must not be reported if the MTD test control values do not meet the criteria above.

D. Target values for amplification cell positive and cell negative controls should be determined in each laboratory by using test results for each batch of prepared controls.

E. Verify that all reagents and materials meet expiration date and QC parameters per NCCLS document M22-A2 (1).

V. PROCEDURE

It is imperative that these cultures be handled in a biological safety cabinet.

A. Equipment preparation
  1. Degas water in sonicator.
  2. Adjust one dry-heat bath to 95°C, one dry-heat or water bath to 60°C, and another dry-heat or water bath to 42°C ($\pm$2°C).
  3. Wipe down work surfaces, equipment, and pipettors with a 1:1 dilution of household bleach (*2.6% NaOCl*) before starting. Cover work surfaces with plastic-backed laboratory bench covers.
  4. Prepare luminometer for operation. Make sure there are sufficient volumes of detection reagents 1 and 2 to complete the tests.

B. Reagent preparation
  1. Reconstitute one vial (25 tests) of lyophilized *M. tuberculosis* amplification reagent with 750 µl of mycobacterium reconstitution buffer. Vortex and let sit at room temperature until clear.
  2. The reconstituted amplification reagent may be stored at 2 to 8°C for 2 months.
  3. The reconstituted amplification reagent should be allowed to come to room temperature before use.

C. Controls
  1. Lysis and amplification controls
    a. Cells used for the cell positive control should be a member of the *M. tuberculosis* complex, such as *M. tuberculosis* ATCC 25177 or ATCC 27294, suspended in sterile water. Cells used for the cell negative control should be mycobacteria other than the tuberculosis complex, such as *M. gordonae* ATCC 14470 or *M. terrae* ATCC 15755.
    b. Preparation and storage of amplification controls.
      (1) Place three to five sterile 3-mm glass beads in a clean screw cap culture tube.
      (2) Add several 1-µl loopfuls of growth from the appropriate culture to 1 to 2 ml of sterile water, cap, and vortex several times.
      (3) Allow the suspension to settle, and transfer the supernatant to a clean culture tube. Adjust turbidity to that of a no. 1 McFarland standard.
      (4) Make a 1:100 dilution (dilution 1) of the suspension into sterile water. Cap and vortex.
      (5) Make a 1:100 dilution (dilution 2) of dilution 1.
      (6) Take 100 µl of dilution 2 and place into 6 ml of sterile water (dilution 3). This contains approximately 25 CFU per 50 µl.
      (7) Plate 50 µl of each dilution onto Lowenstein-Jensen culture medium and freeze the remaining stock at $-$20 or $-$70°C. Test all dilutions using the MTD test.
      (8) The dilutions that give between 25 and 150 CFU per 50 µl on plated culture medium should be thawed, aliquoted, and used as controls.

V. PROCEDURE *(continued)*

(9) The dilutions must be aliquoted into clean 1.5-ml screw-cap microcentrifuge tubes as single-use aliquots (100 µl). The tubes may be stored frozen at −20°C for 6 months or −70°C for 1 year. Frost-free freezers must *not* be used.

c. A single replicate of the positive and negative cell controls must be tested with each run.

d. Each laboratory should determine target values and means for the controls.

2. Hybridization controls

a. Hybridization positive controls and hybridization negative controls are provided in the MTD test kit.

b. A single replicate of each of the hybridization controls should be tested with each run for QC purposes.

3. Specimen inhibition controls

a. When the AFB smear is positive and the MTD test is negative for untreated patients, one must consider the following possibilities.

(1) The specimen is inhibitory.

(2) The specimen contains mycobacteria other than *M. tuberculosis.*

(3) The specimen contains a mixture of high number of nontuberculosis mycobacteria and a low number of *M. tuberculosis* organisms.

b. Testing patient sediments for inhibition in the MTD test

(1) Place 200 µl of specimen dilution buffer into two lysing tubes.

(2) Add 50 µl of amplification positive cell control and 50 µl of sediment from patient specimen to one tube (seeded). Add 50 µl of sediment to the second tube (unseeded). Proceed with the test as usual.

(3) If the luminometer reading of the seeded tube is ≥30,000 RLU, then the sample is not inhibitory to amplification and there apparently was no target available for amplification.

(4) If the number of RLU of the seeded tube is below 30,000, then the sample is inhibitory to amplification, and another sample should be evaluated.

4. Laboratory contamination monitoring control

a. Place 2 ml of sterile water in a clean tube.

b. Wipe the area of bench or equipment to be tested using a premoistened sterile polyester or Dacron swab.

c. Place the swab in the water and swirl gently. Remove the swab and express fluid along the side of the tube.

d. Add 25 µl of the water to an amplification tube containing 25 µl of amplification reagent and 200 µl of oil reagent.

e. Follow the test procedure for amplification and detection.

f. If the results are ≥30,000 RLU, the surface is contaminated and should be decontaminated with bleach.

D. Sample preparation

1. Pipette 200 µl of specimen dilution buffer into labeled lysing tubes.

2. Transfer 50 µl of decontaminated, well-vortexed specimen or cell control from its container to the correspondingly labeled lysing tube.

◩ **NOTE:** The enhanced MTD test uses 500 µl of decontaminated sediment.

3. Cap the tubes and vortex for 3 s.

E. Sample lysis

1. Push the lysing tubes through the sonicator rack so that the reaction mixture in the bottom of the tubes is submerged but the caps are above water. Place sonicator rack on water bath sonicator. Do not allow the tubes to touch the bottom or sides of the sonicator.

V. PROCEDURE *(continued)*

2. Sonicate for 15 min but no more than 20 min. Samples and controls that have been sonicated are referred to as "lysates."

F. Amplification

1. Add 25 μl of reconstituted amplification reagent to each appropriately labeled amplification tube by using a repeat pipettor. Add 200 μl of oil reagent to each tube by using a repeat pipettor.
2. Transfer 50 μl of lysate to the bottom of the appropriately labeled amplification tube using a separate extended-length, hydrophobically plugged pipette tip for each transfer.
3. Incubate tubes at 95°C for at least 15 min, but for no more than 20 min, in the dry-heat bath.
4. Prepare the enzyme mix by adding 1.4 ml of enzyme dilution buffer to the lyophilized enzyme reagent. Swirl to mix. Do not vortex.
5. Transfer the tubes to the 42°C (±1°C) dry-heat bath or water bath and incubate for 5 min. Do not allow tubes to cool at room temperature. Do not cover the water bath.
6. Add 25 μl of enzyme mix to each amplification tube while the tubes are at 42°C (±1°C). Shake to mix. Incubate at 42°C for at least 2 h but no more than 3 h. Sealing cards or snap caps should be used during this step. Do not cover the water bath.
7. After the 2-h incubation, tubes may be placed at 2 to 8°C for up to 2 h or at −20°C overnight.

G. Termination

1. Add 20 μl of termination reagent to each tube. Cover tubes and shake to mix. Incubate at 42°C for 10 min.
2. Tubes may be covered and placed at 2 to 8°C for up to 2 h or at −20°C overnight.

H. Hybridization

1. Reconstitute lyophilized probe reagent with hybridization buffer. Vortex the solution until clear. The reconstituted probe reagent is stable for 1 month at 2 to 8°C.
2. Place 100 μl of hybridization positive control and 100 μl of hybridization negative control into correspondingly labeled 12- by 75-mm polypropylene tubes.
3. Add 100 μl of reconstituted probe reagent to each tube. Cover the tubes and vortex eight times until the reaction mixture is uniformly yellow.
4. Incubate 60°C for at least 15 min, but no more than 20 min, in a dry-heat or water bath.

I. Selection

1. Remove tubes from the 60°C water bath or dry-heat bath and add 300 μl of selection reagent. Cover tubes and vortex three times until the reaction mixture is uniformly pink.
2. Incubate tubes at 60°C for at least 10 min, but no more than 11 min, in a dry-heat or water bath.
3. Remove tubes from water bath or dry-heat bath and cool at room temperature for at least 5 min but no more than 1 h.

J. Detection

1. Select the appropriate protocol from the menu of the luminometer software. Use a 2-s read time.
2. Wipe each tube and insert into the luminometer. Tubes must be read within 1 h of step V.I.3.
3. When the analysis is complete, remove the tubes from the luminometer.
4. Decontaminate tubes and work surfaces with bleach.

V. PROCEDURE *(continued)*

K. Procedure notes

1. Reagents

a. Enzyme reagent should not be held at room temperature for more than 15 min after it is reconstituted.

b. Hybridization buffer may precipitate. Warming and mixing at 60°C will dissolve the precipitate.

2. Temperature

a. The amplification, hybridization, and selection reactions are temperature dependent; ensure that the water bath or dry-heat bath is maintained within the specified temperature range.

b. The tubes must be cooled at 42°C for 5 min before addition of enzyme mix for optimal performance.

c. The temperature is critical for the amplification (42 ± 1°C).

3. Water bath

a. The level of water in the water bath must be maintained so that the entire liquid volume in the tubes is submerged.

b. During the amplification step, water bath covers should not be used to ensure that condensation cannot drip into or onto the tubes.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. If the MTD test result is positive, report the following: "*Mycobacterium tuberculosis* complex rRNA detected. AFB culture pending. Specimen may contain *M. tuberculosis* alone or in combination with a nontuberculosis mycobacterium."

B. If the MTD test is negative, report the following: "No *Mycobacterium tuberculosis* complex rRNA detected. AFB culture pending. Specimen may not contain *M. tuberculosis*, or result may be falsely negative owing to low numbers of *M. tuberculosis* organisms in the presence of other mycobacterial species, or interference with assay detection by specimen inhibitors."

C. Document all testing in hard copy or computerized work card.

D. Call positive results to requesting area and to infection control. Note on the worksheet the name of the person called, date, and time, and initial.

VII. INTERPRETATION

The results of the MTD test are based on a 30,000-RLU cutoff. Samples producing signals greater than or equal to the cutoff value are considered positive. Samples producing signals less than the cutoff value are considered negative.

A. If the controls do not yield the expected results, test results on patient specimens in the same run must not be reported.

B. A value of ≥30,000 RLU is considered positive for *M. tuberculosis.*

C. A value of <30,000 RLU is considered negative for *M. tuberculosis.*

VIII. LIMITATIONS OF TESTING

A. The Gen-Probe MTD test has been validated by using sputum or bronchoalveolar lavage specimens that have been liquefied, concentrated, and decontaminated with either *N*-acetyl-L-cysteine-NaOH or NaOH. Performance with other specimens has not been fully evaluated and may result in false-negative or -positive results.

VIII. LIMITATIONS OF TESTING *(continued)*

B. Detection of *M. tuberculosis* is dependent on the number of organisms present in the specimen. This may be affected by specimen collection methods and patient factors such as age, history of respiratory disease, presence of symptoms, etc. The enhanced MTD test (500 ml of decontaminated sediment) is FDA approved for testing both AFB-positive and -negative respiratory specimens.

C. A negative test does not exclude the possibility of infection.

D. Therapeutic success or failure cannot be determined by using this assay.

REFERENCE

1. **CLSI.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. CLSI, Wayne, PA.

SUPPLEMENTAL READING

Bergmann, J. S., G. Yuoh, G. Fish, and G. L. Woods. 1999. Clinical evaluation of the enhanced Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test for rapid diagnosis of tuberculosis in prison inmates. *J. Clin. Microbiol.* **37:**1419–1425.

Bodmen, T., E. Mockl, K. Michlemann, and L. Matter. 1996. Improved performance of Gen-Probe Amplified Mycobacterium Direct Test when 500 instead of 50 microliters of decontaminated sediment is used. *J. Clin. Microbiol.* **34:**222–223.

Centers for Disease Control and Prevention. 2009. Updated guidelines for the use of nucleic acid amplification tests in the diagnosis of tuberculosis. *Morb. Mortal. Wkly. Rep.* **58:**7–10.

Greco, S., E. Girardi, A. Navarra, and C. Saltini. 2006. Current evidence on diagnostic accuracy of commercially based nucleic acid amplification tests for the diagnosis of pulmonary tuberculosis. *Thorax* **61:**783–790.

Woods, G. L. 2001. Molecular techniques in mycobacterial detection. *Arch. Pathol. Lab. Med.* **125:**122–126.

PART 4

Quantitative Detection of Human Immunodeficiency Virus Type 1 (HIV-1) RNA by Using the Abbott RealTi*m*e HIV-1 Assay

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

In the United States, there are currently four manufacturers of nucleic acid amplification tests for quantitative detection of human immunodeficiency virus type 1 (HIV-1) (Table 12.1–2). A more detailed analysis of the test systems available is presented in Table 12.2.3–2. The procedure described herein features the Abbott RealTi*m*e HIV-1 assay and is intended to provide the reader with a general outline of the methodology and work flow; it does not represent the full operational detail provided by the manufacturer. For the most up-to-date information, the reader is advised to consult the manufacturer and to obtain a package insert (1).

The Abbott RealTi*m*e HIV-1 assay is a real-time nucleic acid amplification test that uses reverse transcription-PCR (RT-PCR) to generate amplified product from the RNA genome of HIV-1 in clinical specimens. Results are reported in a quantitative manner. An RNA sequence that is unrelated to the HIV-1 target sequence is introduced into each specimen at the beginning of sample preparation. This unrelated RNA sequence is simultaneously amplified by RT-PCR and serves as an internal control (IC) to demonstrate that the process has proceeded correctly for each sample. The amount of HIV-1 target RNA sequence that is present at each amplification cycle is measured, through the use of fluorescently labeled oligonucleotide probes, on the Abbott *m*2000rt instrument. The probes do not generate a signal unless they are specifically bound to the amplified product. The amplification cycle at which a fluorescent signal is detected by the Abbott *m*2000rt is proportional to the log of the HIV-1 RNA concentration present in the original sample.

Table 12.2.3–2 Commercially available, FDA-cleared HIV-1 quantitative tests[a]

Manufacturer	Assay	Method	Specimen type	Reportable range	Subtype detection	Instrumentation	IC/quantitation
Abbott Molecular, Inc., Des Plaines, IL	Abbott RealTi*m*e HIV-1	Real-time RT-PCR	Plasma (frozen, EDTA, or ACD-A)	40–10,000,000 copies/ml	Group M subtypes A–H, group O, and group N	*m*2000sp—nucleic acid extraction *m*2000rt—real-time PCR	Internal amplification control added to all specimens Quantitation based on external standard curve
bioMérieux, Inc., Durham, NC	NucliSens HIV-1 QT	NASBA	Plasma (EDTA, citrate, or heparin); whole blood must be centrifuged within 24 h	51–5,390,000 copies/ml	Group M subtypes A–H; only adequately validated with HIV-1 subtype B specimens	NucliSens Reader	Internal quantitative standards (calibrators Qa, Qb, Qc) added to each specimen
Roche Molecular Systems, Inc., Branchburg, NJ	COBAS AmpliPrep/ COBAS TaqMan HIV-1 Test	Real-time RT-PCR	Plasma (EDTA); whole blood must be centrifuged within 6 h	48–10,000,000 copies/ml	Group M subtypes A–H	AmpliPrep—nucleic acid extraction COBAS Taqman Analyzer—real-time PCR	Internal quantitative standard added to each specimen
	Amplicor HIV-1 Monitor Test, version 1.5	RT-PCR	Plasma (EDTA or ACD); whole blood must be centrifuged within 6 h	Routine: 400–750,000 copies/ml Ultrasensitive: 50–100,000 copies/ml	Group M subtypes A–H	Manual extraction Applied Biosystems GeneAmp PCR System 9600 or 2400 thermal cycler COBAS Amplicor—PCR and detection	Internal quantitative standard added to each specimen
Siemens Healthcare Diagnostics, Tarrytown, NY	VERSANT HIV RNA 3.0 Assay	bDNA	Plasma (EDTA, PPT, or ACD-A); whole blood must be centrifuged within 4 h	75–500,000 copies/ml	Group M subtypes A–G	System 340 bDNA Analyzer VERSANT 440 Molecular System	None; quantitation based on external standard curve

[a] This table contains examples of FDA-cleared or -approved nucleic acid tests that are commercially available in the United States. It is not intended to be all-inclusive and does not contain tests used for screening of blood products. Websites of the manufacturer are useful sources of the most up-to-date information. Abbreviations: NASBA, nucleic acid sequence-based amplification; bDNA, branched-chain DNA; PPT, plasma preparation tube.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. The Abbott RealTi*m*e HIV-1 assay is for use with plasma samples only.

2. Blood should be collected in sterile tubes with EDTA or acid citrate dextrose A (ACD-A).

3. When monitoring viral levels in an individual patient, it is advisable to use the same anticoagulant with each draw.

B. Time of specimen collection

There are no particular issues related to timing of collection.

C. Specimen transport

1. Whole blood may be held at 15 to 30°C for up to 6 h or at 2 to 8°C for up to 24 h, prior to centrifugation.

2. After centrifugation, remove plasma from cells and store at −70°C or lower. Multiple freeze-thaw cycles should be avoided and should not exceed three freeze-thaw cycles.

3. Thaw plasma specimens at 15 to 30°C or at 2 to 8°C. Once thawed, if plasma specimens are not being processed immediately, they can be stored at 2 to 8°C for up to 6 h. Ship specimens frozen on dry ice.

4. Fresh plasma has not been validated for use in the assay.

D. Specimen labeling and request submission

The laboratory should determine a mechanism to ensure that the specimen type and time of collection are recorded.

E. Rejection criteria

1. Test only plasma collected in EDTA or ACD-A. Testing of other specimen types or use of other transport media must be validated by the user prior to reporting results (2).

2. Specimens not meeting the stability or storage requirements listed above should be rejected.

3. If an improperly collected, transported, or handled specimen cannot be replaced, testing may be considered after consultation with the health care provider. Document in the final report that specimen quality may have been compromised.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Materials provided

1. Abbott RealTi*m*e HIV-1 amplification reagent kit

2. Abbott RealTi*m*e HIV-1 calibrator kit

3. Abbott RealTi*m*e HIV-1 control kit

B. Materials required but not provided (each available separately)

1. Sample preparation area

a. Abbott *m*2000sp instrument

b. Abbott *m* sample preparation system (4 × 24 preps)

c. 5-ml reaction vessels

d. 13- to 16-mm sample tubes

e. 200-ml reagent vessels

f. 200-μl disposable tips

g. 1,000-μl disposable tips

h. Abbott optical adhesive covers

i. Abbott adhesive cover applicators

j. Abbott splash-free support base

k. Abbott 96-deep-well plate

l. Abbott RealTi*m*e HIV-1 *m*2000 System Combined Application CD-ROM

m. Abbott 96-well optical reaction plate

n. Aerosol barrier pipette tips for 20- to 1,000-μl pipettes

o. Calibrated pipettes capable of delivering 20 to 1,000 μl

p. Centrifuge capable of 2,000 × *g*

q. Master mix tube

r. Vortex mixer

2. Amplification area

a. Abbott *m*2000rt instrument

b. Abbott RealTi*m*e HIV-1 *m*2000 System Combined Application CD-ROM

c. Abbott *m*2000rt optical calibration kit

III. MATERIALS *(continued)*

C. Other materials

1. Biological safety cabinet approved for working with infectious materials
2. Sealable plastic bags
3. RNase-free water
4. 1.7-ml RNase-free microcentrifuge tubes
5. Cotton tip applicators

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Instrument calibration

Optical calibration of the Abbott *m*2000rt instrument is required for the accurate measurement and discrimination of dye fluorescence.

1. The following Abbott *m*2000rt optical calibration plates are used to calibrate the Abbott *m*2000rt instrument for the Abbott RealTi*m*e HIV-1 assay.
   - **a.** FAM plate (carboxyfluorescein)
   - **b.** ROX plate (carboxy-X-rhodamine)
   - **c.** VIC plate (proprietary dye)

B. Assay calibration

For a detailed description of how to perform an assay calibration, refer to the Abbott *m*2000sp and *m*2000rt operations manuals, "Operating Instructions" sections.

1. A calibration curve is required to quantitate the HIV-1 RNA concentration of specimens and controls. Two calibrators are run in replicates of three to generate a calibration curve (HIV-1 concentration versus the threshold cycle [Ct] at which a reactive level of fluorescent signal is detected).
2. The calibration curve slope and intercept are calculated and stored on the instrument. The concentration of HIV-1 RNA in a sample is calculated from the stored calibration curve. Results are automatically reported on the *m*2000rt workstation.
3. Once an Abbott RealTi*m*e HIV-1 calibration is accepted and stored, it may be used for 6 months. During this time, all subsequent samples may be tested without further calibration unless one of the following applies.
   - **a.** An Abbott RealTi*m*e HIV-1 amplification reagent kit with a new lot number is used.
   - **b.** An Abbott *m* sample preparation system (4 × 24 preps) with a new lot number is used.
   - **c.** An Abbott RealTi*m*e HIV-1 application file for a different sample volume is used.
   - **d.** A new Abbott RealTi*m*e HIV-1 application specification file is installed.

C. Detection of inhibition

An IC Ct assay validity parameter is established during a calibration run.

1. A defined, consistent quantity of IC is introduced into each specimen, calibrator, and control at the beginning of sample preparation and detected on the Abbott *m*2000rt instrument to demonstrate proper specimen processing and assay validity.
2. The median amplification cycle at which the IC target sequence fluorescent signal is detected in calibration samples establishes an IC Ct validity range to be met by all subsequent processed specimens and controls.
3. An error is displayed when a specimen or control fails to meet this specification. Refer to the *m*2000rt operations manual for an explanation of the corrective actions for the error.
4. Specimens whose IC Ct exceeds the established range must be retested starting with sample preparation.

IV. QUALITY CONTROL
(continued)

D. Negative and positive controls

1. The negative control, a low positive control, and a high positive control (provided with the kit) must be included in each test run to evaluate run validity. The lot-specific values for the low positive control and high positive control are specified on each Abbott RealTi*m*e HIV-1 control kit card and must be entered into the assay test order when a run is performed.
2. An error is displayed when a kit control result is out of range. Refer to the Abbott *m*2000rt operations manual for an explanation of the corrective actions for the error.
3. If negative or positive kit controls are out of range, all of the specimens and controls from that run must be reprocessed, beginning with sample preparation.
4. External controls may be included in each run. Control material can be previously tested patient samples or commercially available products. An acceptable value range should be established for the control and tracked with each assay run. Values outside the established range should be reviewed prior to reporting patient results.
5. The presence of HIV-1 must not be detected in the negative control. HIV-1 detected in the negative control is indicative of contamination by other samples or by amplified product introduced during sample preparation or during preparation of the Abbott 96-well optical reaction plate. To avoid contamination, clean the Abbott *m*2000sp instrument and the Abbott *m*2000rt instrument and repeat sample processing for controls and specimens.

V. PROCEDURE

Observe standard precautions.

A. Reagent and calibrator preparation

◩ **NOTE:** For a detailed description on how to operate the Abbott *m*2000sp instrument or the Abbott *m*2000rt instrument, refer to the Abbott *m*2000sp and *m*2000rt operations manuals, "Operating Instructions" sections.

1. Thaw assay controls and the IC at 15 to 30°C or at 2 to 8°C. Thaw calibrators at 15 to 30°C or at 2 to 8°C only if performing a calibration run. Once thawed, assay controls, the IC, and calibrators can be stored at 2 to 8°C for up to 24 h before use.
2. Vortex each assay calibrator and each control three times for 2 to 3 s. Ensure that the contents of each vial are at the bottom after vortexing by tapping the vials on the bench to bring liquid to the bottom of the vial. Ensure that bubbles or foam are not generated; if present, remove with a sterile pipette tip, using a new tip for each vial.
3. Thaw amplification reagents at 15 to 30°C or at 2 to 8°C and store at 2 to 8°C until required for the amplification master mix procedure. Once thawed, the amplification reagents can be stored at 2 to 8°C for up to 24 h if not used immediately.

   ◩ **NOTE:** Use one bottle of *m*Lysis buffer, one vial of IC, and one RealTi*m*e HIV-1 amplification reagent pack to support up to 24 reactions. Use a second set of reagents to support 25 to 48 reactions. A maximum of 48 reactions can be performed per run.
4. Gently invert the Abbott *m* sample preparation bottles to ensure a homogeneous solution without generating any bubbles. If crystals are observed in any of the reagent bottles upon opening, allow the reagent to equilibrate at room temperature until the crystals disappear. Do not use the reagents until the crystals have dissolved. Ensure that bubbles or foam are not generated; if present, remove with a sterile pipette tip, using a new tip for each bottle.
5. Vortex each IC vial three times for 2 to 3 s before use. Ensure that bubbles or foam are not generated; if present, remove with a sterile pipette tip, using a new tip for each vial.

V. PROCEDURE *(continued)*

6. Use a calibrated precision pipette *dedicated for IC use only* to add 500 µl of the IC to each bottle of *m*Lysis buffer. Mix by gently inverting the container 5 to 10 times to minimize foaming.

B. Specimen preparation

1. Allow for a maximum of 48 samples for each run. A negative control, a low positive control, and a high positive control are included in each run, therefore allowing a maximum of 45 specimens to be processed per run.
2. Thaw specimens at 15 to 30°C or at 2 to 8°C. Once thawed, specimens can be stored at 2 to 8°C for up to 6 h if not processed immediately.
3. Check sample volume.
   The Abbott RealTi*m*e HIV-1 assay minimum sample volume and associated rack requirements on the Abbott *m*2000sp are described below.
   Caution: *Do not put a 13-mm tube in a 16-mm rack.*

		Abbott RealTi*m*e HIV-1 minimum sample volume: assay application		
Rack	Tube diameter	0.2 ml	0.5 ml	1.0 ml
13 mm	11.6–14.0 mm	0.7 ml	1.0 ml	1.5 ml
16 mm	15.0–16.0 mm	1.0 ml	1.3 ml	1.8 ml

   Caution: *Steps V.B.4 and V.B.5 must be done in the order listed. Vortex the specimens first, and follow with centrifugation. If these two steps are not performed in this order, then invalid results may occur.*
4. Vortex each specimen three times for 2 to 3 s.
5. Centrifuge specimens at 2,000 × *g* for 5 min before loading on the Abbott *m*2000sp worktable.
6. Aliquot each specimen into clean tubes or vials if necessary. Refer to the Abbott *m*2000sp operations manual for tube sizes. Avoid touching the inside of the cap when opening tubes. Take care not to disturb the contents of the tube while removing the tube from the centrifuge so that the bottom of the tube is not touched by the pipette tip. Ensure that the newly aliquoted sample retains the minimum volume indicated in the preceding table.
7. Place the low and high positive controls, the negative control, the calibrators (if applicable), and the patient specimens into the Abbott *m*2000sp sample rack.
8. Place the 5-ml reaction vessels into the *m*2000sp 1-ml subsystem carrier.

C. Reaction plate assembly, amplification, and detection

1. Load the Abbott *m* sample preparation system reagents and the Abbott 96-deep-well plate on the Abbott *m*2000sp worktable as described in the Abbott *m*2000sp operations manual, "Operating Instructions" section.
2. Select the appropriate application file from the Run Sample Extraction screen that corresponds to the sample volume being tested. Initiate the sample extraction protocol as described in the Abbott *m*2000sp operations manual, "Operating Instructions" section.
3. Enter calibrator (needed if a calibration curve has not been stored on the *m*2000rt) and control lot-specific values in the Sample Extraction: Worktable Setup, Calibrator and Control fields. Lot-specific values are specified in each Abbott RealTi*m*e HIV-1 calibrator and control kit card.
   ◩ **NOTE:** The Abbott *m*2000sp master mix addition protocol (step C.7) must be initiated within 1 h after completion of sample preparation.
   ◩ **NOTE:** Change gloves before handling the amplification reagents.

V. PROCEDURE *(continued)*

4. Load the amplification reagents and the master mix vial on the *m*2000sp worktable after sample preparation is completed. Each amplification reagent pack supports up to 24 reactions.
   ◩ **NOTE:** A second amplification reagent pack is required if performing 25 to 48 reactions.
5. Ensure that the contents are at the bottom of the vials prior to opening the amplification reagents by tapping the vials in an upright position on the bench.
6. Remove and discard the amplification vial caps.
7. Select the appropriate deep-well plate from the run master mix addition screen that matches the corresponding sample preparation extraction. Initiate the Abbott *m*2000sp master mix addition protocol. Follow the instructions as described in the Abbott *m*2000sp operations manual, "Operating Instructions" section. The *m*2000rt protocol must be started within 40 min of the initiation of the master mix addition protocol.
8. Switch on and initialize the Abbott *m*2000rt instrument in the amplification area.
   ◩ **NOTE:** The Abbott *m*2000rt instrument requires 15 min to warm up.
   ◩ **NOTE:** Remove gloves before returning to the sample preparation area.
9. Seal the Abbott 96-well optical reaction plate after the Abbott *m*2000sp instrument has completed addition of samples and master mix according to the Abbott *m*2000sp operations manual, "Operating Instructions" section. Export completed PCR plate results to a CD.
10. Place the sealed optical reaction plate into the splash-free support base for transfer to the Abbott *m*2000rt instrument.
11. Place the Abbott 96-well optical reaction plate in the Abbott *m*2000rt instrument. Import the *m*2000sp test order via CD per the import order instructions in the Abbott *m*2000rt operations manual, "Operating Instructions" section.

D. Postprocessing procedures

1. Remove the Abbott 96-deep-well plate from the worktable and dispose of according to the Abbott *m*2000sp operations manual.
2. Place the Abbott 96-well optical reaction plate in a sealable plastic bag and dispose of according to the Abbott *m*2000rt operations manual, along with the gloves used to handle the plate.
3. Clean the splash-free support base before the next use according to the Abbott *m*2000rt operations manual.

E. Calculation

The concentration of viral HIV-1 RNA in a sample or control is calculated from the stored calibration curve.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. The Abbott *m*2000rt instrument automatically reports the results on the Abbott *m*2000rt workstation.

B. The instrument can report assay results as copies/milliliter, log copies/milliliter, international units/milliliter, or log international units/milliliter (1 IU = 0.58 copy; 1 copy = 1.74 IU), with WHO 1st International Standard for HIV-1 RNA (code 97/656).

C. Consistent use of units for reporting of results is important so that changes in viral load can be assessed easily.

VII. INTERPRETATION

A. A result of "not detected" signifies that no target was detected.

B. A result of <1.60, <1.88, or <2.18 log copies/ml indicates that the target was detected but is less than the lower limit of quantitation (LLQ) for the respective input volumes of 1.0, 0.5, and 0.2 ml.

C. For a 1.0-ml input volume, a result of 1.60 to 7.00 log copies/ml indicates that the target was detected and the concentration falls between 1.6 log copies per ml (LLQ) and 7.0 log copies per ml (upper limit of quantitation [ULQ]).

D. For a 0.5-ml input volume, a result of 1.88 to 7.00 log copies/ml indicates that the target was detected and the concentration falls between 1.88 log copies per ml (LLQ) and 7.0 log copies per ml (ULQ).

E. For a 0.2-ml input volume, a result of 2.18 to 7.00 log copies/ml indicates that the target was detected and the concentration falls between 2.18 log copies per ml (LLQ) and 7.0 log copies per ml (ULQ).

F. Note that no interpretation is reported on the *m*2000rt printout when results fall between the LLQ and ULQ.

G. A result of >7.00 log copies/ml indicates that the target was detected and is greater than the ULQ.

Sample volume	Result	Interpretation
1.0 ml	Not detected	Target not detected
	<1.60 log copies/ml (40 copies/ml)	Detected
	1.60 to 7.00 log copies/ml	
	>7.00 log copies/ml	>ULQ
0.5 ml	Not detected	Target not detected
	<1.88 log copies/ml (75 copies/ml)	Detected
	1.88 to 7.00 log copies/ml	
	>7.00 log copies/ml	>ULQ
0.2 ml	Not detected	Target not detected
	<2.18 log copies/ml (150 copies/ml)	Detected
	2.18 to 7.00 log copies/ml	
	>7.00 log copies/ml	>ULQ

VIII. LIMITATIONS OF TESTING

A. A specimen with a result of "target not detected" cannot be presumed to be negative for HIV-1 RNA.

B. As with any diagnostic test, results from the Abbott RealTi*m*e HIV-1 assay should be interpreted in conjunction with other clinical and laboratory findings.

C. To reduce the risk of nucleic acid contamination, clean and disinfect spills of specimens by including the use of a disinfectant such as 1.0% sodium hypochlorite or another suitable disinfectant.

D. It is recommended that the laboratory be monitored for the presence of amplicon at least once a month. Refer to package insert for details.

E. The limits of detection for the Abbott RealTi*m*e HIV-1 assay are 40 copies/ml with a 1.0-ml sample volume, 75 copies/ml with a 0.5-ml sample volume, and 150 copies/ml with a 0.2-ml sample volume.

F. The ULQ is 10 million copies/ml, and the LLQ is equivalent to the limit of detection (*see* VIII.E).

G. The RealTi*m*e HIV assay was evaluated for detection of subtypes/groups with purified RNA transcripts from group M (subtypes A, B, C, D, CRF01-AE, F, CRF02-AG, G, and H), group O, and group N, and by testing 10 clinical specimens of each group M subtype (A, B, C, D, CRF01-AE, F, CRF02-AG, and

VIII. LIMITATIONS OF TESTING *(continued)*

G) and 10 specimens from group O. The results showed that all subtypes and groups tested were detected, and dilution linearity was demonstrated for all groups and subtypes tested (correlation coefficients ranged from 0.997 to 1.000).

H. No interference in the performance of the Abbott RealTi*m*e HIV-1 assay was observed in the presence of the following substances for all positive and negative samples tested: hemoglobin (500 mg/dl), triglycerides (3,000 mg/dl), bilirubin (20 mg/dl), and protein (9 g/dl).

I. Drugs at concentrations in excess of the peak levels in plasma or serum were tested in five pools (see below). No interference in the performance of the Abbott RealTi*m*e HIV-1 assay was observed in the presence of the following drug pools for all positive and negative samples tested.

Drug pool	Drugs tested
1	Zidovudine, saquinavir, ritonavir, clarithromycin, interferon 2a, interferon 2b
2	Abacavir sulfate, amprenavir, peginterferon 2a, peginterferon 2b, ribavirin
3	Tenofovir disoproxil fumarate, lamivudine, indinavir sulfate, ganciclovir, valganciclovir hydrochloride, acyclovir
4	Stavudine, efavirenz, lopinavir, enfuvirtide, ciprofloxacin
5	Zalcitabine, nevirapine, nelfinavir, azithromycin, valacyclovir

REFERENCES

1. **Abbott Molecular Inc.** 2007. Abbott RealTi*m*e HIV-1 package insert, 51-603256/R2 May 2007. Abbott Molecular Inc., Des Plaines, IL.
2. **Federal Register.** 2008. Code of Federal Regulations. Medicare, Medicaid and CLIA Programs; laboratory requirements relating to certain personnel qualifications; final rule. 68 FR 50724, p. 1083–1084. http://edocket.access.gpo.gov/cfr_2008/octqtr/pdf/42cfr493.1253.pdf. Accessed 28 September 2009.

SUPPLEMENTAL READING

Centers for Disease Control and Prevention. 2006. Revised recommendations for HIV testing of adults, adolescents, and pregnant women in health-care settings. *MMWR Morb. Mortal. Wkly. Rep.* **55**(RR-14)**:**1–13.

Clinical and Laboratory Standards Institute/ NCCLS. 2003. *Quantitative Molecular Methods for Infectious Diseases; Approved Guideline MM6-A.* NCCLS, Wayne, PA.

Clinical and Laboratory Standards Institute/ NCCLS. 2006. *Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline MM3-A2.* NCCLS, Wayne, PA.

Panel on Antiretroviral Guidelines for Adults and Adolescents. 3 November 2008. *Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents,* p. 1–139. Department of Health and Human Services, Washington, DC. http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf. Accessed 2 February 2009.

Schutten, M. 2008. Comparison of the Abbott Realtime HIV-1 and HCV viral load assays with commercial competitor assays. *Expert Rev. Mol. Diagn.* **8:**369–377.

Xu, S., A. Song, X. Li, J. Li, Z. Bao, P. Mao, Q. Zhao, and Y. Wang. 2008. Performance of the Abbott RealTime HIV-1 assay for quantification of HIV-1 clades prevalent in China. *J. Clin. Virol.* **41:**305–309.

PART 5

Quantitative Measurement of Human Immunodeficiency Virus Type 1 RNA by Using the Roche Amplicor Ultrasensitive PCR Kit

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The Amplicor HIV-1 Ultrasensitive test is an in vitro nucleic acid amplification test for the quantitation of human immunodeficiency virus type 1 (HIV-1) RNA in human plasma. In order to monitor the effectiveness of the highly active antiretroviral therapy regimens now in use, the sensitivity of the HIV-1 Ultrasensitive test has been increased so that quantitation of viral load down to 40 copies per ml is now possible.

The Amplicor HIV-1 Ultrasensitive test is based on five major processes: specimen preparation, reverse transcription of RNA to generate a cDNA, PCR to target cDNA using HIV-1-specific primers, hybridization of the amplified products to specific oligonucleotide probes, and detection of the probe-bound amplified product by colorimetric determination.

The amount of HIV-1 RNA in each specimen is calculated from the ratio of the total optical density (OD) for the HIV-1-specific well to the total OD for the quantitation standard (QS)-specific well and the input number of QS RNA molecules using the following equation:

$$\left(\frac{\text{Total HIV-1 OD}}{\text{Total QS OD}}\right) \times \text{Input QS copies per PCR} \times 4 = \text{HIV-1 RNA copies/ml}$$

Because of the sensitivity of the PCR method, it is necessary to limit the potential for contamination by performing each step of the process in a separate area of the laboratory and to dedicate equipment to each of the areas.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. Plasma only, collected in acid citrate dextrose (ACD) (yellow top)
2. EDTA (lavender top) tubes are acceptable but will yield test results that are approximately 15% higher than those collected in ACD. If only EDTA blood is available, add the following footnote to the final report: "Specimen collected in EDTA rather than ACD. ACD is the anticoagulant of choice. Use of EDTA anticoagulant will yield result 15% higher than test results obtained with ACD anticoagulant."
3. Store whole blood at 2 to 25°C for no longer than 3 h.
4. Patients must have a previous result by the standard HIV method of <400 copies/ml. For other requests refer to the laboratory director.

B. Timing of specimen collection

There are no particular timing issues to consider in collecting these specimens; however, the time of collection should be recorded.

C. Specimen transport

1. Transport the specimen at room temperature.
2. Within 3 h of blood draw, blood collection tubes must be centrifuged at 800 to 1,600 × *g* for 20 min at room temperature.
3. Make four aliquots by transferring a minimum of 600 μl of plasma to a sterile 1.5-ml polypropylene tube and store at 2 to 8°C for up to 5 days. For longer periods, store at −20 to −80°C.

D. Specimen labeling and submission

The laboratory should determine a mechanism to ensure that the time of collection is recorded.

E. Rejection criteria

1. Test only plasma.
2. Reject heparinized specimens.
3. Reject specimens that arrive in leaky containers.
4. Reject whole-blood specimens that have been refrigerated and/or are received more than 3 h after collection.
5. Request a repeat specimen, or obtain the information, when collection time has not been provided.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

6. If an improperly collected, transported, or handled specimen cannot be replaced, document in the final report that specimen quality may have been compromised.

III. MATERIALS

(See Part 4 above for materials provided.)

Include QC information on reagent container and in QC records.

A. Area 1: reagent preparation
- **1.** Amplicor HIV-1 amplication kit
- **2.** Dedicated lab coat
- **3.** Powder-free gloves
- **4.** 10% Bleach, prepared fresh daily
- **5.** Sanicloth disposable wipes
- **6.** Pipette with plugged pipette tips (100 µl)
- **7.** MicroAmp tray, base, and tray and retainer
- **8.** Repeat pipettor with 1.25-ml sterile Combitips
- **9.** Plastic baggie

B. Area 2: specimen preparation
- **1.** Juon high-speed refrigerated centrifuge
- **2.** Amplicor HIV specimen preparation kit
- **3.** HIV Accurun Control Series 100 (Boston Biomedia)
- **4.** HIV Accurun Control Series 400 (Boston Biomedia)
- **5.** HIV Accurun Negative Control (Boston Biomedia)
- **6.** Dedicated lab coat
- **7.** Powder-free gloves
- **8.** 10% Bleach, prepared fresh daily
- **9.** Sanicloth disposable wipes
- **10.** 95% Ethyl alcohol in glass (Sigma) (freshly diluted to 70% using sterile tissue culture-grade water)
- **11.** Isopropyl alcohol, reagent grade (Fisher)
- **12.** Pipetaide
- **13.** Sterile disposable polystyrene pipettes (5 and 10 ml)
- **14.** 1.5-ml screw-cap microcentrifuge tubes
- **15.** 60 ± 2°C dry-heat block
- **16.** Sterile fine-tip transfer pipettes (graduated)
- **17.** Vortex mixer
- **18.** Test tube racks
- **19.** Microcentrifuge (maximum relative centrifugal force [RCF], 16,000 $\times$ *g*; minimum RCF, 12,500 $\times$ *g*)
- **20.** Pipette with plugged pipette tips (50, 200, and 1,000 µl)
- **21.** MicroAmp tube caps and capping tools

C. Area 3: amplication and detection
- **1.** Amplicor HIV detection kit
- **2.** Dedicated lab coat
- **3.** 10% Bleach, prepared fresh daily
- **4.** Powder-free gloves
- **5.** Thermal cycler with printer
- **6.** Spatula or tool to remove tube caps
- **7.** Multichannel pipette with plugged pipette tips (200 µl)
- **8.** Pipette with plugged pipette tips (25 µl)
- **9.** Disposable reagent reservoirs
- **10.** 1-liter Erlenmeyer flask
- **11.** 100-ml graduated cylinder
- **12.** Type 1 water
- **13.** 37°C (± 2°C) incubator
- **14.** Sterile disposable 96-well plate lid
- **15.** Microwell plate washer
- **16.** Sterile 15-ml polypropylene centrifuge tubes
- **17.** ELISA plate reader

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. For each batch of specimen processed include a low positive control, a high positive control, and one negative control. Process the controls in the exact same manner as the patient specimens.

B. Specimens and controls from separate preparation batches may be amplified and detected at the same time. Each separate specimen batch is validated individually by the set of serum controls for that batch.

C. All test specimens and controls prepared in the same batch should be amplified and detected in adjacent positions in the thermal cycler and on the detection plate. The exact order of the placement is not critical.

D. All controls should yield OD values from the QS that meet the criteria described in V.E and VII.C. If any control has QS OD values that do not meet the criteria, the entire run is invalid.

IV. QUALITY CONTROL
(continued)

E. The negative control must be <40 copies/ml. The high positive control must be between 10,000 and 24,000 copies/ml, and the low positive control must be between 40 and 100 copies/ml.

F. The thermal cycler run QC log must show that there was a successful run.

G. Verify that all reagents and materials meet expiration date and QC parameters as per NCCLS document M22-A2 (1).

V. PROCEDURE

Observe standard precautions.

A. Area 1: reagent preparation

1. Wipe out dead-air box with 10% bleach. Wipe out with a Sanicloth wipe.

2. Prepare working master mix by adding 100 µl of manganese solution to one tube of master mix. Recap the tube and mix well by inverting the tube 10 to 15 times. Discard the remaining manganese solution into an appropriate biosafety container.

3. Determine the number of PCR tubes needed for patient samples and controls, and place in MicroAmp sample tray and lock in place with retainer.

4. Pipette 50 µl of master mix into each PCR tube using a repeat pipettor with sterile Combitip or a micropipette with plugged tips. Inspect the reaction tubes after pipetting to ensure that the same volume was pipetted into each tube. Discard unused working master mix into an appropriate biosafety container.

5. Place tray in baggie and transport to area 2. Store at 2 to 8°C. Amplication must begin within 4 h of preparation of working master mix.

6. Clean out dead-air box with 10% bleach and wipe with a Sanicloth. Turn on UV light for 10 min.

◩ **NOTE:** Do not operate the UV light with the enclosure doors open. Do not place arms or hands in the enclosure when UV light is on. Do not look at the UV bulb while in operation without protective eyewear.

7. Record hood cleaning on hood QC sheet.

B. Area 2: specimen preparation

A precipitate forms in the HIV-1 lysis reagent upon storage at 2 to 8°C. Prior to use, dissolve the precipitate by warming the lysis reagent to 25 to 37°C and mixing thoroughly.

1. Precool the Juon high-speed centrifuge by turning it on, closing the lid, and setting the temperature gauge to 2 to 8°C. It will take approximately 45 min to cool down. To speed up the cooldown process, secure an empty rotor in the centrifuge and run on low speed at 2 to 8°C for 15 to 30 min.

2. Turn on the blower to the biological safety cabinet and allow it to run for 30 min prior to use.

3. Clean out the biological safety cabinet with 10% bleach and wipe with a Sanicloth.

4. Prepare 70% ethanol (mix 11.0 ml of ethanol, in glass, with 4.0 ml of tissue culture-grade water). One milliliter of 70% ethanol is needed for each sample processed.

5. Prepare the Ultrasensitive working lysis reagent.

a. Check that all crystals are dissolved in the lysis reagent.

b. Vortex the QS for at least 10 s.

c. Add 25 µl of QS to one bottle of lysis reagent.

◩ If processing only a partial plate, prepare only enough lysis reagent for the number of specimens being processed (i.e., for six samples add 12.5 µl of QS to 4.5 ml [1/2 bottle] of lysis reagent). Note on the remainder of the lysis reagent the volume that is left and the quantity of QS to add.

d. Mix working lysis reagent.

V. PROCEDURE *(continued)*

6. Label one 1.5-ml microcentrifuge tube for each specimen and control. Make a mark on the side of the tube to help in orientation in the centrifuge.
7. Fill out an HIV-Ultra worksheet. Record lot number and expiration dates. Record number of QS copies added.
8. Thaw controls and patient samples. Controls may be thawed three times without deleterious effects. Vortex all samples and controls for 3 to 5 s when thawed.
9. Spin patient samples and controls briefly to collect sample at the bottom of the tube.
10. Pipette 500 μl of each patient sample and control into the appropriately labeled tube using aerosol barrier pipette tips.
11. Place tubes in the high-speed centrifuge with the orientation mark facing out. Centrifuge for 1.5 h at 16,000 × *g* at 2 to 8°C.
12. Remove the tubes from the centrifuge. Leave the centrifuge lid open to warm up. Wipe out condensation with a paper towel.
13. Using a sterile fine-tip transfer pipette, carefully draw off the supernatant without disrupting the pellet. Maintain a continuous negative pressure as you draw off the liquid. *Up to 25 μl of the supernatant can remain in the tube without affecting the performance of the test.* Leaving fluid may be necessary to not disturb the pellet.
14. Add 600 μl of Ultrasensitive working lysis reagent to each tube. Cap the tubes and vortex for 10 to 15 s.
15. Incubate all tubes for 10 min at room temperature.
16. Add 600 μl of 100% isopropanol to each tube. Mix well by vortexing for 3 to 5 s.
17. Place tubes in the Eppendorf centrifuge with the orientation mark facing out. Centrifuge at 12,500 to 16,000 × *g* for 15 min at room temperature.
18. Using a new sterile fine-tip transfer pipette for each tube, carefully remove and discard the supernatant from each tube, being careful not to disturb the pellet. (Pellets may not be visible at this point.) Remove as much fluid as possible. Do not use vacuum aspiration.
19. Add 1.0 ml of 70% ethanol to each tube.
20. Place the tubes in the Eppendorf centrifuge with the orientation mark facing out. Centrifuge at 12,500 to 16,000 × *g* for 15 min at room temperature.
21. Using a new sterile fine-tip transfer pipette for each tube, carefully remove and discard the supernantant from each tube, being careful not to disturb the pellet. The pellet should be visible at this point. Do not use vacuum aspiration.
22. Place tubes in the Eppendorf centrifuge with the orientation mark facing out. Pulse spin for 1 to 2 s.
23. Remove residual ethanol with an aerosol barrier pipette tip and Pipetman pipettor. *Removing residual ethanol is important, as ethanol may inhibit amplification.*
24. Add 100 μl of specimen diluent to each tube. Recap the tubes and vortex for 10 to 15 s. Some insoluble material will remain.
25. Amplify the processed specimens within 2 h of preparation or store the processed samples at −20°C for up to a week.
26. If samples have been frozen, completely thaw, vortex for 1 to 10 s, and allow particulate material to settle.
27. Pipette 50 μl of prepared samples to the appropriate MicroAmp reaction tube. Do not transfer any precipitated material.
28. Cap the tubes and place in a baggie to transfer to area 3.
29. Clean out the biological safety cabinet with 10% bleach and wipe with a Sanicloth. Allow blower to run for at least 30 min after cleaning.

V. PROCEDURE *(continued)*

30. Record hood cleaning on hood QC sheet.

C. Area 3: amplification

1. Turn the thermal cycler on.

2. Remove MicroAmp tray from base.

3. Place MicroAmp tray into the thermal cycler block. Check to make sure the notch in the sample tray is at the left of the block and that the rim of the tray is seated in the channel around the block.

4. Slide the cover forward.

5. Turn the knob clockwise until hand tight. (The white mark on the cover knob should line up with the white mark on the cover.)

6. Program the thermal cycler as follows.
Program 13, HOLD program: 2 min at 50°C
Program 14, HOLD program: 30 min at 60°C
Program 36, CYCLE program (4 cycles): 10 s at 95°C, 10 s at 55°C, 10 s at 72°C
Program 37, CYCLE program (26 cycles): 10 s at 90°C, 10 s at 60°C, 10 s at 72°C
Program 18, HOLD program: 15 min at 72°C

7. Start the METHOD program (program runs about 1.7 h).

◩ **NOTE:** Specimens must be removed within 15 min of the start of the final HOLD program.

8. Remove the completed PCR sample tray from the thermal cycler and place in tray base. *Do not remove from area 3.*

9. Remove caps carefully to avoid aerosolizing PCR products.

10. *Immediately* add 100 µl of denaturation solution to each PCR tube using a multichannel pipette (program 1 on Amplicor pipettor) with plugged tips. The program will mix the samples up and down five times.

11. Incubate for 10 min at room temperature.
Store the denatured, amplified samples at room temperature only if the detection test will be performed within 1 to 2 h. If not, recap the samples and store at 2 to 8°C for up to 1 week.

12. Review the thermal cycler run parameters for HISTORY FILE and record on RUN QUALITY CONTROL LOG, or print run parameters on printer during run.

D. Area 3: detection

Warm all reagents to room temperature prior to use.

1. Prepare working wash solution by adding 1 volume of wash concentrate (10×) to 9 volumes of type 1 water. Mix well. If necessary, warm the concentrate at 30 to 37°C to redissolve any precipitate. The working wash solution should be made fresh weekly.

2. Allow reagents and microwell plate to warm to room temperature before using.

3. Add 100 µl of hybridization buffer to each well (program 2 on Amplicor pipettor). If the amplified samples were stored at 2 to 8°C, it may be necessary to incubate them at 37°C for 2 to 4 min to reduce viscosity. Rows A to F of the microwell plate are coated with HIV-specific probe; rows G and H are coated with the QS-specific probe.

4. Using plugged pipette tips and a multichannel pipettor (program 3), pipette 25 µl of denatured amplified samples from the amplification tray into row A of the microwell plate. Place the pipette tips at a 45° angle in the corner of the well. Mix by pipetting up and down 10 to 15 times, and then transfer 25 µl to the next row of wells. Repeat this procedure four more times, so as to generate five fivefold dilutions (1:5, 1:25, 1:125, 1:625, and 1:3,125) in rows B to F. Discard 25 µl from the last well after mixing.

V. PROCEDURE *(continued)*

5. Add 25 µl of denatured amplification reaction mixture from the amplification tray into the first row of green-rimmed microwells, row G, using the multichannel pipettor (program 3). Place the pipette tips at a 45° angle in the corner of the well. Mix by pipetting up and down 10 to 15 times, and then transfer 25 µl to the next row of wells. Repeat this procedure one more time, so as to generate one fivefold dilution (1:1, 1:5) in rows G to H. Discard 25 µl from the last well after mixing.
6. Place lid on tray and gently tap the plate 10 to 15 times until the color changes from blue to light yellow.
7. Cover plate and place in 37°C (±2°C) incubator for 1 h. Plates may be incubated for up to 1 h 30 min if time is necessary to stagger plate washing.
8. Wash plate five times manually or by microwell plate washer, using the prepared 1× washing solution.
   a. Manually (do not use squirt bottle):
      (1) Empty contents of plate, invert, and tap dry on paper towel.
      (2) Pipette working wash solution to fill each well (400 to 450 µl).
      (3) Soak for 30 s. Empty contents, invert plate, and tap dry on a paper towel.
      (4) Repeat steps V.D.8.a.(2) and (3) four additional times.
   b. Automated (program washer; Costar):
      (1) Aspirate contents of well.
      (2) Fill each well to top with working wash solution (400 to 450 µl), soak for 30 s, and aspirate dry.
      (3) Repeat step V.D.8.b.(2) four additional times.
      (4) Tap plate dry.
9. Add 100 µl of avidin-horseradish peroxidase conjugate to each well using the multichannel pipettor (program 2). Cover plate and incubate for 15 min at 37°C (±2°C). Do not extend the incubation period beyond 15 min.
10. Wash plate as described in step V.D.8.
11. Prepare working substrate by mixing 2 ml of substrate A and 0.5 ml of substrate B into a polypropylene tube for each two eight-well strips. Prepare substrate no more than 3 h before use, and protect from the light.
12. Pipette 100 µl of prepared working substrate into each well.
13. Allow color to develop for 10 min, at room temperature, *in the dark.* Do not extend the incubation period beyond 10 min.
14. Add 100 µl of stop reagent to each well.
15. Measure the OD at 450 nm within 10 min of adding stop reagent.

E. Calculation of results

1. For each sample, select the highest dilution of the amplicon that gives an OD between 0.2 and 2.0 on the HIV microwells (rows A to F). For the QS, select the highest dilution of the amplicon that gives an OD between 0.3 and 2.0 on the microwells (rows G and H). Enter the OD and the dilution factors for the HIV and QS microwells in the worksheet. These values will be used to calculate the number of HIV copies per ml.
2. To calculate results on the computer, do the following.
   a. Enter OD values and dilution determined as described above into worksheet template on the computer.
   b. Enter the number of QS copies added. This is unique to each lot. Find the value on the test data card included in the kit.
   c. Print a copy of the completed worksheet to keep with patient result worksheet.

V. PROCEDURE *(continued)*

3. To manually calculate the results, follow the instructions below.
- **a.** Choose the appropriate HIV well that has an OD value in the range of 0.2 to 2.0 OD units. If more than one well is in this range, choose the well with the larger dilution factor (i.e., the smaller OD).
- **b.** Subtract the background from the selected HIV OD value. Background = 0.07 OD unit.
- **c.** Calculate the total HIV OD by multiplying the OD background value of the selected HIV well by the dilution factor associated with that well.
- **d.** Choose the appropriate QS well that has an OD value in the range of 0.3 to 2.0 OD units. If more than one well is in the range, choose the well with the larger dilution factor (i.e., the smaller OD).
- **e.** Subtract the background from the selected QS OD value. Background = 0.07 OD unit.
- **f.** Calculate the total QS OD by multiplying the OD background of the selected QS well by the dilution factor associated with that well.
- **g.** Calculate the HIV-1 RNA copies per milliliter of plasma as follows:

$$\left(\frac{\text{Total HIV OD}}{\text{Total QS OD}}\right) \times \text{input copies of QS} \times 4$$

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. The presence of HIV RNA in the sample is determined by relating the absorbance of the unknown specimen to the cutoff value.
B. Report the number of HIV copies per milliliter of specimen.
C. Call positive results to physician of record or designee. Note on the worksheet the name of the person called, date, and time, and initial.
D. Document all testing in hard copy or computerized work card.

VII. INTERPRETATION

A. Quantitative results reported as HIV RNA copies per milliliter serve as an estimate of the viral load of the patient.
B. The linear range of the HIV ultrasensitive test is 40 to 750,000 copies/ml. Results outside this range should be reported as <40 or >750,000 copies/ml.
C. Unexpected results
- **1.** All HIV values are <0.20 OD units. If all HIV wells have OD values less than 0.2, but the QS wells have the expected values, use 0.2 as the OD and 1 as the dilution factor. The result should be reported as <40 copies/ml.
- **2.** All HIV wells are >2.0 OD units. If all HIV wells have OD values greater than 2.0 but the QS wells have the expected values, then either an error occurred in the test or the HIV copy number is above the dynamic range of the assay. Report values as 750,000 copies/ml. If the doctor requests, repeat the test using a 1:50 dilution of the specimen with the negative control plasma. Calculate the results as described above, and then multiply the final result by 50.
- **3.** HIV OD values are out of sequence. If HIV wells do not follow the general pattern of decreasing OD values from row A to row F, an error in dilution may have occurred. The result for that specimen is invalid and the test should be repeated.
- **4.** Both QS values are <0.3 OD unit. If all QS wells are less than 0.3, then either the processed sample was inhibitory to the amplification or the RNA was not recovered during sample preparation. Repeat the entire test, including sample preparation and amplification and detection.

VII. INTERPRETATION
(continued)

5. Both QS values are >2.0 OD units. If all QS wells are greater than 2.0, then an error occurred. Repeat the tests.
6. QS values are out of sequence. If the absorbance of row H is greater than the absorbance in row G, then an error occurred. The result for that specimen is invalid. Repeat the entire test.

REFERENCE

1. **CLSI.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. CLSI, Wayne, PA.

SUPPLEMENTAL READING

Garcia-Lerma, J. G., S. Yamanoto, and M. Gomez-Cano. 1998. Measurement of human immunodeficiency virus type 1 plasma virus load based on reverse transcriptase (RT) activity: evidence of variables in levels of virion-associated RT. *J. Infect. Dis.* **177:**1221–1229.

Ho, D. D. 1996. Viral counts in HIV infection. *Science* **272:**1124–1125.

Lew, J., P. Reichelderfer, M. Fowler, J. Bremer, R. Carrol, S. Cassol, D. Chernoff, R. Coombs, M. Cronin, R. Dickover, S. Fiscus, S. Herman, B. Jackson, J. Kornegay, A. Kovacs, K. McIntosh, W. Meyer, N. Michael, L. Mofenson, J. Moye, T. Quinn, M. Robb, M. Vahey, B. Weiser, and T. Yeghiazarian. 1998. Determination of levels of human immunodeficiency virus type 1 RNA in plasma: reassessment of parameters affecting assay outcome. *J. Clin. Microbiol.* **36:**1471–1479.

Mellors, J. W., C. R. Rinaldo, Jr., P. Gupta, R. M. White, J. A. Todd, and L. A. Kingsley. 1996. Prognosis of HIV-1 infection predicted by the quantity of virus in plasma. *Science* **272:**1167–1170.

PART 6 Detection of Herpes Simplex Virus by Real-Time PCR

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Herpes simplex encephalitis (HSE) is a rare complication of herpetic infection (both herpes simplex virus type 1 [HSV-1] and HSV-2) and is associated with significant morbidity and mortality. Antiviral therapy is successful if administered early in the course of the infection. The clinical presentation of HSE is one of focal encephalitis associated with headache, fever, behavioral disorders, altered levels of consciousness, focal seizures, and hemiparesis. While the clinical picture will lead physicians to treat empirically for HSE, the presentation is not pathognomonic for HSE. Infectious HSV is rarely present in CSF during encephalitis. HSV is cultured from CSF for only about 5% of adult patients and for up to 50% of neonatal cases of HSE. Isolation of HSV from brain tissue after biopsy was originally considered to be the reference standard for the diagnosis of HSE; however, more recently, studies have shown that HSV DNA was detected by PCR in CSF of 53 (98%) of 54 patients with biopsy-proven HSE. In this study the specificity of HSV PCR for the diagnosis of HSE was 94% compared to culture of brain tissue. Thus, PCR detection of HSV DNA should be considered the standard for diagnosis of HSE (2, 6).

This assay targets the HSV glycoprotein G gene; the primers are designed to amplify sequences common to both HSV-1 and HSV-2, but the probes are able to distinguish between the two types. Detection of targeted HSV DNA sequences utilizes TaqMan probe chemistry. This involves the use of a dually labeled probe with a reporter dye at the 5′ end and a quencher at the 3′ end. When the reporter and quencher are both present on an intact probe molecule, any fluorescence energy produced by the reporter is suppressed. However, if the probe binds to a specific target, 5′-to-3′ exonuclease activity of the DNA polymerase will degrade the probe, thus allowing the reporter and quencher to separate, resulting in the generation of a detectable fluorescent signal. The reporter molecule for each analyte exhibits a different fluorescent emission spectrum so that the signals can be differentiated.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

The assay requires the sterile collection of at least 200 µl of spinal fluid, although optimally, 500 µl or more of specimen will be collected to enable repeat analysis if required.

B. Timing of specimen collection

There are no particular timing issues to consider in collecting these specimens; however, the specimen type and time of collection should be recorded.

C. Specimen transport

1. Specimens should be transported to the laboratory at room temperature.
2. Samples are stored at 4°C until testing.
3. An aliquot of 200 µl of specimen is required for testing; the remaining specimen is stored at −70°C for repeat testing if necessary.

D. Specimen labeling and submission

The laboratory should determine a mechanism to ensure that the specimen type and time of collection are recorded.

E. Rejection criteria

1. Other specimen types, such as swabs in viral transport medium or ocular fluids, may be tested if validated by the testing laboratory. However, swab specimens (which may potentially contain high concentrations of virus) should be segregated from CSF samples to minimize the potential for cross-contamination.
2. Reject specimens that arrive in leaking or broken tubes or specimen containers.
3. Testing of volumes of CSF as little as 100 µl brought up to a 200-µl volume with phosphate-buffered saline can be performed pending director approval. Such cases should be noted when reporting results, with the approximate volume entered as a qualifier, as assay sensitivity may be affected.
4. Request a repeat specimen, or obtain the information, when the collection time or specimen type has not been provided.
5. If an improperly collected, transported, and/or handled specimen cannot be replaced, document in the final report that specimen quality may have been compromised.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Supplies and equipment

1. Master mix preparation
- **a.** 2-, 20-, 200-, and 1,000-µl pipettes
- **b.** 2-, 20-, 200-, and 1,000-µl aerosol-resistant pipette tips
- **c.** Spray/squirt bottles—bleach, ethanol
- **d.** −20°C freezer
- **e.** Dead-air box
- **f.** Ziploc bags
- **g.** Sterile 2.0-ml screw-top microcentrifuge tubes
- **h.** Microtube racks
- **i.** 96-well optical plate

2. Sample preparation area
- **a.** MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche)
  - (1) Proteinase K
  - (2) Elution buffer
  - (3) Wash buffer I
  - (4) Wash buffer II
  - (5) Lysis/binding buffer

  NOTE: Avoid mixing lysis/binding buffer with sodium hypochlorite, as toxic gas formation will occur (4).
  - (6) Magnetic glass particle suspension
- **b.** 2-, 20-, 200-, and 1,000-µl pipettes
- **c.** 2-, 20-, 200-, and 1,000-µl aerosol-resistant pipette tips
- **d.** Pipette pump
- **e.** 5-ml serological pipettes
- **f.** Vortex mixer
- **g.** Disposable gloves
- **h.** MagNA Pure LC instrument
- **i.** MagNA Pure LC consumables
  - (1) Sample cartridges
  - (2) Processing cartridges
  - (3) Cartridge seals
  - (4) Reaction tips

III. MATERIALS *(continued)*

(5) Reagent tubs (medium, 20 ml)
(6) Reagent tubs (large)
(7) Drop catcher
(8) Waste bag

j. Ziploc bags
k. Optical plate sealing tape (Applied Biosystems)
l. Plate-sealing tool (Applied Biosystems)
m. Spray/squirt bottles—bleach, ethanol
n. Biosafety cabinet
o. Dead-air box

3. Amplification and detection area
a. Applied Biosystems 7500 Sequence Detection System
b. Spray/squirt bottles—bleach, ethanol

B. Reagents

1. Sodium hypochlorite (bleach) solution
2. 100 proof ethanol
3. 2× universal master mix (Applied Biosystems)
Store in reagent preparation area either refrigerated or at −20°C for longer-term storage. Expiration: do not use beyond manufacturer's control date.
4. TaqMan exogenous internal positive control reagents (Applied Biosystems)
5. 10 mM Tris–1 mM EDTA (TE) solution (pH 8.0) (Ambion or equivalent)
Store at room temperature. Expiration, 1 year.
6. Sterile molecular-biology-grade water (Ambion or equivalent)
Store at room temperature. Expiration, 1 year.
7. HSV-F primer, HSV-R primer, and HSV probe (Biosearch Technologies, Inc., or equivalent) (Table 12.2.3–3)
Depending on the manufacturer, primers may be received as lyophilized pellets, in which case they should be resuspended to a final concentration of 100 μM using TE buffer, pH 8.0. Dilution should be performed in the reagent preparation area. This is the stock solution. Store frozen. Expiration of stock solution, 1 year. If primers and probes are received in liquid form, they should be at a concentration of 100 μM.
◩ **NOTE:** If stock solutions are to be used repeatedly to produce new lots of master mix, they should be stored in 50- to 100-μl aliquots to minimize freeze-thaw cycles.
8. HSV-1 positive control (Acrometrix, Zeptometrix, or equivalent)
9. HSV-2 positive control (Acrometrix, Zeptometrix, or equivalent)
10. Negative control
The negative control can be purchased from a commercial manufacturer, or a clinical matrix (normal human plasma) tested to be negative for the presence of HSV can be utilized.

C. Preparation of reagents

1. Primer-probe mix
a. Prepare final primer-probe mix according to the recipe listed in Table 12.2.3–4. The amount of mix prepared will be based on the routine number of specimens received in the laboratory. A batch of master mix should ideally last at least 6 months. Strict adherence to protocol is essential to avoid contamination of the primer lot. Primers, stock solutions, and primer-probe mixes should be prepared in the reagent preparation area before the technologist performs any tasks in the sample preparation or amplification area. *Never formulate and/or aliquot master mix after working in sample preparation area or amplification area.*
b. Add the components, as listed in Table 12.2.3–4, in a 2.0-ml tube in a dead-air box in the reagent preparation area, and pulse vortex to mix.
c. Aliquot 150 μl of the mixture into sterile 2.0-ml screw-top tubes. Label tubes with the following information: HSV master mix, 10 reactions, date of preparation, batch number, and initials of technologist preparing mixture. Store aliquots at −20°C in area 1 in a non-frost-free refrigerator, and minimize exposure to light. Expiration, 1 year after formulation.
d. Prior to use, add 250 μl of 2× universal master mix per vial of primer-probe mix. This will provide enough master mix for 9 reactions.
◩ **NOTE:** Although the volume is sufficient for 10 reactions, the loss of volume due to

Table 12.2.3–3 PCR primers and probes for the detection of HSV-1 and HSV-2

Oligonucleotide designation	Nucleotide sequence, 5′–3′[a]
HSV-F	TCC TG/CG TTC CTA/C ACG/T GCC TCC C
HSV-R	GCA GIC AC/TA CGT AAC GCA CGC T
HSV-1 probe	6-FAM-CGT CTG GAC CAA CCG CCA CAC AGG T-BHQ-1
HSV-2 probe	QUASAR 670-CGA CCA GAC AAA CGA ACG CCG CCG T-BHQ-2

[a] 6-FAM and QUASAR 670 represent the 5′ reporter fluorophores for the HSV-1 and the HSV-2 probes, respectively. BHQ-1 and BHQ-2 are the quencher molecules for the HSV-1 and the HSV-2 probes, respectively. These reporter and quencher molecules are covalently linked to the oligonucleotides.

Table 12.2.3–4 Primer-probe mix formulations[a]

Component	Final concn	Amt per Rx (µl)	Amt per 100 Rx (µl)
HSV-F (100 µM)	800 nM	0.4	40
HSV-R (100 µM)	800 nM	0.4	40
HSV-1 probe (100 µM)	100 nM	0.05	5
HSV-2 probe (100 µM)	100 nM	0.05	5
Exogenous IPC mix	1×	5	500
Exogenous IPC DNA	1×	1	100
Nuclease-free water		8.10	810

[a] IPC, internal positive control; Rx, reaction.

III. MATERIALS *(continued)*

adherence of liquid to the tube and to pipette tips while aliquoting will result in sufficient reagent to run only 9 reactions.

2. Decontamination solutions
   - **a.** 10% Bleach
     Add 25 ml of concentrated bleach with 225 ml of deionized water passed through a 0.4-µm-pore-size filter in a squirt bottle. A squirt bottle of 10% bleach should be made for each of the three work areas. Expiration, 1 week.
   - **b.** 70% Ethanol solutions
     Mix 480 ml of 100% ethanol with 170 ml of deionized water passed through a 0.4-µm-pore-size filter in a spray bottle. A spray bottle of 70% ethanol should be made for each of the three work areas. Expiration, 1 week.

D. Establishment of control ranges

1. Prior to assay implementation, control ranges must be established for the HSV-1 and HSV-2 positive controls and for the internal positive controls.
2. The average threshold cycle (Ct) values and standard deviations for each control should be established by running at least two replicates of each control over the course of 20 runs.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Run controls

1. One HSV-1 positive control, one HSV-2 positive control, and at least one negative control are included in each run. Each MagNA Pure extraction cartridge should contain one HSV-1 and one HSV-2 positive control and one negative control. Positive and negative controls are taken through the entire extraction and amplification processes. Control ranges should be established for each positive control prior to assay implementation.
2. An internal positive amplification control is included with each specimen. Control ranges should be established for the internal control.

IV. QUALITY CONTROL *(continued)*

B. Establishment of control ranges

1. Prior to assay implementation, control ranges must be established for each control being utilized.
2. The average Ct values and standard deviations for each control should be established by running at least two replicates of each control over the course of 20 runs.

C. Specificity

The primer and probe set used in this reaction is published and has been shown to amplify HSV DNA but not DNA from other herpesviruses or from human sources (5).

D. Contamination prevention

The following steps have been taken to reduce the risk of contamination.

1. Separate areas for assay setup, specimen preparation and amplification, and detection of amplified products
2. Unidirectional work flow
3. The use of dUTP and uracil *N*-glycosylase (UNG) to decrease the risk of carryover contamination
4. Good laboratory technique and a laminar-flow hood during specimen preparation to avoid contamination between specimens

E. Reagent QC

Each batch of master mix is tested as follows.

1. Twenty reactions are performed using water as a template. This is to ensure that the reagents are not contaminated. If any of the reactions are positive, the master mix is rejected.
2. One each of a previous positive and previous negative patient specimen is run with the new batch of master mix along with the positive control. Results must match those obtained with the previous batch or the master mix is rejected.
3. Verify that all reagents and materials meet expiration date and QC parameters as per NCCLS/CLSI document M22-A3 (3).

V. PROCEDURE

Observe standard precautions.

A. Master mix setup

1. In a dead-air box in the reagent preparation area, prepare final PCR master mix by adding 250 µl of 2× universal master mix to a 150-µl aliquot of primer-probe master mix.
2. Add 40 µl of complete master mix to each well of a 96-well optical plate that will be used.
3. Put the optical plate in a Ziploc bag, and transfer it to the sample preparation area. Store at 4°C until extracted samples are ready to load.
4. Clean the area by wiping with 10% bleach solution, followed by wiping with 70% ethanol solution.

B. DNA extraction

1. In a biosafety cabinet in the sample preparation area, load 200 µl of each specimen and of each control into a MagNA Pure sample cartridge. The negative specimen should be the last sample loaded and processed.
2. Reconstitute one bottle of lyophilized proteinase K with 5.0 ml of elution buffer.
3. Open the MagNA Pure LC software.
4. Under sample ordering, choose Total Nucleic Acid Variable Elution.
5. Enter 200 µl as the sample volume.
6. Enter 50 µl as the elution volume.
7. Designate the order of the samples and the negative control in the MagNA Pure LC sample cartridge.

V. PROCEDURE *(continued)*

8. Choose Start Batch.
9. Per the on-screen instructions, place plastic consumables on the MagNA Pure LC stage, place plastic tubs in the tub rack, and add the amounts of reagents (except for the magnetic glass particle suspension) given by the software to the tubs.
   Caution: *Lysis/binding buffer can cause burns. Thoroughly wash exposed areas at once.*
10. Load the tub rack and the sample cartridge containing DNA on the instrument.
11. Click the various on-screen pictures of consumables and reagents to verify placement.
12. Mix the magnetic glass particle suspension with a vortex mixer and add to the corresponding tub on the tub rack.
13. Promptly begin the run by selecting OK.
14. After run completion, remove the sample cartridge containing DNA and store sealed (in a Ziploc bag) at 4°C until use.
15. Dispose of consumables and excess reagents as instructed by the manufacturer.
    Store reconstituted proteinase K at 2 to 8°C for up to 4 weeks. Store remaining kit components at room temperature.
16. Clean the area by wiping with 10% bleach solution, followed by wiping with 70% ethanol solution.
    ◩ **NOTE:** Avoid mixing lysis/binding buffer with sodium hypochlorite, as toxic gas formation will occur. Wipe up all lysis buffer spills before applying bleach (4).

C. PCR setup and sample loading

1. Remove the optical plate containing prealiquoted master mix from the refrigerator and, using the trap map as a guide, pipette 10 µl of extracted specimen or control into each corresponding well.
2. Seal the plate using the optical seal and plate-sealing tool.
3. Clean the area and pipettes with 10% bleach followed by 70% ethanol.
4. Take the PCR plate to the amplification area.

D. PCR run

1. Place the 96-well plate in the sample block, and close the chamber door.
2. Select HSV PCR Program.
   a. The thermal cycling program should be set to have the following parameters.
      (1) 50°C for 2 min
      (2) 95°C for 10 min
      (3) 45 cycles of 95°C for 15 s, followed by 58°C for 60 s
   b. Data should be collected for the FAM channel (target amplification) and for the VIC channel (internal control amplification).
3. Following the run, remove the plate from the instrument, and clean the area with 10% bleach followed by 70% ethanol.

E. Data analysis

1. Click Analyze, and select each sample to view results. Any positive sample must have a valid amplification curve (showing exponential increase), along with a displayed Ct value less than 45, to be considered a positive amplification. Samples for which a Ct value is given but that do not have a clear exponential growth phase should be referred to the laboratory director for interpretation.
2. Results should be viewed for all three detectors corresponding to HSV-1, HSV-2, and the internal control.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. For each specimen, report HSV-1 result as one of the following.
  1. HSV-1 DNA detected
  2. HSV-1 DNA not detected
  3. Inhibitors present

B. For each specimen, report HSV-2 result as one of the following.
  1. HSV-2 DNA detected
  2. HSV-2 DNA not detected
  3. Inhibitors present

C. Call results to physician of record or designee. Note on the worksheet the name of the person called, date, and time, and initial.

D. Document all testing in hard copy or computerized work card.

E. Include, "This test was developed and its performance characteristics determined by (laboratory name). It has not been cleared or approved by the Food and Drug Administration." As recommended by the CAP, add, "The FDA has determined that such clearance or approval is not necessary. This test is used for clinical purposes. It should not be regarded as investigation or for research. This laboratory is certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA-88) as qualified to perform high-complexity clinical laboratory testing" (1).

F. As applicable, include, "This test is performed pursuant to a license agreement with Roche Molecular Systems, Inc."

VII. INTERPRETATION

A. Results should not be reported if either positive control falls out of the specified range or if the negative control is positive.

B. If run controls are valid, report results for each analyte (HSV-1 and HSV-2).

C. For each analyte, report as "detected" if the sample has a Ct value less than 45 with a valid amplification curve.

D. For each analyte, report as "not detected" only if the sample result is "Undet" (no Ct value was generated) *and* if the internal positive control amplification is within the specified range.

E. If an analyte was not detected (no Ct value generated) but the internal control was out of range, PCR inhibitors may have been present, or improper assay setup may have occurred for that particular sample. The sample should be rerun. If, after the sample is rerun, the internal control still falls out of range, the report should indicate that PCR inhibition was observed and that an alternate specimen should be submitted if clinically warranted.

VIII. LIMITATIONS OF TESTING

A. A negative result for HSV DNA by PCR does not rule out the possibility that HSV DNA is present in the specimen at a concentration below the level of sensitivity of the assay.

B. A positive result for HSV DNA by PCR may be the result of contamination rather than a true positive result. This protocol employs several steps to decrease the risk of contamination, including separate areas for assay setup, specimen preparation, and amplification and detection of amplified products; unidirectional work flow; the use of dUTP and UNG to decrease the risk of carryover contamination; good laboratory technique; and the use of a laminar-flow hood during specimen preparation to avoid contamination between specimens.

C. The performance characteristics of this assay must be established by each laboratory.

REFERENCES

1. **College of American Pathologists.** 2009. Laboratory Accreditation Program, Microbiology Checklist, rev. 06/15/2009. College of American Pathologists, Northfield, IL.
2. **Lakeman, F. D., R. J. Whitley, and the National Institute of Allergy and Infectious Diseases Collaborative Antiviral Study Group.** 1995. Diagnosis of herpes simplex encephalitis: application of polymerase chain reaction to cerebrospinal fluid from brain-biopsied patients and correlation with disease. *J. Infect. Dis.* **171:**857–863.
3. **NCCLS/CLSI.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. CLSI, Wayne, PA.
4. **Roche Applied Science.** July 2007. MagNA Pure LC Total Nucleic Acid Isolation Kit instruction manual. Roche Applied Science, Indianapolis, IN.
5. **Ryncarz, A. J., J. Goddard, A. Wald, M. Huang, B. Roizman, and L. Corey.** 1999. Development of a high-throughput quantitative assay for detecting herpes simplex virus DNA in clinical samples. *J. Clin. Microbiol.* **37:**1941–1947.
6. **Whitley, R. J., and F. Lakeman.** 1995. Herpes simplex virus infections of the central nervous system: therapeutic and diagnostic considerations. *Clin. Infect. Dis.* **20:**414–420.

PART 7

Detection of *Mycoplasma pneumoniae* in Respiratory Specimens by PCR

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Mycoplasma pneumoniae is a common cause of lower respiratory tract infection. *M. pneumoniae* causes about 50% of cases of pneumonia in school-age children and between 2 and 4% of community-acquired pneumonia requiring hospitalization among adults. Because culture and serologic tests for diagnosis of *M. pneumoniae* infections may be unreliable and results may not be available in a timely fashion, PCR-based assays for *M. pneumoniae* infection have been developed. PCR results can be made available in 1 day, and PCR has been shown to be approximately 85% sensitive and 98% specific for diagnosis of *M. pneumoniae* infection (1, 3–5, 6–8). Testing can be performed on throat swab specimens, as well as sputum and bronchoalveolar lavage (BAL) specimens. The target of *M. pneumoniae* amplification is a 144-bp fragment of genomic DNA (1). The primer pair is specific for *M. pneumoniae* and does not amplify DNA from other species of *Mycoplasma,* oral microbiota, or other respiratory pathogens. Detection is performed using a specific probe in an ELISA-type format (Table 12.2.3–5).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. The appropriate specimens for *M. pneumoniae* PCR include throat swab, sputum, or BAL specimens.
2. The required specimen volumes are 500 μl for sputum and 1.5 ml for BAL specimens.
3. Dacron swabs should be used for collecting throat swab specimens. Materials in other types of swabs can be inhibitory to PCR.
4. The Dacron swab should be placed in viral transport medium and briskly mixed in the medium, and the shaft of the swab should be broken off, leaving the swab in the transport container.

Table 12.2.3–5 PCR primers and probe for detection of *Mycoplasma pneumoniae* in respiratory specimens

Primer or probe designation	Nucleotide sequence, 5′–3′	Product size (bp)
Primer MP5-1	GAA GCT TAT GGT ACA GGT TGG	144
Primer MP5-2	ATT ACC ATC CTT GTT GTA AGG	144
Probe MP5-4[a]	CGT AAG CTA TCA GCT ACA TGG AGG	144

[a] Label probe 5′ end with biotin.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

B. Timing of specimen collection

There are no particular timing issues to consider in collecting these specimens; however, the specimen type and time of collection should be recorded.

C. Specimen transport

1. Specimens should be transported to the laboratory at room temperature.
2. Throat swab specimens must be transported in viral transport medium. Sputum and BAL specimens do not require transport medium and should be collected and transported in sterile containers.
3. Specimens should be stored in a refrigerator if they cannot be transported within a few hours.
4. Specimens should not be frozen before transport.
5. Once specimens are received in the laboratory they should be stored at 4°C until they are tested.
6. After testing is completed, store the extracted specimens at −70°C.
7. Store any remaining primary specimen from positive patients at −70°C.

D. Specimen labeling and submission

The laboratory should determine a mechanism to ensure that the specimen type and time of collection are recorded.

E. Rejection criteria

1. Specimens other than throat swabs, sputum, or BAL fluid (e.g., nasal wash, lung tissue, or CSF) require director approval.
2. Reject throat swabs that are not submitted in transport medium.
3. Reject throat swabs made of material other than Dacron.
4. Reject specimens that arrive in leaky containers.
5. Request a repeat specimen, or obtain the information, when collection time or specimen type has not been provided.
6. If an improperly collected, transported, or handled specimen cannot be replaced, document in the final report that specimen quality may have been compromised.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Special supplies and equipment

1. Area 1
- **a.** Repeat pipettor with tips
- **b.** Perkin-Elmer 9600 reaction tray and retainer sets
- **c.** Perkin-Elmer 200-µl reaction tubes
- **d.** Ziploc baggies
- **e.** Dead-air box
- **f.** 2.0-ml Sarstedt screw-top tubes
- **g.** Low-speed microcentrifuge
- **h.** Rainin Gilson P20 and P200 Pipetman
- **i.** 20- and 200-µl plugged DNase-free aerosol-resistant pipette tips
- **j.** Spray/squirt bottles—bleach, ethanol
- **k.** Refrigerator
- **l.** −20°C freezer
- **m.** Microtube racks
- **n.** 1.7-ml microcentrifuge flip-top tubes
- **o.** 2-ml microcentrifuge tubes
- **p.** Microtube racks
- **q.** QIAamp blood and tissue kit (catalog no. 29106; QIAGEN)
- **r.** Coats and gloves dedicated to area 1

2. Area 2
- **a.** Sarstedt 1.7-ml screw-top tubes
- **b.** Aluminum foil
- **c.** Perkin-Elmer reaction tube cap strips (8 and 12 strips)
- **d.** Floaters
- **e.** Capping instrument
- **f.** −70°C freezer
- **g.** 50-ml conical tubes
- **h.** Eppendorf microcentrifuge
- **i.** Rainin Gilson P20, P200, and P1000 Pipetman
- **j.** 20-, 200-, and 1,000-µl plugged DNase-free aerosol-resistant pipette tips
- **k.** Extended-length pipette tips (p200)
- **l.** Spray/squirt bottles—bleach, ethanol
- **m.** Refrigerator
- **n.** −20°C freezer

III. MATERIALS *(continued)*

o. 1.7-ml microcentrifuge flip-top tubes
p. 2-ml microcentrifuge tubes
q. Microtube racks
r. Water bath
s. Biosafety cabinet
t. Microcentrifuge cap locks
u. Heraeus 17 RS centrifuge
v. Coats and gloves dedicated to area 2

3. Area 3
a. Electrophoresis gel box, tray and combs, and power source
b. Perkin-Elmer thermal cycler, model 9600
c. 200-ml Erlenmeyer flasks
d. Stir bars
e. UV-protective face shield or goggles
f. UV illuminator box
g. Camera
h. Rainin Gilson P20, P200, and P1000 Pipetman
i. Automated multichannel pipette
j. 20-, 200-, and 1,000-µl plugged DNase-free aerosol-resistant pipette tips
k. Spray/squirt bottles—bleach, ethanol
l. Refrigerator
m. 1.7-ml microcentrifuge flip-top tubes
n. Microtube rack
o. Disposable reagent reservoirs
p. Low-electroendosmosis agarose (catalog no. A-6013; Sigma)
q. Loading dye
r. Chemical fume hood
s. 37°C incubator
t. Microwell automated plate washer
u. Microwell automated plate reader
v. Detection reagents (GEN-ETI-K DEIA #PS0001, DNA EIA kit; DiaSorin)
w. Deionized water
x. Coats and gloves dedicated to area 3

B. Reagents

1. Deoxynucleoside triphosphates (dNTPS)
Stock dNTPs (Perkin-Elmer) dATP, dCTP, dGTP, and dTTP (10 mM each) and dUTP (100 mM; Pharmacia Biotech). Store at −20°C in area 1. Expiration per manufacturer's control date.

2. Molecular-biology-grade water, diethyl pyrocarbonate (DEPC) treated (catalog no. 9920; Ambion), 500 ml. Expiration, 1 year.
3. Deionized ultrafiltered water (Fisher W2-20), 20 liters. Expiration, 1 year.
4. Enzymes
a. AmpliTaq DNA polymerase (Perkin-Elmer), 250 U/tube; 5 U/µl; 50 µl. Store at −20°C in area 1. Expiration per manufacturer's control date.
b. AmpErase—uracil *N*-glycosylase (UNG) (Perkin-Elmer), 100 µl; 1 U/µl. Store at −20°C in area 1. Expiration per manufacturer's control date.
c. QIAGEN protease
(1) QIAGEN protease should be stored at 4°C until resuspended. Expiration of powdered enzyme is 1 year.
(2) Add 7 ml of H_2O to the vial of protease provided by the manufacturer. Mix thoroughly and dispense into 200-µl aliquots; freeze at −20°C. Aliquots are single use. Expiration, 6 months. Store in area 1.

5. Buffers
a. 10× PCR buffer II (Perkin-Elmer)—500 mM KCl, 100 mM Tris-HCl (pH 8.3), 0.01% (wt/vol) gelatin. Expiration per manufacturer's control date. Store at −20°C in area 1.
b. TBE buffer, 10× stock solution, 10× TBE buffer, 1 liter (catalog no. V4251; Promega).
(1) 10× stock—890 mM Tris acetate, 890 mM boric acid, 20 mM EDTA
(2) Expiration of stock solution, 1 year
(3) Do not use if the buffer contains a precipitant.
(4) Store at room temperature in area 3.
c. Detection washer buffer
(1) 25×; 40-ml volume. Add 40 ml of 25× wash buffer to 960 ml of deionized water (or 20 ml to 480 ml of water, or 10 ml to 240 ml of water, depending on the number of detection reactions).

III. MATERIALS *(continued)*

(2) Store at room temperature in area 3; use the buffer the day it is prepared.

d. Agarose gel loading dye
(1) Mix the following components in a 50-ml conical tube: 20 g of sucrose and 125 mg of bromophenol blue. Bring up to 50 ml with deionized water passed through a 0.2-μm-pore-size filter.
(2) Store at room temperature in area 3.
(3) Expiration of gel loading dye, 6 months.

e. AW buffer for QIAamp purification
(1) Add 190 ml of 100% ethanol to the buffer as provided by QIAGEN.
(2) Mix thoroughly; store in 7- to 8-ml single-use aliquots at room temperature in area 1.
(3) Expiration, 1 year.

f. AL buffer for QIAamp purification
(1) Mix reagent AL1 into AL buffer (reagent AL2); both are provided in the kit.
(2) Mix thoroughly by shaking. Aliquot 1.5 ml into microcentrifuge tubes (single use); store in the dark at room temperature in area 1. Expiration, 1 year.

g. TE buffer, 100× stock solution (Tris-EDTA buffer, catalog no. T-9285; Sigma)
(1) 1.0 M Tris-HCl (pH 8.0), 0.1 M EDTA
(2) Store at room temperature.
(3) Expiration, 1 year.
(4) Mix 1 ml of 100% TE buffer with 99 ml of water (final concentration: 10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Mix and store at room temperature in area 1.

6. Oligonucleotides

a. *M. pneumoniae* primers: MP5-1 and MP5-2 (Gibco BRL)
(1) Primers are received as lyophilized pellets. Suspend these in 50 μl of DNase- and RNase-free water *in area 1;* this is the stock solution. Expiration of stock solution, 1 year.
(2) *Note:* Strict adherence to protocol is essential to avoid contamination of primer lot. Primers should be diluted in area 1 before the technologist performs any tasks in area 2 or 3. *Never dilute primers after working in area 2 or 3.*
(3) Preparation of the probe stock solution should be done a day in advance of the next anticipated use of the primer pair to ensure that they are totally resuspended.

b. Human DNA control primers (DQA primers): DQA-1 and DQA-2 (Gibco BRL)
(1) Human DNA control to evaluate specimen quality for throat swab specimen only
(2) Primer sequences (214-bp product)
(a) DQA-1: 5′-GTG CTG CAG GTG TAA ACT TGT ACC AG-3′
(b) DQA-2: 5′-CAC GGA TCC GGT AGC AGC GGT AGA GTT G-3′
(3) Primers are received as lyophilized pellets. Suspend these in 50 μl of DNase- and RNase-free water *in area 1;* this is the stock solution. Expiration of stock solution, 1 year.
(4) *Note:* Strict adherence to protocol is essential to avoid contamination of primer lot. Primers should be diluted in area 1 before the technologist performs any tasks in area 2 or 3. *Never dilute primers after working in area 2 or 3.*
(5) Preparation of the primer stock solution should be done a day in advance of the next anticipated use of the primer pair to ensure that they are totally resuspended.

III. MATERIALS *(continued)*

(6) Dilute an aliquot of the stock solution to 20 pmol/µl of working solution with required amount of double-distilled H_2O (ddH_2O). The amount of primer in each lot is variable, so different amounts of ddH_2O will be required to dilute the stock solution to 20 pmol/µl. Do not make more primer working solution than is needed for the master mix described below (dilute primers are not stable). Stock solution should be stored at −20°C in area 1. Expiration, 6 months.

7. $MgCl_2$ solution for PCR (25 mM solution; Perkin-Elmer). Store at −20°C in area 1. Expiration per manufacturer's control date. The $MgCl_2$ stock solution is included when the TAQ polymerase is ordered.

8. Preparation of reagents

a. *M. pneumoniae* master mix

(1) Mix the following components in area 1 dead-air box (800 reactions):

10× PCR buffer II .....4.0 ml
$MgCl_2$ (25 M) .....4.8 ml
dNTPs
dATP .....800 µl
dCTP .....800 µl
dGTP .....800 µl
dTTP .....800 µl
dUTP .....160 µl
primer MP5-1 .....1.0 ml
primer MP5-2 .....1.0 ml
ddH_2O ..... 21.24 ml

(2) Aliquot the mixture into sterile flip-top microcentrifuge tubes. The amounts given above allow for 28 aliquots of 293 µl (6.5 reactions) and 70 aliquots of 383 µl (8.5 reactions)

(3) There may be a few microliters of mixture left; discard it. Label tubes with the following information: master mix, number of reactions (six or eight), date of preparation, batch number, and initials of technologist preparing mixture. Store the tubes at −20°C in area 1.

(4) Prior to use, add TAQ (0.25 µl/reaction) and UNG (0.5 µl/reaction) to the master mix. To the 6-reaction aliquot add 3.0 µl of UNG and 1.5 µl of TAQ. To the 8-reaction aliquot add 4.0 µl of UNG and 2.0 µl of TAQ.

(5) For each individual reaction the final concentrations of the master mix components are as follows.

Component	Volume/ reaction (50 µl)	Final concentration
10× PCR buffer	5 µl	1×
$MgCl_2$ (25 mM)	6 µl	3 mM
dNTPs	1 µl (A, C, G, T)	0.2 mM each
dUTP	0.2 µl	0.4 mM
Primer MP5-1	1.25 µl	0.5 µM
Primer MP5-2	1.25 µl	0.5 µM
UNG	0.5 µl	0.5 U
TAQ	0.25 µl	1.25 U
ddH_2O	26.55 µl	
Specimen	5 µl added in area 2	

(6) Master mix expiration, 6 months.

b. DQA Master mix

(1) Mix the following components in area 1 dead-air box (120 reactions).

10× PCR buffer II .....600 µl
$MgCl_2$ (25 mM) .....720 µl
dNTPs
dATP .....120 µl
dCTP .....120 µl
dGTP .....120 µl
dTTP .....120 µl
dUTP ..... 24 µl
primer DQA-1 ..... 60 µl
primer DQA-2 ..... 60 µl
ddH_2O ..... 3.366 ml

(2) Aliquot the mixture into sterile flip-top microcentrifuge tubes. The amounts given above allow for 15 aliquots of 203 µl (4.5 reactions) and 7 aliquots of 293 µl (6.5 reactions).

III. MATERIALS *(continued)*

(3) There may be a few microliters of mixture left; discard it. Label tubes with the following information: master mix, number of reactions (four or six), date of preparation, batch number, and initials of technologist preparing mixture. The tubes are stored at −20°C in area 1.

(4) Prior to use, add TAQ (0.25 µl/reaction) and UNG (0.5 µl/reaction) to the master mix. To the four-reaction aliquot add 2.0 µl of UNG and 1.0 µl of TAQ. To the six-reaction aliquot add 3.0 µl of UNG and 1.5 µl of TAQ.

(5) For each individual reaction the final concentrations of the master mix components are as follows:

Component	Volume/ reaction (50 µl)	Final concentration
10× PCR buffer II	5 µl	1X
$MgCl_2$ (25 mM)	6 µl	3 mM
dNTPs	1 µl (A, C, G, T)	0.2 mM each
dUTP	0.2 µl	0.4 mM
Primer DQA-1	0.5 µl	0.2 µM
Primer DQA-2	0.5 µl	0.2 µM
UNG	0.5 µl	0.5 U
TAQ	0.25 µl	1.25 U
ddH_2O	28.5 µl	
Specimen	5 µl added in area 2	

(6) Master mix expiration, 6 months

c. Preparation of TBE buffer 10× working solution

(1) Add 2 liters of 10× TBE stock solution into the 20-liter carboy. Fill to the 20-liter mark with deionized water passed through a 0.2-µm-pore-size filter.

(2) Store at room temperature in area 3.

(3) Expiration date of TBE buffer, 12 months from date of preparation.

9. Decontamination solutions

a. 10% Bleach. Add 25 ml of concentrated bleach with 225 ml of deionized water passed through a 0.4-µm-pore-size filter in a squirt bottle. A squirt bottle of 10% bleach should be made for each of the three work areas. Expiration, 1 week.

b. 70% Ethanol solutions. Mix 480 ml of 100% ethanol with 170 ml of deionized water passed through a 0.4-µm-pore-size filter in a spray bottle. A spray bottle of 70% ethanol should be made for each of the three work areas. Expiration, 1 week.

10. Positive controls

a. *M. pneumoniae* positive control

Stock solution. Obtain *M. pneumoniae* from ATCC (ATCC29085), and resuspend the lyophilized material in 1 ml of PPLO broth. Store 5-µl aliquots in screw-top microcentrifuge tubes at −70°C. Expiration, 1 year.

b. DQA positive control

(1) Human placental DNA (catalog no. D4642; 5 U; 20 U/mg; Sigma)

(2) Stock solution. Resuspend the 5 U (250 µg) of DNA in 250 µl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Concentration of stock solution is 1 µg/ul. Store 5-µl aliquots in screw-top microcentrifuge tubes at −70°C. Expiration, 1 year.

(3) Working solution. Make a 1:1,000 dilution of the stock solution and store at −70°C. Concentration of working solution is 1 ng/µl. Expiration, 1 year.

(4) Use 3 µl as positive control for the DQA assay.

III. MATERIALS *(continued)*

11. DNA molecular weight standard for agarose gel electrophoresis
Use Ready Load (100-bp DNA ladder, catalog no. 10380-012; Gibco BRL). The solution is premixed with loading dye. Concentration is 0.1 μg/μl. Add 5 μl of the DNA ladder solution to the first lane (on the left) of all agarose gels. Expiration, 1 year.

12. Preparation of microwell plates coated with *M. pneumoniae* MP5-4 probe
- **a.** Preparation of microwell plates is done in area 1. Prepare one or two full (96-well) plates.
- **b.** Dilute probe to a final concentration of 50 pg/μl.
- **c.** Using a multichannel pipettor, pipette 100 μl of diluted probe into each microwell. Cover plate, place in a Ziploc bag, and incubate overnight at 4°C.
- **d.** The following day pick up concentrated wash buffer from cold room. *Do not take wash buffer from area 3.*
- **e.** Prepare 1× wash solution, 480 ml of Fisher water (must be Fisher water), and 20 ml of 25× wash buffer. This will wash two plates. If washing one plate, make one-half the volume.
- **f.** In area 1, aspirate probe from the microwells, add 300 μl of 1× wash solution, soak for 30 s, and aspirate contents of each well. Repeat this process four more times (total of five washes). After rinsing, turn strips over and gently tap on blotting paper.
- **g.** Store strips at 4°C in area 3. Expiration date, 1 month.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. One positive control is included in each run. *M. pneumoniae* DNA is used as the template (*see* item III.b) for the positive control reaction.

B. Multiple negative controls (at least three) are included in each run. One negative control (using phosphate-buffered saline [PBS]) is prepared using the QIAGEN protocol.

C. Inhibition controls are performed on each clinical specimen, except BAL specimens. An aliquot of specimen is "spiked" with positive control DNA. This spiked control must be positive to ensure that there were no inhibiting substances in the specimen.

D. To evaluate the quality of the specimen, a human DNA control is performed on throat swab specimens. If no human DNA is detected, a disclaimer is included in the report of the result.

E. The primer pair used in this reaction is published and has been shown to amplify *M. pneumoniae* DNA. The primers do not amplify other species of *Mycoplasma*, organisms in the oral microbiota, or other organisms causing upper respiratory tract infection or pneumonia (1).

F. The following steps have been taken to reduce the risk of contamination.
- **1.** Separate areas for assay setup, specimen preparation and amplification, and detection of amplified products
- **2.** Unidirectional work flow
- **3.** The use of dUTP and UNG to decrease the risk of carryover contamination
- **4.** Good laboratory technique and a laminar-flow hood during specimen preparation to avoid contamination between specimens
- **5.** After amplification reaction, tubes are opened in a chemical fume hood in area 3.
- **6.** Periodic wipe tests of all three laboratory areas

G. Each batch of master mix is tested as follows.
- **1.** Twenty reactions are performed using water as a template. This is to ensure that the reagents are not contaminated. If any of the reactions are positive, the master mix is rejected.

IV. QUALITY CONTROL *(continued)*

2. The *M. pneumoniae* positive control, a previous positive patient specimen, and a previous negative patient specimen are tested with the new master mix. This is to ensure that the sensitivity of the assay is acceptable. Results must match those obtained with the previous batch of master mix, or the new batch of master mix is rejected.

H. Detection plates are evaluated by running a previous positive and previous negative specimen and six negative controls (water). Patient results must match results obtained on the previous run and negative controls must be negative, or the plate is rejected.

I. Verify that all reagents and materials meet expiration date and QC parameters as per CLSI document M22-A3 (2).

V. PROCEDURE

Observe standard precautions.

A. Master mix preparation (area 1)

1. Remove required combination of six- and/or eight-reaction aliquots of *M. pneumoniae* master mix from −20°C freezer. Allow to thaw.

Number of patient specimens	Master mix aliquots
1	6 reactions
2	8 reactions
3	12 reactions (6 + 6)
4	14 reactions (6 + 8)

2. Remove the required number of reaction aliquots of DQA master mix from the −20°C freezer. Allow to thaw.
3. Turn off UV light and wipe down dead-air box surface with 10% bleach and then 70% ethanol.
4. Assemble tray and tray retainer set with corresponding number of 200-µl reaction tubes.
5. After master mix is thawed, remove TAQ and AmpErase (UNG) from the freezer. Pipette appropriate amount of TAQ and UNG into the master mix (0.5 µl of UNG per reaction, 0.25 µl of TAQ per reaction; *see* below). If using more than one tube of master mix (i.e., four patients require six- and eight-reaction aliquots), combine aliquots of master mix before adding TAQ and UNG.

   ◩ *Do not vortex enzymes.* Mix by inverting and then centrifuge briefly at low speed to bring solution out of cap.

Master mix	TAQ	UNG
M. pneumoniae—6	1.5 µl	3 µl
M. pneumoniae—8	2.0 µl	4 µl
DQA—4	1.0 µl	2 µl
DQA—6	1.5 µl	3 µl

6. Return TAQ and UNG to the freezer.
7. Pipette 45 µl of the master mix-TAQ aliquots into each reaction tube.
8. Place tray in Ziploc baggie and set aside.
9. Wipe dead-air space surface with 10% bleach and then 70% ethanol. Before leaving area 1 turn UV light timer to 15 min.
10. Bring bagged tray and tray map to area 2.
11. Bring necessary QIAGEN reagents from area 1 (columns, microcentrifuge tubes, water, alcohol, AL and AW buffers, and protease) to area 2.

B. Specimen preparation and loading (area 2)

1. Place bagged reaction in tray in 4°C refrigerator until ready to use.
2. Set up rack for specimen preparation (QIAamp).

V. PROCEDURE *(continued)*

3. Each patient sample requires two microcentrifuge tubes, one spin column, and three spin collection tubes.
4. Perform specimen preparation as follows.
   a. For throat swab specimens, vortex the swab in the transport medium, express fluid from the swab, and discard. Transfer 1.5 ml to a microcentrifuge tube and centrifuge for 15 min at 23,000 × *g*. Resuspend the pellet in 200 μl of PBS.
   b. For BAL specimens, place 1 to 2 ml of specimen in a microcentrifuge tube and centrifuge for 15 min at 23,000 × *g*. Resuspend the pellet in 200 μl of PBS.
   c. Pipette 200 μl of sputum, concentrated throat swab specimen, or concentrated BAL specimen into microcentrifuge tube using extended-length pipette tips; put remaining specimen in the refrigerator.
   d. Add 25 μl of QIAGEN protease and 200 μl of AL buffer; vortex thoroughly.
   e. Incubate at 70°C for 30 min (may incubate for up to 3 to 4 h) in a water bath using a flotation rack. Sample should not be viscous; reincubate for 10 to 20 min if needed.
   f. Add 210 μl of 100% ethanol and vortex.
   g. Transfer lysate to a QIAamp spin column which is already inserted into a 2-ml collection tube. Centrifuge for 1 min. Use the microcentrifuge at 6,000 × *g* (8,000 rpm, setting 8). Place QIAamp spin column in a clean 2-ml collection tube.
   h. Add 500 μl of buffer AW and centrifuge for 1 min.
      (1) Use the microcentrifuge at 6,000 × *g* (8,000 rpm).
      (2) Place QIAamp spin column in a clean 2-ml collection tube.
   i. Add 500 μl of AW buffer and centrifuge for 1 min.
      (1) Use the microcentrifuge at 6,000 × *g* (8,000 rpm). Then centrifuge in microcentrifuge at full speed for 2 min.
      (2) Place QIAamp spin column in a 1.7-ml microcentrifuge tube labeled with the patient's specimen number and date.
   j. Elute DNA by pipetting 200 μl of distilled water prewarmed to 70°C onto the QIAamp spin column. Incubate at 70°C for 5 min in a water bath.
   k. Centrifuge spin column at 6,000 × *g* for 1 min (8,000 rpm, setting 8).
   l. Remove and discard spin column. Specimen may be frozen at −20°C, or continue with protocol.
   m. Cap and vortex specimen; put on blue boiling caps.
   n. Boil specimens for 10 min.
5. Clean biosafety cabinet with 10% bleach and then 70% ethanol.
6. Remove tray from refrigerator; using worksheet as a guide, pipette 5 μl of prepared specimen into the test well, the inhibition control well, and the DQA well if needed.
7. Pipette 5 μl of distilled water or QIAGEN processed water into the appropriate negative control wells.
8. Cap test wells. *Test wells should be capped before the positive control is brought into the biosafety cabinet.*
9. Dilute positive control for use as follows.
   a. For *M. pneumoniae*, dilute a 5-μl aliquot of stock solution 1:10 (add 45 μl of distilled H_2O (dH_2O) to the tube and mix well). Use 2 μl of *Mycoplasma* DNA as positive control.
   b. For DQA, use 3 μl of the working solution (1 ng/μl) as positive control.

V. PROCEDURE *(continued)*

10. Pipette 3 µl of *M. pneumoniae* positive control into each inhibition control tube ("spiked") and the positive control well. Pipette 3 µl of DQA control into the positive control well.

11. Cap the remaining wells.

12. Place tray into thermal cycler and run program 126 (Perkin-Elmer 9600). Thermal cycler parameters:
HOLD 1: 20°C for 10 min
HOLD 2: 94°C for 10 min
35 cycles: 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s
HOLD 3: 95°C for 15 min

13. Clean biosafety cabinet and pipettes with 10% bleach followed by 70% ethanol; turn on the UV light.

14. After program is finished, *immediately* immerse tubes *completely* in ice. This will denature the DNA. The tubes must be at 95°C and placed directly in ice. The DQA tubes do not need to be placed in ice.

15. Print run record.

16. Bring tray and tray map into area 3.

C. Detection (area 3)

1. Agarose gel detection for DQA reactions

a. Fill out gel map.

b. Wipe down area 3 fume hood with 10% bleach and then 70% ethanol.

c. Wear gloves at all times when handling electrophoretic equipment, photographic equipment, and agarose gels.

d. Pour 2% agarose gel approximately 40 min before thermal cycler is finished.

(1) Weigh out 2 g of low-EEO agarose and put into a 100-ml Erlenmeyer flask.

(2) Add 50 ml of H_2O and 50 ml of TBE buffer.

(3) Add magnetic stir bar and stopper flask with Kimwipes.

(4) Place on hot plate. Set heat and stir controls to 4.

(5) Bring solution to a gentle boil.

(6) While the agarose solution is heating, place tape at either end of gel tray to make a tight seal and insert two combs.

(7) After agarose has come to a boil, add 2.5 µl of ethidium bromide (10 mg/ml; Bio-Rad) to agarose solution. Swirl to mix.

(8) ***Caution:*** *Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye.*

(9) Pour agarose solution into tray and insert combs. Let stand to cool for 20 to 30 min.

(10) Fill gel box with 300 ml of 1× TBE and 300 µl of dH_2O.

(11) Remove tape and combs and place gel tray in gel box; buffer solution should cover gel. Place the tray in the gel box with the holes farthest away from the positive charge (red).

e. Work with the amplified material in the chemical fume hood.

f. Remove cap strips from DQA tubes carefully to avoid any aerosolization. (Do this while caps are still warm and malleable.)

g. Add 5 µl of loading dye to each DQA tube, and pipette up and down to mix; use a new tip for each tube.

h. Pipette 35 µl of each sample (using a P200 pipette) into appropriate gel wells. Use a new pipette tip for each tube.

i. Add 5 µl of DNA molecular weight standard to the first lane (on the left) in each row of the gel.

V. PROCEDURE *(continued)*

j. Place protective lid on gel box, and connect leads. Let gel run for approximately 25 min at 150 to 160 V.

k. The remaining amplified material should be stored at 4°C.

l. Remove gel tray from gel box. Carefully slide gel from gel tray onto UV box (gels fragment very easily). Put on UV-protective face shield and then turn UV box on to *high* intensity setting and photograph.
Caution: *Use protective eyeglasses or face shield when viewing gels on UV box.*

m. Photograph gel. Camera settings: shutter speed, 2 (0.5 s); aperture, 5.6. Develop the photo for 30 to 60 s.

n. Discard gel in infectious-substance boxes. Empty and rinse gel box into the sink. Wipe down UV box with water. Wipe down fume hood with 10% bleach and then 70% ethanol.

o. Trim picture and tape to worksheet; label lanes on the gel.

p. Record in result area of the worksheet and have a supervisor or director review and sign.

2. Probe detection of *M. pneumoniae* reactions

a. Bring detection reagents, including microwell strips, to room temperature.

b. Add 100 μl of hybridization buffer to each microwell; use plugged pipette tips.

c. Add 20 μl of denatured amplified sample to the appropriate microwell. Seal the micotiter wells, and mix by tapping the plate gently.

d. Incubate for 1 h ± 5 min at 50°C (± 1°C).

e. Just before end of the incubation, prepare working anti-double-stranded DNA (anti-dsDNA) antibody solution. Make a 1:50 dilution of the anti-dsDNA antibody solution using the anti-dsDNA diluent.

Number of strips	Anti-dsDNA (μl)	Anti-dsDNA diluent (μl)
1	20	980
2	40	1,960
3	60	2,940
4	80	3,920

f. Prepare 500 ml of wash buffer; add 20 ml to 480 ml of Fisher water (must be Fisher water). Store unused buffer for 1 week at 4°C. After refrigeration, warm buffer at 37°C to resuspend any crystals. Wash plate five times in an automated plate washer (program 9) (add 350 μl of wash buffer, soak for 30 s, and aspirate).

g. Add 100 μl of diluted anti-dsDNA antibody to each well. Cover and incubate for 30 ± 2 min at room temperature.

h. Wash plate as described in step V.C.2.f.

i. Prepare working enzyme tracer solution. Make a 1:50 dilution of the tracer with tracer diluent.

Number of strips	Enzyme tracer (μl)	Tracer diluent (μl)
1	20	980
2	40	1,960
3	60	2,940
4	80	3,920

j. Pipette 100 μl of diluted enzyme tracer into each well. Cover and incubate for 30 ± 2 min at room temperature.

k. Prepare the working chromagen-substrate solution before the end of the incubation period.

V. PROCEDURE *(continued)*

(1) Add equal volumes of chromagen and substrate

Number of strips	Chromagen (ml)	Substrate (ml)
1	0.5	0.5
2	1.0	1.0
3	1.5	1.5
4	2.0	2.0

(2) Store chromogen-substrate in the dark until ready to use.

l. Wash plate as described in step V.C.2.f.

m. Pipette 100 µl of chromogen-substrate into each well. Cover and incubate for 30 ± 2 min at room temperature in the dark.

n. Pipette 100 µl of blocking reagent into each well.

o. Measure optical density at 450 nm (OD_{450}) just after adding the blocking reagent.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report results as positive, low positive, or negative for *M. pneumoniae* DNA.

B. Call positive results to requesting area. Note on the worksheet the name of the person called, date, and time, and initial.

C. Document all testing in hard copy or computerized work card.

VII. INTERPRETATION

A. *M. pneumoniae* PCR

1. The cutoff for a positive result is an OD_{450} of ≥ 1.0.

2. An OD_{450} between 0.5 and 1.0 is reported as a low positive.

3. An OD_{450} of <0.5 is negative.

4. The cutoff was determined by calculating the mean and standard deviation (SD) of the OD values of 116 negative patients. The cutoff is set at the mean + 10 SDs.

5. If any of the negative control reactions are positive (OD > cutoff), the run is invalid and must be repeated.

6. If the *M. pneumoniae* positive control reaction does not have an OD above 1.0, the run is invalid and must be repeated.

7. A clinical specimen is considered negative for *M. pneumoniae* DNA if the OD value of the specimen is below the cutoff and if the spiked control has an OD value greater than the cutoff value. For BAL specimens there is no inhibition control.

8. If the clinical specimen is negative for *M. pneumoniae* DNA (OD < cutoff) and the spike control is also negative, the test is invalid and testing of the specimen must be repeated. If similar results are obtained on repeat testing, the test should be reported as "uninterpretable; specimen contains a substance that inhibits amplification."

9. If the patient result is low positive or positive and the spike is negative, the specimen should be retested and the result discussed with the supervisor, senior technologist, or director before reporting.

VII. INTERPRETATION
(continued)

B. DQA PCR

1. If the negative control reaction is positive, the run is invalid and must be repeated.

2. If there is not a 214-bp product amplified in the positive control reaction, the run is invalid and must be repeated.

3. If the clinical specimen does not contain a 214-bp band, the result should include following disclaimer: "Interpret with caution; no human DNA detected."

VIII. LIMITATIONS OF TESTING

A. A negative result for *M. pneumoniae* DNA by PCR does not rule out the possibility that *M. pneumoniae* DNA is present in the specimen at a concentration below the level of sensitivity of the assay. The assay is capable of detecting 200 CFU of *M. pneumoniae* per ml of specimen. It is also possible that there is inhibition of the PCR which is not detected by this protocol.

B. A positive result for *M. pneumoniae* DNA by PCR may be the result of contamination rather than a true positive result. This protocol employs several steps to decrease the risk of contamination, including separate areas for assay setup, specimen preparation, and amplification and detection of amplified products; unidirectional work flow; the use of dUTP and UNG to decrease the risk of carryover contamination; good laboratory technique and the use of a laminar-flow hood during specimen preparation to avoid contamination between specimens; opening of reaction tubes under a fume hood in area 3 after amplification; and periodic wipe tests of work areas.

C. The presence of *M. pneumoniae* DNA in the lower respiratory tract may not always correlate with clinical disease. The DNA may be present from a previous (resolved) upper respiratory tract infection unrelated to the current illness.

REFERENCES

1. **Bernet, C., M. Garret, B. deBarbeyrae, C. Bebear, and J. Bonnet.** 1989. Detection of *Mycoplasma pneumoniae* by using the polymerase chain reaction. *J. Clin. Microbiol.* **27:**2492–2496.
2. **CLSI.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. CLSI, Wayne, PA.
3. **Jacobs, E.** 1993. Serologic diagnosis of *Mycoplasma pneumoniae* infections: a critical review of current procedures. *Clin. Infect. Dis.* **17**(Suppl. 1)**:**S79–S82.
4. **Kessler, H. H., D. E. Dodge, and K. Pierer.** 1997. Rapid detection of *Mycoplasma pneumoniae* by an assay based on PCR and probe hybridization in a nonradioactive microwell plate format. *J. Clin. Microbiol.* **35:**1592–1594.
5. **Luneberg, E., J. S. Jensen, and M. Frosch.** 1993. Detection of *Mycoplasma pneumoniae* by polymerase chain reaction and nonradioactive hybridization in microtiter plates. *J. Clin. Microbiol.* **31:**1088–1094.
6. **Ramirez, J. A., S. Ahkee, and A. Tolentino.** 1996. Diagnosis of *Legionella pneumophila, Mycoplasma pneumoniae,* or *Chlamydia pneumoniae* lower respiratory infection using the polymerase chain reaction on a throat swab specimen. *Diagn. Microbiol. Infect. Dis.* **24:**7–14.
7. **Tjhie, J. H. T., F. J. M. van Kuppeveld, and R. Roosendahl.** 1994. Direct PCR enables detection of *Mycoplasma pneumoniae* in patients with respiratory tract infections. *J. Clin. Microbiol.* **32:**11–16.
8. **van Kuppeveld, F. J., K. Johansson, and J. M. Galama.** 1994. 16S rRNA based polymerase chain reaction compared with culture and serological methods for diagnosis of *Mycoplasma pneumoniae* infection. *Eur. J. Clin. Microbiol. Infect. Dis.* **13:**401–405.

PART 8

Detection of *Bordetella pertussis* and *Bordetella parapertussis* by Real-Time PCR

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Pertussis, or whooping cough, caused by *Bordetella pertussis* or *Bordetella parapertussis* has reemerged as an important disease in children and adults. As *B. pertussis* is a fastidious pathogen and requires specialized media for culture, PCR amplification has been adopted as a strategy for diagnosis. Direct immunofluorescence assay and microbiological culture have reduced sensitivities compared to PCR-based detection, as determined by multiple studies. Endpoint and real-time PCR methods are commonly used for the detection of *B. pertussis* or *B. parapertussis* DNA in nasopharyngeal swabs and aspirates from patients with disease symptoms.

Real-time PCR assays have been developed for the multiplex detection of *B. pertussis* and *B. parapertussis* (2, 7). Both Cepheid (Sunnyvale, CA) and Roche Diagnostics (Indianapolis, IN) offer ASRs for the detection of *B. pertussis.* The Roche ASR assay detects both *B. pertussis* and *B. parapertussis* on the LightCycler. The primers are designed to amplify a 234-bp fragment of the IS*481* gene of *B. pertussis* and a 200-bp fragment of the IS*1001* gene of *B. parapertussis* (6). Hybridization probes are used to target each of the amplicons, and a melting curve analysis is used to differentiate between *B. pertussis* and *B. parapertussis.* An internal recovery template is also included to control for PCR inhibition (6).

A lower limit of detection of one genome equivalent has been observed for both *B. pertussis* and *B. parapertussis.* The sequences targeted by this assay represent multicopy, relatively species-specific targets that provide a high level of sensitivity and specificity. Importantly, the IS*481* target for *B. pertussis* has cross-reactivity with *Bordetella holmesii* and *Bordetella bronchiseptica* in human infections, so species specificity is not absolute (3, 7).

◩ **NOTE:** Performance of PCR requires licensure from Roche Molecular Systems, Inc.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. Specimen: nasopharyngeal material
2. Collection device: rayon-tipped, flexible nasopharyngeal swab in Amies charcoal
3. Label: patient identifiers, date, and time
4. Timing of collection: not applicable

◩ **NOTE:** Alternative collection devices must be tested to ensure compatibility with the protocol. To prevent inhibition of PCR, avoid nasopharyngeal swabs with calcium alginate tips.

B. Specimen transport and storage

1. Transport temperature: room temperature (immediately to lab)
2. Storage temperature: 4°C

C. Rejection criteria

Unlabeled specimen

◩ **NOTE:** Notify the ordering clinician if a new specimen is needed.

III. MATERIALS

Include QC information on reagent container and in QC records.

(All MagNA Pure and LightCycler reagents and equipment are from Roche Diagnostics.)

A. DNA extraction

1. MagNA Pure LC DNA isolation kit III (bacteria, fungi)
   - **a.** Proteinase K
   - **b.** Elution buffer
   - **c.** Wash buffer I
   - **d.** Wash buffer II
   - **e.** Wash buffer III
   - **f.** Lysis/binding buffer
   - **g.** Magnetic glass particle suspension
   - **h.** Bacterial lysis buffer

   ◩ **NOTE:** Avoid mixing lysis/binding buffer with sodium hypochlorite, as toxic gas formation results (4).
2. Specimen collected on rayon nasopharyngeal swab (Mini Tip Amies Charcoal, Flexible Twisted Wire; Copan Diagnostics)
3. 1.5-ml microcentrifuge tubes

III. MATERIALS *(continued)*

4. P2, P20, and P200 pipettes
5. 2-, 20-, and 200-µl pipette tips (with aerosol barriers)
6. Heating block (65 and 95°C)
7. Pipette pump
8. 5- and 10-ml serological pipettes
9. Vortex mixer
10. Disposable gloves
11. MagNA Pure LC instrument
12. MagNA Pure LC consumables
    a. Sample cartridges
    b. Processing cartridges
    c. Cartridge seals
    d. Reaction tips
    e. Reagent tubs (medium, 20 ml)
    f. Reagent tubs (large)
    g. Drop catcher
    h. Waste bag
    i. Waste bottle

◩ **NOTE:** The appropriate performance of alternative DNA extraction methods with the PCR assay must be satisfactorily demonstrated.

B. PCR setup

1. LightCycler FastStart DNA master hybridization probes (deoxynucleotide triphosphates, DNA polymerase, $MgCl_2$, buffer)
2. LightCycler *Bordetella* IS*481/1001* recovery template
3. PCR-grade water
4. LightCycler *Bordetella* IS*481/1001* primer/hybridization probes

◩ **NOTE:** This mix contains two separate primer and hybridization probe pairs specific for *B. pertussis* (IS*481*) and *B. parapertussis* (IS*1001*), and a hybridization probe pair specific for the internal control (recovery template). One of each of the hybridization probes for *B. pertussis* and *B. parapertussis* is labeled with fluorescein and the other with LightCycler Red 640. The hybridization probes for the recovery template are labeled with fluorescein or LightCycler Red 705.

5. LightCycler *Bordetella* IS*481/1001* template DNA
6. LightCycler capillaries with stoppers
7. LightCycler carousel
8. LightCycler carousel centrifuge

C. Amplification and melting curve analysis

1. LightCycler color compensation set
2. LightCycler 1.0 instrument thermal cycler

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Controls

1. The positive control (LightCycler *Bordetella* IS*481/1001* template DNA) demonstrates that appropriate melting curves are produced for *B. pertussis* and *B. parapertussis*.
2. The negative (reagent) control demonstrates that reagents are free of target or amplicon contamination.
3. The inhibition control (LightCycler *Bordetella* IS*481/1001* recovery template) monitors for PCR inhibitors.

B. Do not use expired reagents.

C. Check new reagent lots by comparison with the old lot.

V. PROCEDURE

Observe standard precautions.

A. DNA extraction

1. Reconstitute one bottle of lyophilized proteinase K with 1.2 ml of elution buffer.
2. For each specimen, mix 130 µl of lysis buffer and 20 µl of proteinase K in a 1.5-ml microcentrifuge tube.
   Caution: *Lysis/binding buffer can cause burns. Thoroughly wash exposed areas at once.*
3. With every extraction run, include a reagent control composed of 130 µl of lysis buffer and 20 µl of proteinase K in a 1.5-ml microcentrifuge tube.
4. Add patient swabs to the respective microcentrifuge tubes.
5. Incubate patient sample tubes and the negative control tube at 65°C for 10 min.

V. PROCEDURE *(continued)*

6. Press swabs against the sides of microcentrifuge tubes to remove excess liquid.
7. Incubate patient sample tubes and the negative control tube at 95°C for 10 min.
8. Move lysates and the negative control to respective wells of a MagNA Pure LC sample cartridge.
9. Open the MagNA Pure LC software.
10. Under sample ordering, choose DNA III Bacteria-Standard Protocol.
11. Enter 100 µl as the sample volume.
12. Enter 100 µl as the elution volume.
13. Designate the order of the samples and the negative control in the MagNA Pure LC sample cartridge.
14. Choose Start Batch.
15. Per the on-screen instructions, place plastic consumables on the MagNA Pure LC stage, place plastic tubs in the tub rack, and add the amounts of reagents (except for the magnetic glass particle suspension) given by the software to the tubs.
16. Load the tub rack and the sample cartridge containing DNA on the instrument.
17. Click the various on-screen pictures of consumables and reagents to verify placement.
18. Mix the magnetic glass particle suspension with a vortex mixer and add to the corresponding tub on the tub rack.
19. Promptly begin the run by selecting OK.
20. Remove the sample cartridge containing DNA and store sealed (with a cartridge seal) at 4°C until use.
21. Dispose of consumables and excess reagents as instructed by the manufacturer.

◩ **NOTE:** Store reconstituted proteinase K at 2 to 8°C for up to 4 weeks. Store remaining kit components at room temperature.

B. PCR setup

1. Prepare the LightCycler FastStart DNA master hybridization probes by combining vials 1a and 1b (as labeled in the kit) and mixing by pipetting.
2. Dilute sufficient LightCycler *Bordetella* IS*481/1001* recovery template 1:10 with PCR-grade water.
3. Prepare PCR master mix by adding these volumes multiplied by the number of samples plus controls (include 10% extra for pipetting error) to a 1.5-ml tube and mixing by pipetting:

Reagent	Volume
PCR-grade water	10 µl
LightCycler FastStart DNA master hybridization probes	2 µl
LightCycler *Bordetella* IS*481/1001* primer/hybridization probes	2 µl
LightCycler *Bordetella* IS*481/1001* recovery template (diluted)	1 µl

◩ **NOTE:** Keep reagents on a cooling block during master mix preparation.

4. To each capillary, add 15 µl of master mix.
5. For each specimen, add 5 µl of DNA to the respective capillary.
6. To the positive control capillary, add 5 µl of LightCycler *Bordetella* IS*481/1001* template DNA.
7. To the negative control capillary, add 5 µl of the negative control.
8. Close capillaries with stoppers.

V. PROCEDURE *(continued)*

9. Using the LightCycler carousel centrifuge, spin the capillaries at 3,000 rpm.

◩ **NOTE:** Store mixed LightCycler FastStart DNA master hybridization probes at −20°C for up to 3 months or 2 to 8°C for up to 1 week. Store remaining reagents at −20°C. Avoid freeze-thaw cycles. Avoid exposure of LightCycler *Bordetella* IS*481/1001* primer/hybridization probes to light.

◩ **NOTE:** Avoid touching the sides of capillaries.

C. Amplification and melting curve analysis

1. Load the color compensation file (as per the LightCycler manual).

2. Enter the parameters in Table 12.2.3–6 into the LightCycler software.

◩ **NOTE:** Before performing the PCR, save these entered parameters for future use.

3. Enter sample and control identifiers in the specimen fields of the LightCycler software.

4. Load the carousel onto the LightCycler.

5. Begin the run.

6. Select fluorescence channel 2 (F2)/1 or F2/back F1.

7. Upon completion of the run, review melting curves using channel F2 for *B. pertussis/parapertussis* and channel F3 for the internal control.

8. Dispose of capillaries, taking care not to break them during removal from the carousel.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report positive results as "Positive for *Bordetella pertussis*" or "Positive for *Bordetella parapertussis*."

B. Report negative results as "*Bordetella pertussis* and *Bordetella parapertussis* not detected."

C. Notify the ordering clinician and the local or state health department of specimens positive for *B. pertussis*.

D. Include, "This test was developed and its performance characteristics determined by (laboratory name). It has not been cleared or approved by the Food and Drug Administration." As recommended by the CAP, add, "The FDA has determined that such clearance or approval is not necessary. This test is used for clinical purposes. It should not be regarded as investigation or for research. This laboratory is certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA-88) as qualified to perform high-complexity clinical laboratory testing" (1).

E. As applicable, include, "This test is performed pursuant to a license agreement with Roche Molecular Systems, Inc."

Table 12.2.3–6 Thermal cycler parameters for *B. pertussis/B. parapertussis* real-time PCR

Phase	Denaturation	Amplification			Melting curve analysis				Cooling
Cycles	1	45			1				1
Analysis mode	None	Quantification			Melting curve				None
Temp target segment	1	1	2	3	1	2	3	4	1
Target temp (°C)	95	95	55	72	95	59	45	85	45
Incubation time (h:min:s)	00:10:00	00:00:10	00:00:15	00:00:15	0	00:00:20	00:00:20	0	00:00:10
Temp transition rate (°C/s)	20.0	20.0	20.0	20.0	20.0	20.0	0.2	0.1	20.0
Acquisition mode	None	None	Single	None	None	None	None	Cont.[a]	None

[a] Cont., continuous.

VII. INTERPRETATION

A. The positive control should produce a melting peak at 69 ± 2°C, corresponding to *B. pertussis*, and at 62 ± 2°C, corresponding to *B. parapertussis.*

B. The negative control should not produce an amplification curve or melting peaks.

C. The inhibition (internal) control for each sample should produce a melting peak at 71.3 ± 2°C.

D. A positive specimen produces a melting peak corresponding to *B. pertussis* or *B. parapertussis* (Fig. 12.2.3–2).

E. A negative specimen does not produce an amplification curve or a melting curve.

F. Failure of positive or negative controls invalidates the run.

G. Failure of the inhibition control invalidates the result for the corresponding specimen, except when a melting peak for *B. pertussis* or *B. parapertussis* is present in the F2 channel.

H. For patient samples, amplification curves with thresholds beyond 35 cycles may represent nonspecific product formation.

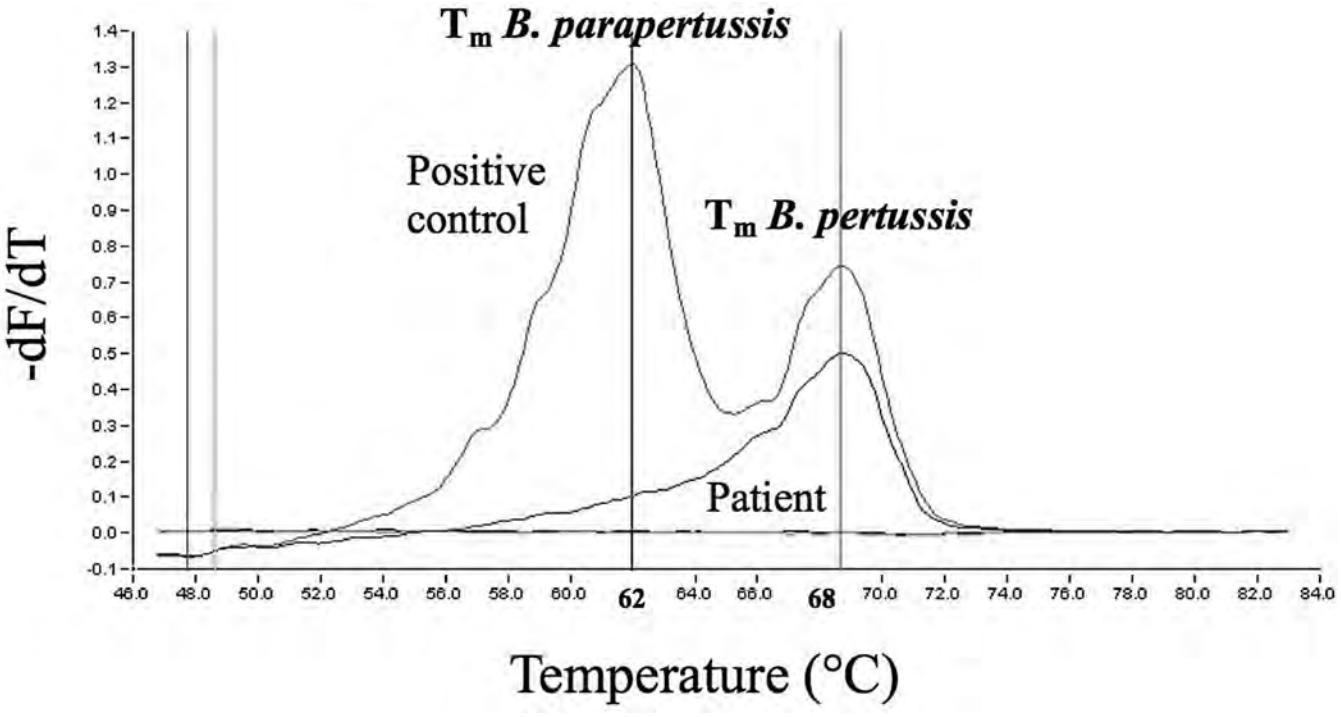

Figure 12.2.3–2 Melting peaks corresponding to *B. parapertussis* and *B. pertussis.*

VIII. LIMITATIONS

A. The performance characteristics of this assay must be established by each laboratory.

B. IS*481* for *B. pertussis* may also be present in *B. holmesii* and *B. bronchiseptica*, and assays based on IS*481* may yield false-positive results.

REFERENCES

1. **College of American Pathologists.** 2009. Laboratory Accreditation Program, Microbiology Checklist, rev. 06/15/2009. College of American Pathologists, Northfield, IL.
2. **Kösters, K., U. Reischl, J. Schmetz, M. Riffelmann, and C. H. Wirsing von König.** 2002. Real-time LightCycler PCR for detection and discrimination of *Bordetella pertussis* and *Bordetella parapertussis. J. Clin. Microbiol.* **40:**1719–1722.
3. **Muyldermans, G., O. Soetens, M. Antoine, S. Bruisten, B. Vincart, F. Doucet-Populaire, N. K. Fry, P. Olcén, J. M. Scheftel, J. M. Senterre, A. van der Zee, M. Riffelmann, D. Piérard, and S. Lauwers.** 2005. External quality assessment for molecular detection of *Bordetella pertussis* in European laboratories. *J. Clin. Microbiol.* **43:**30–35.
4. **Roche Applied Science.** July 2004. *MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) (Cat. No. 03 264 785 001) Instruction Manual.* Roche Applied Science, Indianapolis, IN.
5. **Roche Diagnostics.** October 2003. *LightCycler* Bordetella IS481/1001 *Primer/Hybridization Probes Instruction Manual*, version 1. Roche Applied Science, Indianapolis, IN.
6. **Roche Diagnostics.** October 2003. *LightCycler* Bordetella *IS*481/1001 *Template Set Instruction Manual*, version 1. Roche Applied Science, Indianapolis, IN.
7. **Sloan, L. M., M. K. Hopkins, P. S. Mitchell, E. A. Vetter, J. E. Rosenblatt, W. S. Harmsen, F. R. Cockerill, and R. Patel.** 2002. Multiplex LightCycler PCR assay for detection and differentiation of *Bordetella pertussis* and *Bordetella parapertussis* in nasopharyngeal specimens. *J. Clin. Microbiol.* **40:**96–100.

PART 9 Quantitative Detection of Human Cytomegalovirus by Real-Time PCR

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Real-time PCR represents an example of a laboratory-developed quantitative assay. There are a number of different methods by which quantitation can be achieved, including extrapolation from standard curves and comparison to internal or external quantitative standards. In addition, there are various algorithms that can be used to detect a positive signal in a reaction. An in-depth description of these quantitation and detection methods is provided in the supplemental reading section. The method described herein utilizes an external calibration curve for quantitative analysis, along with assignment of a detection cycle based on a threshold crossing algorithm.

Human cytomegalovirus (CMV) is a herpesvirus that infects a large proportion of the population. However, although up to 80% of adults in the United States may be seropositive for CMV, the majority of infections are subclinical. Nevertheless, new infections or reactivation of latent infections can cause serious disease in immunocompromised patients, including transplant recipients, in whom it may cause pneumonitis, retinitis, hepatitis, and colitis. Quantitative PCR is an important tool for monitoring transplant patients. Viremia has been shown to correlate with clinical disease, and monitoring viral loads after transplant surgery enables the monitoring of CMV disease progress or relapse and allows for the administration of effective preemptive antiviral therapy (1, 4, 6).

This assay targets a 283-bp segment of the CMV UL83 gene. The primers chosen will not amplify and detect DNA from other herpesviruses, and there is no cross-reactivity observed with human DNA (5). Detection of the targeted CMV DNA sequence utilizes TaqMan probe chemistry. This involves the use of a dually labeled probe with a reporter dye at the 5′ end and a quencher at the 3′ end. When the reporter and quencher are both present on an intact probe molecule, any fluorescence energy produced by the reporter is suppressed. However, if the probe binds to a specific target, 5′-to-3′ exonuclease activity of the DNA polymerase will degrade the probe, thus allowing the reporter and quencher to separate, resulting in the generation of a detectable fluorescent signal.

The amount of fluorescence produced is proportional to the starting quantity of template DNA, so monitoring the fluorescence produced during each PCR cycle enables a determination of the amount of

target template DNA that is present in the sample. In this assay, the amount of DNA in a patient's specimen is determined by assessing the point at which fluorescence production in a reaction exceeds a predetermined threshold value. The earlier that this fluorescent signal is detected, the larger the amount of template DNA. The specific quantity is calculated by interpolation from a standard curve using a curve equation generated by assaying dilutions of a quantified CMV virus stock.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. The assay requires the sterile collection of at least 200 µl (0.20 ml) of specimen, although optimally, 500 µl or more of specimen will be collected to enable repeat analysis if required.

2. Specimens may include plasma, serum, or other specimen types that have been validated by the performing laboratory. Blood samples should be anticoagulated using EDTA or acid citrate dextrose only.

B. Timing of specimen collection

There are no particular timing issues to consider in collecting these specimens; however, the specimen type and time of collection should be recorded.

C. Specimen transport

1. Separate plasma within 3 h by centrifugation at 1,000 × *g* for 20 min at room temperature.

2. Plasma samples may be transported at 2 to 8°C or frozen.

3. Samples may be stored at 2 to 8°C for up to 7 days or frozen for longer-term storage.

4. An aliquot of 200 µl of specimen is required for testing; the remaining specimen is stored frozen for repeat testing if necessary.

D. Specimen labeling and submission

The laboratory should determine a mechanism to ensure that the specimen type and time of collection are recorded.

E. Rejection criteria

1. Test only validated specimen types.

2. Reject specimens for which heparin was used as an anticoagulant.

3. Reject specimens that arrive in leaking or broken tubes or specimen containers.

4. Request a repeat specimen, or obtain the information, when the collection time or specimen type has not been provided.

5. If an improperly collected, transported, and/or handled specimen cannot be replaced, document in the final report that specimen quality may have been compromised.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Supplies and equipment

1. Master mix preparation

- **a.** 2-, 20-, 200-, and 1,000-µl pipettes
- **b.** 2-, 20-, 200-, and 1,000-µl aerosol-resistant pipette tips
- **c.** Spray/squirt bottles—bleach, ethanol
- **d.** −20°C freezer
- **e.** Dead-air box
- **f.** Ziploc bags
- **g.** Sterile 2.0-ml screw-top microcentrifuge tubes
- **h.** Microtube racks
- **i.** 96-well optical plate

2. Sample preparation area

- **a.** MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche)
  - (1) Proteinase K
  - (2) Elution buffer
  - (3) Wash buffer I
  - (4) Wash buffer II
  - (5) Lysis/binding buffer

    ◩ **NOTE:** Avoid mixing lysis/binding buffer with sodium hypochlorite, as toxic gas formation will occur (7).
  - (6) Magnetic glass particle suspension

III. MATERIALS *(continued)*

b. 2-, 20-, 200-, and 1,000-µl pipettes
c. 2-, 20-, 200-, and 1,000-µl aerosol-resistant pipette tips
d. Pipette pump
e. 5-ml serological pipettes
f. Vortex mixer
g. Disposable gloves
h. MagNA Pure LC instrument
i. MagNA Pure LC consumables
(1) Sample cartridges
(2) Processing cartridges
(3) Cartridge seals
(4) Reaction tips
(5) Reagent tubs (medium, 20 ml)
(6) Reagent tubs (large)
(7) Drop catcher
(8) Waste bag
j. Ziploc bags
k. Optical plate sealing tape (Applied Biosystems)
l. Plate-sealing tool (Applied Biosystems)
m. Spray/squirt bottles—bleach, ethanol
n. Biosafety cabinet
o. Dead-air box

3. Amplification and detection area
a. Applied Biosystems 7500 Sequence Detection System
b. Spray/squirt bottles—bleach, ethanol

B. Reagents

1. Sodium hypochlorite (bleach) solution
2. 100 proof ethanol
3. 2× universal master mix (Applied Biosystems)
Store in reagent preparation area either refrigerated or at −20°C for longer-term storage. Expiration: do not use beyond manufacturer's control date.
4. TaqMan exogenous internal positive control reagents (Applied Biosystems)
5. 10 mM Tris–1 mM EDTA (TE) solution (pH 8.0) (Ambion or equivalent)
Store at room temperature. Expiration, 1 year.
6. Sterile molecular-biology-grade water (Ambion or equivalent)
Store at room temperature. Expiration, 1 year.
7. CMV-F primer, CMV-R primer, and CMV probe (Biosearch Technologies, Inc., or equivalent) (Table 12.2.3–7)
Depending on the manufacturer, primers may be received as lyophilized pellets, in which case they should be resuspended to a final concentration of 100 µM using TE buffer, pH 8.0. Dilution should be performed in the reagent preparation area. This is the stock solution. Store frozen. Expiration of stock solution, 1 year. If primers and probes are received in liquid form, they should be at a concentration of 100 µM.
◩ **NOTE:** If stock solutions are to be used repeatedly to produce new lots of master mix, they should be stored in 50- to 100-µl aliquots to minimize freeze-thaw cycles.
8. CMV high positive control (Acrometrix, Zeptometrix, or equivalent)
9. CMV low positive control (Acrometrix, Zeptometrix, or equivalent)
10. Negative control
The negative control can be purchased from a commercial manufacturer, or a clinical matrix (normal human plasma) tested to be negative for the presence of CMV virus can be utilized.
11. CMV stock solutions for standard curve preparation (Zeptometrix or equivalent): 400 to 100,000 copies/ml

C. Preparation of reagents

1. Primer-probe mix
a. Prepare final primer probe mix according to the recipe listed in

Table 12.2.3–7 PCR primers and probe for quantitation of human CMV

Oligonucleotide designation	Nucleotide sequence, 5′–3′
CMV-F	GTCAGCGTTCGTGTTTCCCA
CMV-R	GGGACACAACACCGTAAAGC
CMV probe	6-FAM-CCCGCAACCCGCAACCCTTCATG-TAMRA[a]

[a] 6-FAM and TAMRA represent the 5′ reporter fluorophore and the 3′ quencher, respectively. These molecules are covalently linked to the oligonucleotide.

III. MATERIALS *(continued)*

Table 12.2.3–8. The amount of mix prepared will be based on the routine number of specimens received in the laboratory. A batch of master mix should ideally last at least 6 months. Strict adherence to protocol is essential to avoid contamination of the primer lot. Primers, stock solutions, and primer-probe mixes should be prepared in the reagent preparation area before the technologist performs any tasks in the sample preparation or amplification area. *Never formulate and/or aliquot master mix after working in sample preparation area or amplification area.*

b. Add the components, as listed in Table 12.2.3–8, in a 2.0-ml tube in a dead-air box in the reagent preparation area, and pulse vortex to mix.

c. Aliquot 150 µl of the mixture into sterile 2.0-ml screw-top tubes. Label tubes with the following information: CMV master mix, 10 reactions, date of preparation, batch number, and initials of technologist preparing mixture. Store aliquots at −20°C in area 1 in a non-frost-free refrigerator, and minimize exposure to light. Expiration, 1 year after formulation.

d. Prior to use, add 250 µl of 2× universal master mix per vial of primer-probe mix. This will provide enough master mix for 9 reactions.

◩ **NOTE:** Although the volume is sufficient for 10 reactions, the loss of volume due to adherence of liquid to the tube and to pipette tips while aliquoting will result in sufficient reagent to run only 9 reactions.

2. Decontamination solutions

a. 10% Bleach
Add 25 ml of concentrated bleach with 225 ml of deionized water passed through a 0.4-µm-pore-size filter in a squirt bottle. A squirt bottle of 10% bleach should be made for each of the three work areas. Expiration, 1 week.

b. 70% Ethanol solutions
Mix 480 ml of 100% ethanol with 170 ml of deionized water passed through a 0.4-µm-pore-size filter in a spray bottle. A spray bottle of 70% ethanol should be made for each of the three work areas. Expiration, 1 week.

D. Establishment of standard curve

1. Prior to assay implementation, a standard curve must be generated.

2. Establish the standard curve by using a test procedure to run quantified CMV dilutions. For materials purchased from Zeptometrix, concentrations will be 100,000, 50,000, 10,000, 5,000, and 400 copies/ml.

a. Run a minimum of five replicates of each virus concentration in each of at least five runs.

b. Calculate the average threshold cycle (Ct) value for each dilution, and plot the log concentration values against the average Ct values obtained. The resulting curve equation can be used to calculate the quantities of unknown samples. For unknown samples, using the curve equation $y = mx + b$, the x value (concentration of the unknown) is solved by using the generated Ct value as the y value and by using the slope *(m)* and intercept *(b)* values that were derived during the establishment of the standard curve.

Table 12.2.3–8 Primer-probe mix formulation[a]

Component	Final concn	Amt per Rx (µl)	Amt per 100 Rx (µl)
CMV-F (100 µM)	200 nM	0.1	10
CMV-R (100 µM)	200 nM	0.1	10
CMV probe (100 µM)	100 nM	0.05	5
10× exogenous IPC mix	1×	5	500
50× exogenous IPC DNA	1×	1	100
Nuclease-free water		8.75	875

[a] IPC, internal positive control; Rx, reaction.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Run controls

1. One high positive control, one low positive control, and at least one negative control are included in each run. Each MagNA Pure extraction cartridge should contain one high positive, one low positive, and one negative control. Positive and negative controls are taken through the entire extraction and amplification processes. Control ranges should be established for each positive control prior to assay implementation.
2. An internal positive amplification control is included with each specimen. Control ranges should be established for the internal control.

B. Establishment of control ranges

1. Prior to assay implementation, control ranges must be established for the high positive, low positive, and internal positive controls.
2. The average Ct values and standard deviations for each control should be established by running at least two replicates of each control over the course of 20 runs.

C. Specificity

The primer and probe set used in this reaction is published and has been shown to amplify CMV DNA but not DNA from other herpesviruses or from human sources (5).

D. Contamination prevention

The following steps have been taken to reduce the risk of contamination.

1. Separate areas for assay setup, specimen preparation and amplification, and detection of amplified products
2. Unidirectional work flow
3. The use of dUTP and uracil *N*-glycosylase (UNG) to decrease the risk of carryover contamination
4. Good laboratory technique and a laminar-flow hood during specimen preparation to avoid contamination between specimens

E. Reagent QC

Each batch of master mix is tested as follows.

1. Twenty reactions are performed using water as a template. This is to ensure that the reagents are not contaminated. If any of the reactions are positive, the master mix is rejected.
2. One each of a previous positive and previous negative patient specimen is run with the new batch of master mix along with the positive control. Results must match those obtained with the previous batch or the master mix is rejected.
3. Verify that all reagents and materials meet expiration date and QC parameters as per CLSI document M22-A3 (2).

V. PROCEDURE

Observe standard precautions.

A. Master mix setup

1. In a dead-air box in the reagent preparation area, prepare final PCR master mix by adding 250 µl of 2× universal master mix to a 150-µl aliquot of primer-probe master mix.
2. Add 40 µl of complete master mix to each well of a 96-well optical plate that will be used.
3. Put the optical plate in a Ziploc bag, and transfer it to the sample preparation area. Store at 4°C until extracted samples are ready to load.
4. Clean the area by wiping with 10% bleach solution, followed by wiping with 70% ethanol solution.

V. PROCEDURE *(continued)*

B. DNA extraction

1. In a biosafety cabinet in the sample preparation area, load 200 µl of each specimen and of each control into a MagNA Pure sample cartridge. The negative specimen should be the last sample loaded and processed.
2. Reconstitute one bottle of lyophilized proteinase K with 5.0 ml of elution buffer.
3. Open the MagNA Pure LC software.
4. Under sample ordering, choose Total Nucleic Acid Variable Elution.
5. Enter 200 µl as the sample volume.
6. Enter 50 µl as the elution volume.
7. Designate the order of the samples and the negative control in the MagNA Pure LC sample cartridge.
8. Choose Start Batch.
9. Per the on-screen instructions, place plastic consumables on the MagNA Pure LC stage, place plastic tubs in the tub rack, and add the amounts of reagents (except for the magnetic glass particle suspension) given by the software to the tubs.
   Caution: *Lysis/binding buffer can cause burns. Thoroughly wash exposed areas at once.*
10. Load the tub rack and the sample cartridge containing DNA on the instrument.
11. Click the various on-screen pictures of consumables and reagents to verify placement.
12. Mix the magnetic glass particle suspension with a vortex mixer and add to the corresponding tub on the tub rack.
13. Promptly begin the run by selecting OK.
14. After run completion, remove the sample cartridge containing DNA and store sealed (in a Ziploc bag) at 4°C until use.
15. Dispose of consumables and excess reagents as instructed by the manufacturer.
    Store reconstituted proteinase K at 2 to 8°C for up to 4 weeks. Store remaining kit components at room temperature.
16. Clean the area by wiping with 10% bleach solution, followed by wiping with 70% ethanol solution.
    ◩ **NOTE:** Avoid mixing lysis/binding buffer with sodium hypochlorite, as toxic gas formation will occur. Wipe up all lysis buffer spills before applying bleach.

C. PCR setup and sample loading

1. Remove the optical plate containing prealiquoted master mix from the refrigerator and, using the trap map as a guide, pipette 10 µl of extracted specimen or control into each corresponding well.
2. Seal the plate using the optical seal and plate-sealing tool.
3. Clean the area and pipettes with 10% bleach followed by 70% ethanol.
4. Take the PCR plate to the amplification area.

D. PCR run

1. Place the 96-well plate in the sample block, and close the chamber door.
2. Select CMV PCR Program.
   a. The thermal cycling program should be set to have the following parameters.
      (1) 50°C for 2 min
      (2) 95°C for 10 min
      (3) 45 cycles of 95°C for 15 s, followed by 60°C for 60 s
   b. Data should be collected for the FAM channel (target amplification) and for the VIC channel (internal control amplification).

V. PROCEDURE *(continued)*

3. Following the run, remove the plate from the instrument, and clean the area with 10% bleach followed by 70% ethanol.

E. Data analysis

1. Click Analyze, and select each sample to view results. Any positive sample must have a valid amplification curve (showing exponential increase), along with a displayed Ct value less than 45, to be considered a positive amplification. Samples for which a Ct value is given but that do not have a clear exponential growth phase should be referred to the laboratory director for interpretation.
2. Results should be viewed for both detectors corresponding to CMV detection and the internal control detection.
3. For samples with valid amplification curves and for which a Ct value has been generated, the Ct value should be input into the standard curve equation to derive a quantitation value.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report result as one of the following.

1. CMV DNA, <400 copies/ml
2. CMV DNA, >100,000 copies/ml
3. CMV DNA, *x* copies/ml, where *x* is the actual quantity detected
4. Inhibitors present

B. Call results to physician of record or designee. Note on the worksheet the name of the person called, date, and time, and initial.

C. Document all testing in hard copy or computerized work card.

D. Include, "This test was developed and its performance characteristics determined by (laboratory name). It has not been cleared or approved by the Food and Drug Administration." As recommended by the CAP, add, "The FDA has determined that such clearance or approval is not necessary. This test is used for clinical purposes. It should not be regarded as investigation or for research. This laboratory is certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA-88) as qualified to perform high-complexity clinical laboratory testing" (3).

E. As applicable, include, "This test is performed pursuant to a license agreement with Roche Molecular Systems, Inc."

VII. INTERPRETATION

A. Results should not be reported if either positive control falls out of the specified range or if the negative control is positive.

B. If run controls are valid, report results as listed below.

1. If the internal positive control value for a specimen is valid (in range) and a Ct value has been generated for a sample, report the given quantity value for that specimen.
   - **a.** Ct values will be input into the standard curve equation.
   - **b.** For samples with quantities falling within the reportable range of the assay (400 to 100,000 copies/ml), the actual quantity value will be reported.
   - **c.** For samples that have quantities of <400 copies/ml, report as "<400 copies/ml."
   - **d.** For samples that have quantities of >100,000 copies/ml, report as ">100,000 copies/ml."

VII. INTERPRETATION *(continued)*

2. For each sample with an internal positive control Ct value that is within range but for which detection of the CMV analyte was listed as "Undet" in the instrument report (no Ct value generated), report as "<400 copies/ml."
3. For samples for which the CMV analyte was listed as "Undet" on the instrument report (no Ct value was generated) but for which the internal positive control amplification is *not* within the specified range, the following apply.
   a. Inhibitors may have been present, or improper assay setup may have occurred for that particular sample. The sample should be rerun.
   b. If, after the sample is rerun, the internal control still falls out of range, the report should indicate that PCR inhibition was observed and that an alternate specimen should be submitted if clinically warranted.

VIII. LIMITATIONS OF TESTING

A. A positive detection of the analyte may be the result of contamination rather than a true positive result. This protocol employs several steps to decrease the risk of contamination, including separate areas for assay setup, specimen preparation, and amplification and detection of amplified products; unidirectional work flow; the use of dUTP and UNG to decrease the risk of carryover contamination; good laboratory technique; and the use of a laminar-flow hood during specimen preparation to avoid contamination between specimens.

B. This test is not to be used for specimen types such as tissue or bronchial washes, for which the significance of a quantitative result has not been established.

C. The performance characteristics of this assay must be established by each laboratory.

D. Quantitated values of calibration materials obtained from external vendors should be verified.

E. The reportable range of the assay is limited to values between the highest and lowest calibrators. Additional calibrators with higher and/or lower quantities than those described can be used to extend the reportable range.

REFERENCES

1. **Boeckh, M., and G. Boivin.** 1998. Quantitation of cytomegalovirus: methodologic aspects and clinical applications. *Clin. Microbiol. Rev.* **11:**533–554.
2. **CLSI.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. CLSI, Wayne, PA.
3. **College of American Pathologists.** 2009. Laboratory Accreditation Program, Microbiology Checklist, rev. 06/15/2009. College of American Pathologists, Northfield, IL.
4. **Drew, W. L.** 2007. Laboratory diagnosis of cytomegalovirus infection and disease in immunocompromised patients. *Curr. Opin. Infect. Dis.* **4:**408–411.
5. **Gault, E., Y. Michel, A. Dehee, C. Belabani, J. Nicolas, and A. Garbarg-Chenon.** 2001. Quantification of human cytomegalovirus DNA by real-time PCR. *J. Clin. Microbiol.* **39:**772–775.
6. **Roberts, T. C., D. C. Brennan, R. S. Buller, M. Gaudreault-Keener, M. A. Schnitzler, K. E. Sternhell, K. A. Garlock, G. G. Singer, and G. A. Storch.** 1998. Quantitative polymerase chain reaction to predict occurrence of symptomatic cytomegalovirus infection and assess response to ganciclovir therapy in renal transplant recipients. *J. Infect. Dis.* **178:**626–635.
7. **Roche Applied Science.** July 2007. MagNA Pure LC Total Nucleic Acid Isolation Kit instruction manual. Roche Applied Science, Indianapolis, IN.

12.3.1 Introduction

Bacteria and fungi can be identified by using nucleic acid hybridization techniques. Nucleic acid probes can be used to confirm the identification of culture isolates that have been tested by presumptive identification methods. Alternatively, probes can be used as the primary method for identifying isolated organisms. Culture identification by probe hybridization is not dependent on the ability to detect minute quantities of nucleic acid, and thus sensitivity is not a limiting factor in this application of molecular technology. The advantage of probe-based identification is greatest for slow-growing organisms like the mycobacteria or for organisms for which convenient commercial identification systems are not available. Although the specificity of the available commercial probes is high and they facilitate rapid identification of a number of pathogens (Table 12.1–3), misidentifications do occur and serve to emphasize the need for caution in using any single characteristic in identification of a species. It should also be emphasized that at this point identification by probe hybridization is more expensive than conventional techniques for many organisms.

SUPPLEMENTAL READING

Cormican, M. G., and M. A. Pfaller. 2001. Molecular pathology of infectious diseases, p. 1241–1253. *In.* J. B. Henry (ed.), *Clinical Diagnosis and Management by Laboratory Methods,* 20th ed. W. B. Saunders Company, Philadelphia, PA.

Nolte, F. S., and A. M. Caliendo. 2003. Molecular detection and identification of microorganisms, p. 234–256. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology,* 8th ed. ASM Press, Washington, DC.

Tenover, F. C. 2007. Rapid detection and identification of bacterial pathogens using novel molecular technologies: infection control and beyond. *Clin. Infect. Dis.* **44:**418–423.

Woods, G. L. 2001. Molecular techniques in mycobacterial detection. *Arch. Pathol. Lab. Med.* **125:**122–126.

12.3.2 Identification of Bacteria and Fungi by Using Nucleic Acid Probes

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Bacteria and fungi can be identified by using nucleic acid probes. Nucleic acid probes can be used to confirm the identification of isolates that have been tested by presumptive identification methods. Alternatively, probes can be used as the primary method for identifying isolated organisms. The AccuProbe system (Gen-Probe, Inc., San Diego, CA) is currently the only DNA probe system available commercially. DNA probes are available for the organisms listed in Table 12.1–4. The sample preparation procedure for mycobacterial and fungal probes differs from that for bacterial probes and is described in procedure 12.3.3.

◩ **NOTE:** Sample preparation methods are identical for mycobacteria and fungi.

The AccuProbe system is based on nucleic hybridization of a DNA probe (oligomer) that is complementary to the rRNA of the target organism. The probes are labeled with an acridinium ester. The organism is treated to release the rRNA and then incubated with the labeled single-stranded DNA probe. If the rRNA and DNA probes are complementary, a stable DNA-RNA hybrid forms. A hydrolyzing reagent is added to selectively remove unbound DNA probe. The chemiluminescence of the DNA-RNA hybrids is quantitated in relative light units (RLU) with a luminometer.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

Test growth from appropriate solid media with morphology suggestive of the organism in question. Samples may be tested as soon as growth is visible, but they should be less than 48 h old. If a single colony is to be tested, it should be at least 1 mm in diameter. Alternatively, several (three or four) smaller colonies can be tested. Do not use confluent growth.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

1. Identification reagent kit (provided)
   - **a.** Reagent 1 (lysis reagent)
   - **b.** Reagent 2 (hybridization buffer)
   - **c.** Reagent 3 (selection reagent)
2. Detection reagent kit (provided)
   - **a.** Detection reagent 1
   - **b.** Detection reagent 2
3. Organism-specific probe kit (provided)

B. Materials required but not provided

1. 1-µl plastic sterile inoculating loops, applicator sticks
2. Reference strains for controls
3. Incubator or water bath (35 to 37°C; for gram-positive bacteria)
4. Water bath or heating block (60°C)
5. Micropipettes (50 and 300 µl)
6. Repipettors (50 and 300 µl)
7. Vortex mixer
8. Leader 1 luminometer or AccuLDR luminometer (Gen-Probe)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that all reagents and materials meet expiration date and QC parameters per CLSI document M22-A3 (1).

B. Include in each run appropriate reference strains representing a positive and negative control for each organism being tested.

C. Negative and positive control strains should yield the following values.

	AccuLDR (RLU)	Leader (RLU)
Negative control	<600	<20,000
Positive control	>1,500	>50,000

V. PROCEDURE

Observe standard precautions.

A. Equipment preparation

1. Adjust the incubator or water bath to 35 to 37°C if testing gram-positive bacteria.
2. Adjust the water bath or heating block to 60 ± 1°C.
3. Prepare the luminometer for operation. Make sure there are sufficient volumes of detection reagents 1 and 2.

B. Sample preparation

1. Label the probe reagent tubes. Remove and retain the caps.
2. Pipette 50 µl of reagent 1 (lysis reagent) into each probe reagent tube.
3. Transfer one or several colonies from solid medium into probe reagent tubes. Avoid taking any of the solid medium with the cells. Twirl the loop or stick in reagent 1 to remove the cells, and mix thoroughly.
4. For gram-positive organisms, recap the probe reagent tubes and incubate them at 35 to 37°C for 5 min. Remove the tubes from the water or dry bath, and then remove and retain the caps.

C. Hybridization

1. Pipette 50 µl of reagent 2 (hybridization buffer) into each probe reagent tube.
2. Recap the tubes, and mix the contents by shaking or vortexing.
3. Incubate for 15 min at 60°C in a water bath or heating block.

D. Selection

1. Remove the probe reagent tubes from the water or dry bath. Remove and retain the caps. Pipette 300 µl of reagent 3 (selection reagent) into each tube. Recap the tubes, and vortex them to mix the contents completely.
2. Incubate the probe reagent tubes for 5 min at 60°C.
3. Remove the tubes from the water or dry bath, and leave them at room temperature for at least 5 min. Remove and discard the caps. Read results in the luminometer within 30 min (within 1 h for *Streptococcus pneumoniae*) after removing the tubes from the water bath or heating block.

E. Detection

1. Select the appropriate protocol from the menu of the luminometer software.
2. Using a damp tissue or paper towel, wipe each tube to ensure that no residue is present on the outside of the tube, and insert the tube into the luminometer according to the instrument instructions.
3. When the analysis is complete, remove the tube from the luminometer. The sample cannot be read again.

F. Procedure notes

1. A precipitate may form in the hybridization buffer. Warming and mixing the solution at 35 to 60°C will dissolve the precipitate.
2. The hybridization and selection reactions are temperature dependent. Therefore, it is imperative that the incubator, water bath, or heating block be

V. PROCEDURE *(continued)*

maintained within the specified temperature. The entire liquid reaction volume in the probe reagent tube must be exposed to the required temperature.

3. With gram-positive bacteria, the hybridization reaction should be started within 30 min of adding cells and reagent 1 to the probe reagent tubes.
4. The hybridization and selection reactions are time dependent. Hybridize for at least 20 min. Incubate the probe reaction tubes during the selection step for at least 5 min but no more than 6 min.
5. It is critical to have a homogenous mixture during the selection (vortex) step, specifically after the addition of reagent 3.
6. Troubleshooting
   a. Elevated negative control values of more than 20,000 RLU in the Leader 1 luminometer or more than 600 photometric light units (PLU) in the AccuLDR luminometer (formerly PAL) can occur. If they do, streak a portion of the growth onto the appropriate agar medium and incubate it to check for multiple colony types.
   b. Low positive control values (less than 50,000 RLU in the Leader 1 or less than 1,500 PLU in the AccuLDR) can be caused by insufficient cell numbers, by testing mixed or aged cultures, or, for gram-positive organisms, by leaving cells exposed to the lysis reagent for more than 30 min before hybridization reagents are added.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report results as genus and species as indicated.

B. Document all testing in hard copy or computerized work card.

VII. INTERPRETATION

A. The results of the AccuProbe culture identification test are based on the following cutoff values. Samples producing signals higher than or equal to the cutoff values are considered positive. Samples with signals lower than these values are considered negative. Results from samples in the indeterminate range must be retested.

	AccuLDR (RLU)	Leader (RLU)
Cutoff value	1,500	50,000
Repeat range	1,200–1,499	40,000–49,000

B. Performance characteristics

1. Sensitivity, 100%
2. Specificity, 100%
3. Percent agreement with reference identification, 100%

REFERENCE

1. **CLSI.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. CLSI, Wayne, PA.

SUPPLEMENTAL READING

Daily, J. A., N. L. Clifton, K. C. Seskin, and W. M. Gooch III. 1991. Use of rapid, nonradioactive DNA probes in culture confirmation tests to detect *Streptococcus agalactiae, Haemophilus influenzae,* and *Enterococcus* spp. from pediatric patients with significant infections. *J. Clin. Microbiol.* **29:**80–82.

Denys, G. A., and R. B. Carey. 1992. Identification of *Streptococcus pneumoniae* with a DNA probe. *J. Clin. Microbiol.* **30:**2725–2727.

Gen-Probe, Inc. 1995. AccuProbe culture identification test, package insert. Gen-Probe, Inc., San Diego, CA.

Kohne, D. E. 1990. The use of DNA probes to detect and identify microorganisms, p. 11–35. *In* B. Kleger et al. (ed.), *Rapid Methods in Clinical Microbiology*. Plenum Press, New York, NY.

Lewis, J. S., D. Krnaig-Brown, and D. A. Trainor. 1990. DNA probe confirmatory test for *Neisseria gonorrhoeae. J. Clin. Microbiol.* **28:**2349–2350.

Stockman, K., K. A. Clark, J. M. Hunt, and G. D. Roberts. 1993. Evaluation of commercially available acridinium ester-labeled chemiluminescent DNA probes for culture identification of *Blastomyces dermatitidis, Coccidioides immitis, Cryptococcus neoformans,* and *Histoplasma capsulatum. J. Clin. Microbiol.* **31:**845–850.

12.3.3 DNA Probes for the Identification of Mycobacteria

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

A commercially manufactured system, the AccuProbe culture confirmation test, is available for identification of isolates of several species of mycobacteria. The system is based on the use of DNA probes that are complementary to species-specific rRNA. The mycobacterial cells are lysed by sonication, heat killed, and exposed to DNA that has been labeled with a chemiluminescent tag. The labeled DNA probe combines with the organism's rRNA to form a stable DNA-RNA hybrid. A selection reagent "kills" the signal on all unbound DNA. Chemiluminescence produced by DNA-RNA hybrids is measured in a luminometer.

Probes for the identification of *Mycobacterium tuberculosis* complex, *Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium gordonae, M. avium-M. intracellulare* complex, and *Mycobacterium kansasii* are available. Nucleic acid probes provide the most rapid identification of mycobacteria of any identification systems.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

It is imperative that these cultures be handled in a biological safety cabinet.

Any actively growing culture less than 1 month old recovered on any solid or broth medium such as Middlebrook 7H9, 7H10, 7H11, or 7H11 selective or Lowenstein-Jensen medium can be used.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents supplied by manufacturer (Table 12.3.3–1)

◩ Indicate the expiration date on the label and in the work record or on the manufacturer's label.

B. Supplies (required but not supplied)

1. Micropipettes (100 µl)
2. Micropipettor (300 µl)
3. Repipettor (300 µl)
4. Repipettor syringes
5. Plastic sterile inoculating loops (1 µl)

C. Equipment (required but not supplied)

1. Luminometer (Leader 1, Leader 250, or PAL)
2. Vortex mixer
3. Water or dry bath, 90°C (±5°C)
4. Water or dry bath, 60°C (±1°C)
5. Water bath sonicator

Table 12.3.3–1 Reagents for AccuProbe culture confirmation

Reagent[a]	Description	Storage temp (°C)
Lysing tubes	Lyophilized glass beads and buffer	23–25
Probe reagent	Tubed lyophilized species-specific DNA (in foil pouches)[b]	4
Reagent 1	Specimen diluent	23–25
Reagent 2	Probe diluent	23–25
Reagent 3	Selection reagent	23–25
Detection I	H_2O_2 in nitric acid solution containing 1 N NaOH	23–25
Detection II	H_2O_2 in nitric acid solution containing 1 N NaOH	23–25
Ultrasonic enhancer	Detergent-like solution to increase sonication power	23–25

[a] All reagents are stable for approximately 1 year from date of manufacture.

[b] After pouch is opened, stable for 2 months.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that all reagents and materials meet expiration date and QC parameters per CLSI document M22-A3 (1).

B. Frequency and tolerance of controls

1. Run a positive and negative control with each batch of organisms tested.

2. If controls are not within limits, check age of culture (should be less than 1 month old), level of water in sonicator, quality of sonicator (by observing waving water patterns while sonicator is on; water should "dance"), and temperature of hybridization; if necessary, run a tritium standard.

C. Control organisms (Table 12.3.3–2)

V. PROCEDURE

It is imperative that these cultures be handled in a biological safety cabinet.

A. Degas the water for 15 min. Turn on 95°C water bath (or heat block) and 60°C water bath (or heat block).

B. Add 100 µl of reagent 1 (specimen diluent) and 100 µl of reagent 2 (probe diluent) (Table 12.3.3–1) to each lysing tube.

C. Working in a biosafety cabinet, transfer a loopful (1 µl) of test organism into the lysing tube. Twirl the loop against the side of the tube to remove the entire inoculum.

Table 12.3.3–2 Control organisms for AccuProbe mycobacterial culture confirmation tests

Probe to be tested	Control	
	Positive	Negative
M. avium	*M. avium* ATCC 25291	*M. intracellulare* ATCC 13950
M. intracellulare	*M. intracellulare* ATCC 13950	*M. avium* ATCC 25291
M. tuberculosis complex	*M. tuberculosis* ATCC 25177	*M. avium* ATCC 25291
M. gordonae	*M. gordonae* ATCC 14470	*M. scrofulaceum* ATCC 19073
M. avium complex	*M. intracellulare* ATCC 13950	*M. gordonae* ATCC 14470
M. kansasii	*M. kansasii* ATCC 12478	*M. tuberculosis* ATCC 25177

V. PROCEDURE *(continued)*

D. Place the lysing tubes in the sonicator. Ensure that the water level is high enough to cover the contents of the tube. Do not allow the tubes to touch the sides of the sonicator. Sonicate at room temperature for 15 min.

E. Place the lysing tubes in the 95°C water bath (or heat block) for 15 min.

F. Allow the tubes to cool at room temperature for 5 min.

G. Pipette 100 µl of the killed lysate into the probe reaction tube.

H. Incubate the tubes for 15 min at 60°C in the water bath (or heat block).

I. Pipette 300 µl of reagent 3 (selection reagent) (Table 12.3.3–1) into each tube. Recap and vortex the tubes, and immediately place them back into the 60°C water bath (or heat block). Incubate for 5 min for *M. avium* complex and *M. gordonae,* 8 min for *M. kansasii,* and 10 min for *M. tuberculosis* complex.

J. Prepare the luminometer for operation by completing a wash cycle. Wipe each tube with a damp tissue before inserting it into the luminometer. Read each tube and controls. The luminometer records relative light units (RLU).

K. Procedure notes

1. It is important to maintain the 60°C (±1°C) dry bath, because annealing of the DNA-RNA hybrid is temperature sensitive.

2. The water level of the sonicator should be high enough to cover the hybridization buffer, beads, and organisms.

3. Other sources of error include failure to vortex after addition of the organism-buffer mixture to the probe tube and after addition of reagent 3, which inactivates the acridinium ester not bound to rRNA.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Report results as genus and species as indicated.

B. Document all testing in hard copy or computerized work card.

VII. INTERPRETATION

A. A positive result is >30,000 RLU.

B. Signals below 30,000 RLU are considered negative.

C. Repeat any test with a result between 20,000 and 29,999 RLU.

VIII. LIMITATIONS OF THE PROCEDURE

A. This method cannot be used directly on fresh clinical specimens. It is for culture identification only.

B. A small number of biochemically determined *M. avium* complex isolates will not produce a positive result with the *M. avium* complex probe. The taxonomic status of these strains is currently uncertain.

C. The *M. tuberculosis* complex culture confirmation test does not differentiate among *M. tuberculosis, Mycobacterium bovis, M. bovis* BCG, *Mycobacterium africanum,* and *Mycobacterium microti.* Additional tests such as niacin and nitrate reductase are required to differentiate *M. tuberculosis* from other members of the group.

D. The *M. avium* complex culture confirmation test does not differentiate between *M. avium* and *M. intracellulare.*

E. The sample preparation for the identification of fungal pathogens *Histoplasma capsulatum, Blastomyces dermatitidis,* and *Coccidioides immitis* is identical to that of the mycobacteria. These organisms may be identified using the appropriate Gen-Probe kits and reagents.

REFERENCE

1. **CLSI.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. CLSI, Wayne, PA.

SUPPLEMENTAL READING

Badak, F. Z., S. Goksel, and R. Sertoz. 1999. Use of nucleic acid probes for identification of *Mycobacterium tuberculosis* directly from MB/BacT bottles. *J. Clin. Microbiol.* **37:**1602–1605.

Devallois, A., K. S. Goh, and N. Rastogi. 1997. Rapid identification of mycobacteria to species level by PCR-restriction fragment length polymorphism analysis of the hsp65 gene and proposition of an algorithm to differentiate 34 mycobacterial species. *J. Clin. Microbiol.* **35:**2969–2973.

Ellner, P. D., T. E. Kiehn, R. Cammarata, and M. Hosmer. 1988. Rapid detection and identification of pathogenic mycobacteria by combining radiometric and nucleic acid probe methods. *J. Clin. Microbiol.* **26:**1349–1352.

Evans, K. D., A. S. Nakasone, P. A. Sutherland, L. M. dela Maza, and E. M. Peterson. 1992. Identification of *Mycobacterium tuberculosis* and *Mycobacterium avium-M. intracellulare* directly from primary BACTEC cultures by using acridinium-ester-labeled DNA probes. *J. Clin. Microbiol.* **30:**2427–2431.

Gen-Probe, Inc. 1995. AccuProbe culture confirmation test for mycobacteria, package insert. Gen-Probe, Inc., San Diego, CA.

Goto, M., S. Oka, K. Okuzumi, S. Kimur, and K. Shimada. 1991. Evaluation of acridinium ester-labeled DNA probes for identification of *Mycobacterium tuberculosis* and *Mycobacterium avium-Mycobacterium intracellulare* complex in culture. *J. Clin. Microbiol.* **29:**2473–2476.

Musial, C. E., L. S. Tice, L. Stockman, and G. P. Roberts. 1988. Identification of mycobacteria from culture by using the Gen-Probe rapid diagnostic system for *Mycobacterium avium* complex and *Mycobacterium tuberculosis* complex. *J. Clin. Microbiol.* **26:**2120–2123.

Richter, E., S. Niemann, S. Rüsch-Gerdes, and S. Hoffner. 1999. Identification of *Mycobacterium kansasii* by using a DNA probe (AccuProbe) and molecular techniques. *J. Clin. Microbiol.* **37:**964–970.

12.3.4 Identification of Bacteria and Yeasts by Using AdvanDx Peptide Nucleic Acid Fluorescence In Situ Hybridization Kits

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The AdvanDx PNA FISH assays use a hybridization technique employing peptide nucleic acid probes coupled with fluorescence in situ hybridization (PNA FISH). PNA probes are charge neutral, which provides for better binding to either target DNA or RNA. The fluorochrome-labeled PNA probes hybridize to unique rRNA inside of the organisms, allowing the identification of pathogens directly in smears made from positive blood cultures. The probes are added to the smears and hybridized at 55°C for 90 min. The hybridization is followed by a wash at 55°C for 30 min with a stringent wash solution. Finally, the smears are mounted with mounting medium and examined by fluorescence microscopy. Microorganisms are identified as bright fluorescent cells in multiple fields of view.

Blood cultures in a clinical laboratory that are flagged as positive are typically Gram stained to provide quick information on the possible organism. Depending on the Gram stain reaction and morphology, specimens are stained with a specific PNA FISH probe kit. Identification can occur within approximately 2.5 h of the blood culture becoming positive, thus giving a definitive identification to the doctor, enabling faster and more accurate patient management with antimicrobial agents.

There are several configurations of PNA FISH reagents currently FDA approved, such as *Candida albicans/Candida glabrata*, *Staphylococcus aureus* single probe, *S. aureus*/coagulase-negative staphylococci (CoNS), *Enterococcus* sp./*Enterococcus faecalis*, and Yeast Traffic Light. Yeast Traffic Light is a multicolor PNA FISH assay that can detect *C. albicans* and/or *Candida parapsilosis*, *Candida tropicalis*, and *C. glabrata* and/or *Candida krusei*. The assay does not differentiate between *C. glabrata* and *C. krusei*, and it does not differentiate between *C. albicans* and *C. parapsilosis*.

All kits available at the time of this writing are presented in Table 12.1–3. Others are under development. The procedure below describes the *S. aureus*/CoNS PNA FISH test, which identifies both *S. aureus* and CoNS. The procedures for testing with all kits are similar. For the most up-to-date information, the reader is advised to consult the manufacturer and to obtain a package insert.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Collect blood cultures according to institutional and manufacturer requirements.

B. PNA FISH is approved for testing using bottles from three blood culture systems.
1. bioMérieux BacT/ALERT, including the FAN bottles
2. Becton Dickinson BACTEC, including resin bottles
3. Trek ESP

C. Positive blood cultures may be tested as soon as growth is detected by continuously monitoring blood culture systems. The smears should be Gram stained initially to prompt selection of the appropriate PNA FISH kit.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Materials provided

PNA FISH kit components
1. Fixation solution—3 ml of phosphate-buffered saline with detergent
2. *S. aureus*/CoNS PNA FISH—1.5-ml PNA probes in hybridization solution; contains 30% formaldehyde
3. 60× wash solution—50 ml of Tris-buffered saline with detergent

III. MATERIALS *(continued)*

4. Mounting medium—3 ml of photobleaching inhibitor in glycerol

B. Materials required and available from AdvanDx

1. One-well microscope slides (catalog no. AC001)
2. Coverslips (catalog no. AC002)
3. Slide warmer (PNA FISH workstation) (55 ± 1°C)
4. Water bath (55 ± 1°C)
5. Dual band filter—fluorescein isothiocyanate/Texas red (catalog no. AC0003)

C. Materials required but not provided

1. Ethanol, 80 or 96%
2. Water, deionized or distilled
3. Staining dish with cover and slide holder
4. Fluorescence microscope equipped with a 60× or 100× oil objectives
5. Measuring cylinder
6. Sterile airway needle/subculture units
7. ATCC QC strains or control slides
8. Immersion oil; must comply with the microscope objective and be nonfluorescent

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Positive and negative controls should be tested with each batch of slides that are stained.

1. Prepare smears from positive and negative control organisms as described below. Stock strains of *S. aureus* and *Staphylococcus epidermidis* should be used for the positive control. For the negative control, use a stock strain of either *Streptococcus* or *Enterococcus.*
   - **a.** Use standard aerobic blood culture bottles and label them as positive and negative controls for PNA FISH as well as the respective organism name.
   - **b.** Resuspend a colony of the control organism strain in approximately 1 ml of sterile saline.
   - **c.** Inoculate the 1 ml of resuspended positive control organism, *S. aureus,* into a positive control bottle.
   - **d.** Inoculate the 1 ml of resuspended *S. epidermidis* into a separate positive control bottle.
   - **e.** Inoculate the 1 ml of resuspended *Enterococcus* sp. or *Streptococcus* sp. into a negative control bottle.
   - **f.** Place the bottles on the laboratory blood culture system and incubate until the bottles signal positive, as indicated by the system.
   - **g.** Prepare control smear as indicated in the procedure for patient samples. Slides prepared in this way can be prepared in bulk and used as control slides.
   - **h.** If PNA FISH assays with more than one organism being detected are being used, the separate positive control organisms, if prepared in-house, can be added to a single slide or to individual slides. ATCC isolates can be used for QC.
2. Alternatively, control slides can be purchased from AdvanDx.

B. The positive control smears should contain bright fluorescent organisms in multiple fields morphologically similar to the Gram stain of the original positive blood culture bottle.

C. The negative control slides should not contain fluorescent organisms.

V. PROCEDURE

Observe standard precautions.

A. Prior to testing

1. Bring kit components to room temperature prior to use. All steps, unless otherwise specified, are performed at room temperature.
2. Adjust the temperature of the water bath and incubator to 55 ± 1°C.
3. Make sure that the fluorescence microscope is turned on at least 15 min prior to use.

V. PROCEDURE *(continued)*

4. Prepare working-strength wash solution by adding 1 part (4 ml) 60× wash solution concentrate to 59 parts (236 ml) fresh deionized or distilled water. Prepare fresh wash solution as required for each run. Do not reuse the wash solution. Store remaining concentrate at 2 to 8°C.
5. Pour the prepared working-strength wash solution into a covered staining dish and start preheating in the 55 ± 1°C water bath.

B. Sample preparation

1. Preparation of smears
   - **a.** Using AdvanDx microscope slides, label slides for control and patient samples.
   - **b.** Place 1 drop of fixation solution in the well on the microscope slide.
   - **c.** Using a ventilation needle, transfer a small drop of positive blood culture directly from the positive blood culture bottle into the fixation solution.
   - **d.** Mix gently and thoroughly using a sterile tip such as an inoculation loop or wooden stick. Make sure to cover the whole circle on the slide.
2. Fixation
   - **a.** Heat slide for 20 min at 55 to 80°C (heat fixation). Slides can be air dried and fixed by methanol fixation or flame fixation. Most laboratories have removed burners so a heating block or methanol fixation can be used.
   - **b.** Immerse the slide in 80 or 96% ethanol for 5 to 10 min. The slide should be air dried for approximately 10 min or until the slide is dry.

C. Hybridization

1. Add 1 drop of *S. aureus*/CoNS PNA probe to the well on the microscope slide with the smear. Include control slides for each staining batch. Do not allow dropper bottle to touch the smear to prevent cross-contamination between specimens.
2. Add coverslip to the slide. Avoid air bubbles during this step.
3. Incubate for 90 ± 5 min at 55 ± 1°C.
4. Place probes back in refrigerator before proceeding.

D. Stringent wash

1. Immerse slide in preheated wash solution at 55°C; in doing so, carefully remove the coverslip. The coverslip should be made to slide off by gently agitating the slide while it is submersed in the wash solution. At times the coverslip must be pushed off with forceps.
2. Incubate for 30 ± 5 min at 55 ± 1°C.
3. Allow the slide to air dry.

E. Mounting

1. After the smear is dry, add 1 drop of mounting medium to the smear.
2. Add coverslip to the slide. Avoid air bubbles during this step.

F. Reading slides

The slide should be examined within 2 h after staining is completed.

1. The slide should not be exposed to direct sunlight or another light source. This may lead to a quenching of the fluorescence and a possible false-negative test result.
2. Examine slides with a fluorescence microscope using the dual filter at a magnification of either ×60 or ×100.
3. On a slide positive for *S. aureus*, organisms will appear as bright green fluorescent cocci in clusters in multiple fields of view.
4. On a slide positive for CoNS, organisms will appear as bright red fluorescent cocci in clusters in multiple fields of view.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. Positive results should be reported as follows.
- **1.** *S. aureus* identification by PNA FISH
- **2.** CoNS identification by PNA FISH
- **3.** The report can include the sensitivity and specificity data for PNA FISH derived from the package inserts: sensitivity, 99%; specificity, 99%.

B. Negative results should be reported as follows.
- **1.** Negative for *S. aureus* and CoNS by PNA FISH. Culture identification to follow.
- **2.** The report can include the sensitivity and specificity data for PNA FISH derived from the package inserts: sensitivity, 99%; specificity, 99%.

C. Each laboratory must determine whether to report the Gram stain result (gram-positive cocci suggesting staphylococci) prior to the PNA FISH result or to hold reporting until the PNA FISH is completed. This may depend on how the PNA FISH is run: batch testing or as each blood culture signals positive.

D. Laboratories should also determine whether to call the PNA FISH results or advise providers when the Gram stain is called that the PNA FISH results will be available later in the day.

E. Additional reporting comments may be added for *S. aureus* positivity, to include information on the rate of methicillin resistance for *S. aureus* at the reporting institution.

VII. INTERPRETATION

A. Prior to reporting patient results, examine control slides for proper staining and results.
- **1.** The positive control smears should contain bright fluorescent organisms in multiple fields morphologically similar to the Gram stain of the original positive blood culture bottle.
  - **a.** *S. aureus* organisms are identified as multiple bright green fluorescent clusters of cocci in multiple fields.
  - **b.** CoNS are identified as multiple bright red fluorescent clusters of cocci in multiple fields.
- **2.** The negative control slides should not contain fluorescent organisms. The smear should appear generally reddish, with no fluorescence detected.
- **3.** If the PNA FISH result is negative for a specimen that had rare or few organisms on the Gram stain, report the PNA FISH result as "Unable to confirm identification of organism by PNA FISH."
- **4.** Mixed slide—the presence of fluorescent and nonfluorescent morphologically consistent microorganisms is indicative of a mixed culture.

B. Patient slides should be read and interpreted as described for control slides.

C. The positive blood culture will need to be subcultured to solid medium to obtain an isolate for susceptibility testing and to determine if mixed cultures are present.

VIII. LIMITATIONS OF THE PROCEDURE

A. False-positive results (green fluorescence) with *Staphylococcus schleiferi* may occur with the *S. aureus* PNA FISH probe due to a single base mismatch.

B. The following rarely occurring *Staphylococcus* species are negative (no fluorescence) by *S. aureus*/CoNS PNA FISH: *S. caseolyticus, S. felis,* and *S. simulans.*

C. Results from the PNA FISH kits should be interpreted in conjunction with other laboratory and clinical data available to the clinician.

VIII. LIMITATIONS OF THE PROCEDURE *(continued)*

D. It is important to use the dual band filter suggested in the package insert. Failure to do so may result in false-positive controls and/or samples.

E. The sensitivity of PNA FISH is related to the level of rRNA present in the cells. Slides from positive blood cultures should be prepared within a reasonable period after signaling on the blood culture instrument. No time frame is given in the manufacturer's package insert, so the time frame should be established by each laboratory. Initial data generated in my laboratory suggest that 24 h may be applicable (unpublished data).

F. The procedure has not been validated for specimens other than blood cultures.

G. Note that each individual package insert lists limitations for each PNA FISH assay. Please refer to each package insert for detailed information.

SUPPLEMENTAL READING

Alexander, B. D., E. Dodds-Ashley, L. B. Reller, and S. D. Reed. 2006. Cost savings with implementation of PNA FISH testing for identification of Candida albicans in blood cultures. *Diagn. Microbiol. Infect. Dis.* **54:**277–282.

Forrest, G. N., S. Mehta, E. Weekes, D. P. Lincalis, J. K. Johnson, and R. A. Venezia. 2006. Impact of rapid in situ hybridization testing on coagulase-negative staphylococci positive blood cultures. *J. Antimicrob. Chemother.* **58:**143–158.

Oliveira, K., S. M. Brecher, A. Durbin, D. S. Shapiro, D. R. Schwartz, P. C. De Girolami, J. Dakos, G. W. Procop, D. Wilson, C. S. Hanna, G. Haase, H. Peltroche-Llacsahuanga, K. C. Chapin, M. C. Musgnug, M. H. Levi, C. Shoemaker, and H. Stender. 2003. Direct identification of *Staphylococcus aureus* from positive blood culture bottles. *J. Clin. Microbiol.* **41:**889–891.

Oliveira, K., G. W. Procop, D. Wilson, J. Coull, and H. Stender. 2002. Rapid identification of *Staphylococcus aureus* directly from blood cultures by fluorescence in situ hybridization with peptide nucleic acid probes. *J. Clin. Microbiol.* **40:**247–251.

Rigby, S., G. W. Procop, G. Haase, D. Wilson, G. Hall, C. Kurtzman, K. Oliveira, S. Von Oy, J. J. Hyldig-Nielsen, J. Coull, and H. Stender. 2002. Fluorescence in situ hybridization with peptide nucleic acid probes for rapid identification of *Candida albicans* directly from blood culture bottles. *J. Clin. Microbiol.* **40:**2182–2186.

Sogarrd, M., D. S. Hansen, M. J. Fiandaca, H. Stender, and H. C. Schonheyder. 2007. Peptide nucleic acid fluorescence in situ hybridization for rapid detection of *Klebsiella pneumoniae* from positive blood cultures. *J. Med. Microbiol.* **56:**914–917.

Wilson, D. A., M. J. Joyce, L. S. Hall, L. B. Reller, G. D. Roberts, G. S. Hall, B. D. Alexander, and G. W. Procop. 2005. Multicenter evaluation of a *Candida albicans* peptide nucleic acid in situ hybridization probe for characterization of yeast isolates from blood cultures. *J. Clin. Microbiol.* **43:**2909–2921.

12.4.1 Introduction

The ability to identify specific strains within a species of pathogen is an important aid in the rational development of effective measures to prevent and control nosocomial infections. The efforts of both microbiologists and hospital epidemiologists are facilitated greatly by the availability of the newer molecular epidemiologic typing techniques. The variety of molecular epidemiologic tools available at present is considerable, and based on current experience the methods that appear to be the most practical and useful for both large- and small-scale epidemiologic studies are the DNA-based methods such as pulsed-field gel electrophoresis (Table 12.1–5). Although these methods clearly have limitations, they generally are a significant improvement over the more conventional typing methods, many of which are too cumbersome, insensitive, and time-consuming to be of practical value for epidemiologic evaluations. It is important to understand that no single technique is universally applicable and that the choice for a particular application is related to the species studied, the scope of the question posed, and the convenience of the technique. The techniques of molecular epidemiology are useful in answering real clinical and infection control questions and are not limited to research uses. Examples include distinguishing between relapse and reinfection in an individual patient and in tracking the spread of an individual strain of a bacterium or fungus within the hospital environment (1–3).

REFERENCES

1. **Li, W., D. Raoult, and P. E. Fournier.** 2009. Bacterial strain typing in the genomic era. *FEMS Microbiol. Rev.* **33:**892–916.
2. **Olive, D. M., and P. Bean.** 1999. Principles and applications of methods for DNA-based typing of microbial organisms. *J. Clin. Microbiol.* **37:**1661–1669.
3. **Pfaller, M. A.** 1999. Molecular epidemiology in the care of patients. *Arch. Pathol. Lab. Med.* **123:**1007–1010.
4. **Soll, D. R., C. Pujol, and S. R. Lockhart.** 2007. Laboratory procedures for the epidemiological analysis of microorganisms, p. 129–151. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.

12.4.2 Plasmid Fingerprinting of Gram-Negative Organisms

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Plasmids are extrachromosomal circles of DNA that are present in many but not all species of bacteria. Many organisms, particularly those that are resistant to multiple antimicrobial agents, may harbor two or more plasmids. The presence of similarly sized plasmids in a series of isolates collected as part of an epidemiological investigation can be used in conjunction with other typing schemes to indicate the presence of an epidemic strain in a hospital or community. However, two or more isolates of the same species that are devoid of plasmid DNA cannot be assumed to be the same strain. Isolates of the same species each showing a single plasmid of similar molecular size should be investigated further by cleaving the plasmid DNA with restriction endonucleases to confirm the similarity of the plasmids.

Plasmid fingerprinting techniques focus on the recovery of the supercoiled, covalently closed, circular form of the molecule. Lysis of the cell wall with detergent at high pH allows denaturation of chromosomal DNA without irreparable harm to plasmid DNA. Precipitation of plasmid DNA with high concentrations of NaCl and isopropanol results in preparations relatively free of chromosomal DNA. Although plasmids normally have a molecular size ranging from 1 to 100 kb, some *Pseudomonas* plasmids may be as large as 400 kb. It is important to run molecular size standards consisting of supercoiled plasmids and not restriction endonuclease fragments for accurate determinations of plasmid size.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Most enteric gram-negative bacilli and *Pseudomonas* species grown on a nonselective agar medium for 18 to 24 h will yield plasmid DNA with this procedure.

III. MATERIALS

Include QC information on reagent container and in QC records.

◩ **NOTE:** Autoclave all centrifuge tubes and pipette tips before use to destroy any nucleases that may be present.

- **A.** Sterile microcentrifuge tubes (1.5 ml) with caps
- **B.** Pipettor, variable volume (0 to 20 μl)
- **C.** Pipettor, variable volume (20 to 200 μl)
- **D.** Sterile pipette tips
- **E.** Disposable 5-ml pipettes
- **F.** Microcentrifuge (maximum speed, 12,000 × *g*)
- **G.** Wet ice in bucket
- **H.** Vacuum line with trap
- **I.** TE buffer (50:10), pH 8.0 (50 mM Tris, 10 mM EDTA)
- **J.** Lysis buffer, pH 12.4 (stable for 1 month): Trizma base-Na_2 EDTA-sodium dodecyl sulfate (SDS)-NaOH
- **K.** 2 M Tris, pH 7.0 (stable for 6 months)
- **L.** 5 M NaCl (indefinite stability)
- **M.** 100% Ethanol
- **N.** Stop mix (running dye) (stable for 1 year): bromophenol blue-SDS-Ficoll type 400

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that all materials and reagents meet expiration date and QC parameters per CLSI document M22-A3 (1).

B. A standard strain containing well-characterized plasmids should be lysed in parallel with each plasmid screen to ensure the quality of the lysis buffer. *Escherichia coli* V517, which contains eight plasmids, is a good QC strain and also serves as an excellent source of molecular size standards (it is available from the ATCC [ATCC 37514]). Supercoiled standards also can be purchased from several commercial sources.

V. PROCEDURE

Observe standard precautions.

A. Pipette 40 μl of TE buffer (50:10) into a 1.5-ml microcentrifuge tube.

B. Suspend one-fourth loopful (0.01-ml inoculating loop) of bacteria taken from an 18- to 24-h growth on a nonselective agar plate in the buffer.

C. Add 0.6 ml of lysis buffer, and mix by inverting the tube 20 times. Do not vortex.

D. Incubate at 37°C in a water bath for 20 min.

E. Add 30 μl of 2 M Tris (pH 7.0), and invert the tube 10 times to mix.

F. Add 160 μl of 5 M NaCl, and invert the tube 10 times.

G. Place tubes on wet ice for at least 20 min. Incubation may continue for up to 1 h.

H. Centrifuge tubes at 12,000 × *g* in a microcentrifuge for 5 min. Pour supernatant into a fresh (sterile) 1.5-ml centrifuge tube.

I. Add 0.55 ml of cold absolute isopropanol (store at −20°C), and mix by inverting the tube.

J. Freeze at −20°C for at least 20 min. Tubes can be left in the freezer overnight.

K. Remove tubes from freezer, thaw at room temperature, and centrifuge at 12,000 × *g* for 3 min. Discard supernatant, and cautiously remove remaining alcohol with a Pasteur pipette connected to a vacuum line. Do *not* remove pellet at bottom of tube. Place inverted tubes on a paper towel for 30 min. Make sure that all alcohol is removed.

L. Suspend DNA in 30 μl of TE buffer (10:1). Add 6 μl of stop mix. Allow at least 1 h for DNA to suspend.

M. Electrophorese 15-μl samples through a 0.7% agarose gel at 200 V/h.

N. Stain gel in ethidium bromide (0.2 μg/ml of water).

◩ **NOTE:** Wear gloves when handling ethidium bromide.

O. Photograph gel with orange filter and mid-range UV transilluminator.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

The manner in which the results of plasmid fingerprinting investigations are reported depends on three factors: why the tests were ordered, who ordered them, and the expertise and comfort level of the microbiologist performing the test. Physicians and many infection control practitioners who request such tests will need to be given both the results and an interpretation of the results. The following guidelines are suggested.

A. Provide a duplicate picture of the gel for the record. If you are not comfortable releasing a copy of the gel, you probably are not comfortable with the results, and the procedure should be deemed inconclusive. Some isolates of *Pseudomonas* spp. are difficult to lyse, and a report of "inconclusive" is better than a guess.

VI. REPORTING RESULTS *(continued)*

B. Include in your report the sizes of the plasmids, if possible, and a comment such as "Plasmid patterns of isolates A, B, and C suggest that they are the same strain" or "Plasmid patterns suggest the presence of three unrelated isolates."

C. Report a series of isolates that each have a single plasmid, all of approximately the same size, with a caution that further studies may be warranted. Whether further studies are done depends on the strength of the other data collected as part of the epidemiologic investigation. For example, if the isolates were collected within a short time from patients on the same ward with a common health care practitioner and if the antibiograms for all of the isolates are identical and have an unusual resistance pattern, this is strong supporting evidence of strain identity, and further information is probably not required. The strength of the data should be reflected in the report. If, on the other hand, the isolates were collected from patients on different wards, have antibiograms showing nothing but susceptibility, were collected over the course of 2 months, and do not have a substantial epidemiologic link, additional typing data should be sought, and the report should indicate that this is being done.

D. Results generated for infection control investigations do not necessarily have to be entered on a patient's chart. Provide a copy of the gel to the infection control practitioner, and discuss the results in the context of what is known about the outbreak.

VII. INTERPRETATION

Some chromosomal DNA fragments will be present in plasmid preparations and will be seen on agarose gels, indicating that the bacteria have lysed. The chromosomal DNA band, which tends to be diffuse, will migrate to the 12- to 15-kb area of the gel. Plasmids produce sharp, well-delineated bands on agarose gels. Plasmids $>$15 kb in size will migrate more slowly than the chromosomal band, while smaller plasmids will migrate more quickly than the chromosomal band. Isolates with multiple plasmids producing identical or very similar plasmid migration profiles after electrophoresis are likely to represent multiple isolates of the same strain. Plasmids placed in the outside lanes of a gel may migrate slightly more slowly (smile effect). This problem can be reduced by running stop mix in the two outside lanes of the gel. Two isolates determined to be devoid of plasmids cannot be assumed to be the same strain on this basis. If several isolates each contain only a single plasmid, all similar in size, other typing procedures or restriction enzyme analysis of the plasmids should be performed. Typing of isolates should be done only within a defined epidemiologic situation or to assess the relatedness of isolates from a single patient.

A. Organisms such as *Stenotrophomonas maltophilia, Serratia marcescens,* and some species of *Acinetobacter,* all of which are frequently involved in outbreaks of nosocomial infections, are often devoid of plasmid DNA. The presence of a chromosomal DNA band in the absence of plasmid DNA indicates that the technique is working and that the isolates did undergo lysis. In this situation, alternative typing methods must be used.

B. The presence of a large bright band in the pocket of the gel without indications of DNA elsewhere in the gel suggests that the isolates did not lyse well and that the DNA has not migrated out of the pocket of the gel. In this situation, the pH of the lysis buffer should be checked.

VIII. LIMITATIONS OF TESTING

A. Not all organisms have plasmid DNA. Large plasmids tend to cluster in the upper portions of the gel, and it is difficult to distinguish the sizes of plasmids that are >100 kb. Exercise caution when determining the relatedness of large plasmids. Because plasmids of identical size can be totally unrelated to one another, always exercise caution when isolates with a single plasmid each are encountered.

B. Whereas plasmid profiling is a relatively simple and rapid means of assessing organism relatedness, and remains quite useful especially in the context of antimicrobial resistance studies, it has been largely replaced as an epidemiologic typing method by chromosomal DNA-based typing methods.

REFERENCE

1. **CLSI.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. CLSI, Wayne, PA.

SUPPLEMENTAL READING

Olive, D. M., and P. Bean. 1999. Principles and applications of methods for DNA-based typing of microbial organisms. *J. Clin. Microbiol.* **37:**1661–1669.

Pfaller, M. A. 1999. Molecular epidemiology in the care of patients. *Arch. Pathol. Lab. Med.* **123:**1007–1010.

Tenover, F. C., K. Phillips, and L. G. Carlson. 1994. Plasmid fingerprinting of gram-negative organisms, p. 10.4.1–10.4.4. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook,* vol. 2, Supplement 1. American Society for Microbiology, Washington, DC.

12.4.3 Plasmid Fingerprinting of Staphylococci

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Plasmid analysis is based on the fact that different bacterial strains often carry different types or numbers of plasmids. This is a useful epidemiologic tool for investigations of strain relatedness. Approximately 80 to 90% of the clinical isolates of staphylococci carry one or more plasmids. Plasmid DNA is isolated from the bacterial cells, digested with a restriction endonuclease, and electrophoresed through agarose. Different plasmids display different patterns of fragment sizes. For initial analysis, the restriction endonucleases *Eco*RI and *Hin*dIII are recommended.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Most staphylococci grown on a nonselective agar medium for 18 to 24 h will yield plasmid DNA with this procedure.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Stocks and purchased chemicals

Indicate the expiration date on the label and in the work record or on the manufacturer's label. All materials are stable for 2 years at room temperature unless otherwise noted. Stock solutions that must be autoclaved or filter sterilized are noted.

1. 1 M Tris, pH 8.0 (autoclave)
2. 1 M Tris, pH 7.0 (autoclave)
3. Sodium dodecyl sulfate (SDS), 20% (filter sterilize)
4. 0.5 M EDTA, pH 8.0 (autoclave)
5. 4 M NaCl (autoclave)
6. 2 N NaOH
7. Sucrose (50%) (filter sterilize)
8. Ethanol (95%)
9. Agarose
10. Mueller-Hinton broth (MHB) for coagulase-negative staphylococci
11. TSB for *Staphylococcus aureus*
12. Restriction endonucleases *Eco*RI and *Hin*dIII and buffers (New England BioLabs, Inc., Beverly, MA)
13. Molecular size standards (New England BioLabs, Inc.); use *Eco*RI or *Hin*dIII digests of bacteriophage lambda
14. 3 M sodium acetate (autoclave)
15. Lysostaphin stock (1 mg/ml [Applied Microbiology, New York, NY])
16. Lysozyme stock (20 mg/ml)
17. RNase A stock solution (10 mg/ml [New England BioLabs, Inc.])
18. RNase dilution buffer (New England BioLabs, Inc.): 1 M Tris (pH 8.0), 1 M Tris (pH 7.0), 4 M NaCl
19. Dye mixture: 20 mM EDTA, 50% glycerol, 0.1% bromophenol blue, 1% xylene cyanol
20. 10× TBE buffer: Tris base, boric acid, Na_2EDTA

B. Working solutions

Indicate the expiration date on the label and in the work record or on the manufacturer's label. Working solutions are made from stock solutions and are stable for 1 year at room temperature.

1. TES-SDS: 100 mM Tris, 70 mM EDTA, 50 mM NaCl, and 1.5% SDS
2. TES-sucrose: 100 mM Tris, 70 mM EDTA, 50 mM NaCl, and 5% sucrose

III. MATERIALS *(continued)*

3. 0.375 M EDTA, pH 8.0
4. TE buffer (10:0.1): 10 mM Tris and 0.1 mM EDTA
5. 0.4% SDS–0.4 N NaOH
6. RNase A working solution. Dilute RNase A stock solution 1:100 in 10 mM Tris (pH 7.5)–15 mM NaCl. Boil in water bath for 15 min. Cool gradually to room temperature. Aliquot, and store at −20°C.
7. Loading dye, 1:1 mixture. Mix the dye and 1% SDS in a 1:1 ratio.
8. Loading dye, 1:1:4 mixture. Mix 1 part dye mixture, 1 part 1% SDS, and 4 parts distilled water.
9. DNA wash solution: 10 mM Tris, 100 mM EDTA, 75 mM sodium acetate

C. Supplies and equipment

1. Power supply
2. Gel box for electrophoresis
3. Microcentrifuge
4. Microcentrifuge tube (1.5 ml) (supplier not critical)
5. Shaking water bath or shaking platform in incubator
6. Vacuum line or vacuum unit (for aspiration of liquids)
7. Transilluminator (mid-range UV)
8. Camera unit (e.g., Polaroid MP-4 or handheld Polaroid-type Fotodyne FCR-10)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that all materials and reagents meet expiration date and QC parameters per CLSI document M22-A3 (1).

B. A staphylococcal strain of known plasmid content should be run in parallel with each screening procedure. *S. aureus* SM818-73 and coagulase-negative strain SM734-41 are available from one of the authors (M. A. Pfaller) of this procedure as controls.

V. PROCEDURE

Observe standard precautions.

A. DNA preparation

1. Using three to five well-isolated colonies, inoculate one tube of growth medium for each test and control strain to be screened. For *S. aureus,* use 10 ml of TSB; for coagulase-negative staphylococci, use 20 ml of MHB divided into two 10-ml cultures (for convenience in processing).
2. Incubate with shaking at 37°C for 16 to 24 h.
3. Centrifuge broth for 15 min at 7,000 × *g*, and remove supernatant by aspiration.
4. Suspend cells in 1 ml of sterile saline, and transfer them to a sterile 1.5 ml microcentrifuge tube (supplier not critical).
5. Centrifuge at top speed (12,000 × *g*) for 15 s. Carefully aspirate supernatant.
6. Suspend cells in 100 μl of TES-sucrose. For *S. aureus,* add 50 μl of dilute lysostaphin. Make dilute lysostaphin by mixing 5 μl of lysostaphin stock with 45 μl of TES-sucrose. For coagulase-negative staphylococci, add 15 μl of lysostaphin stock and 50 μl of lysozyme stock. Mix well.
7. Incubate tubes in water bath at 37°C for 20 min for *S. aureus* and 60 min for coagulase-negative staphylococci.
8. Add 50 μl of 0.375 M EDTA (pH 8.0) to each tube. Mix by inverting the tube 10 to 15 times. Solution may become very viscous.
9. Incubate on ice for 15 min.
10. Add 100 μl of TES-SDS (SDS must be in solution with no precipitate). Mix by inverting the tube 10 to 15 times.
11. Place on ice for at least 30 min. Hold for additional time if necessary.
12. Centrifuge at 12,000 × g for 15 min. Use of a refrigerated microcentrifuge or a centrifuge located in a cold room is optimal but not mandatory. Centrifugation at room temperature will also work.

V. PROCEDURE *(continued)*

13. Remove 300 µl of supernatant, and place it in a new microcentrifuge tube. Avoid the pellet and any viscous material. If less than 300 µl is available, make up difference with TES-sucrose. Discard pellet in appropriate biosafety container.
14. Add 150 µl of 0.4% SDS–0.4 N NaOH. Mix by inverting the tube 10 times. Quickly place on ice for 5 min.
15. Add 150 µl of 3 M sodium acetate (pH 5.4), and invert the tube 10 times. Return to ice quickly, and incubate for 60 min or longer.
16. Centrifuge at 12,000 × *g* for 15 min. Remove supernatant to a fresh tube. Avoid white pellet. Discard pellet in appropriate biosafety container.
17. Add 1 ml of 95% cold ethanol to supernatant. Mix by inverting the tube 20 times, until contents are well mixed. This can be stored overnight at −20°C.
18. Centrifuge at 12,000 × *g* for 30 min. (Shorter centrifugation times will reduce yield.) Remove supernatant by aspiration with drawn-glass pipette, taking care to save the pellet (may be difficult to see).
19. Suspend pellet in 500 µl of DNA wash solution. Incubate at 37°C for 20 min. Vortex gently (low setting) at 10 min and again at 20 min.
20. Centrifuge at 12,000 × *g* for 10 min.
21. Transfer supernatant to a new microcentrifuge tube containing 1 ml of cold 95% ethanol. Mix by inverting the tube 20 times. This mixture can be stored overnight at −20°C.
22. Centrifuge at 12,000 × *g* for 30 min and remove supernatant, taking care to save the pellet. Centrifuge an additional 5 min, and aspirate remaining ethanol.
23. Dry under vacuum for 30 min. This is sufficient time for 20 to 30 tubes.
24. Remove 3 µl of plasmid preparation, and combine it with 10 µl of the 1:1:4 dye mixture. Electrophorese through an agarose gel to screen for whole-plasmid DNA. Electrophoresis times will vary depending on the size of the gel and the apparatus used. Alternatively, the samples can be stored at −20°C. DNA is stable in a −20°C non-frost-free freezer (defrosting cycle is hard on DNA samples) for approximately 6 months.

B. Restriction analysis of plasmid DNA

1. Place 3 µl of the plasmid preparation in each of two fresh tubes for restriction enzyme digestion (one with *Eco*RI, the other with *Hin*dIII), and store the remainder at −20°C.
2. To the 3-µl DNA sample add the following: 4.5 µl of sterile water; 1.0 µl of 10× restriction buffer, per manufacturer; 1.0 µl of RNase A working solution; and 0.5 µl of restriction enzyme (*Eco*RI or *Hin*dIII).
3. Centrifuge at 12,000 × *g* for 15 s. Place in 37°C water bath for 2 h. Add 3 µl of the 1:1 mixture of SDS and dye.
4. Prepare 0.7% agarose gel by using 1× TBE buffer. Load the entire 13-µl sample into a well of the gel. Use a *Hin*dIII or *Eco*RI digest of phage lambda loaded onto a separate well as molecular size standards for restriction digests.
5. Electrophorese for 4 h at 100 V or for 1.5 h at 130 V (slower is better). The dark blue dye should move >3 cm. Photograph using a mid-range (260 nm) transilluminator and orange filter (Kodak 22A Wrattan or equivalent).

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A report of the results of plasmid analysis, detailing which isolates are related or distinct, should be issued to the service that generated the request for typing (*see* legend to Fig. 12.4.3–1 for an example). Photographs of the results may accompany the report at the discretion of the laboratory.

VII. INTERPRETATION

One of the most difficult aspects of interpreting plasmid patterns is determining whether there was adequate lysis of the cells during the protocol to provide DNA for analysis. In preparations with no detectable plasmid DNA, small amounts of chromosomal DNA must be present for the preparation to be considered adequate for analysis. If no chromosomal DNA is present (it usually appears as a faint smear in the molecular size range of 12 to 15 kb) and no plasmid DNA is detectable, repeat the procedure.

Two or more isolates are presumed to be the same strain if all of the bands in both the *Hin*dIII and *Eco*RI restriction digests are identical. If multiple bands do not match between isolates, they are presumed to represent different strains.

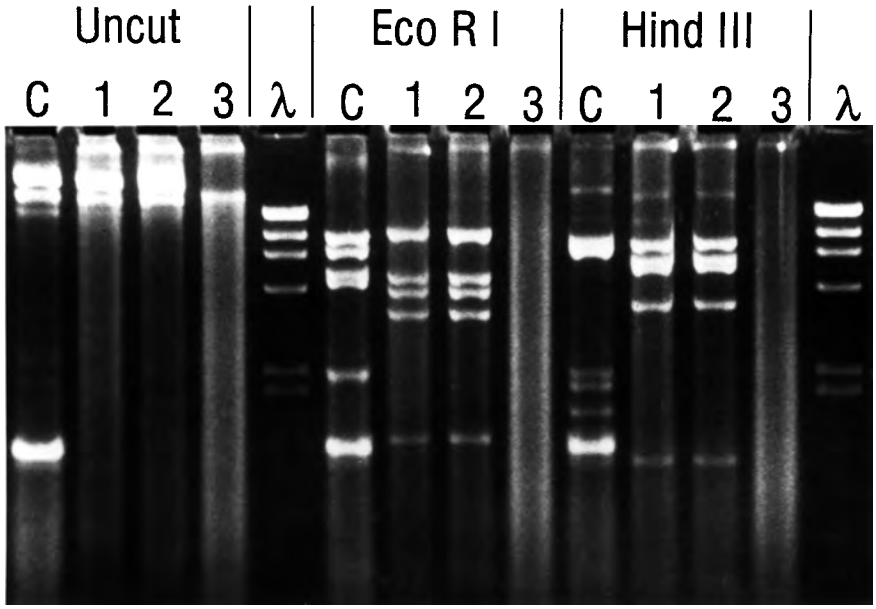

Figure 12.4.3–1 *S. aureus* plasmid DNA preparations. Uncut, undigested plasmid DNA; *Eco*RI, DNA digested with *Eco*RI; *Hin*dIII, plasmid DNA digested with *Hin*dIII. Lanes: C, control strain SM818-73; 1, 2, and 3, isolates collected from a single patient. Isolate 3 does not have detectable plasmid DNA. The report for this group of isolates stated, "Based on restriction endonuclease analysis of plasmid DNA, these strains were grouped as follows: isolates 1 and 2 are identical to each other; isolate 3 is not related to isolates 1 and 2."

REFERENCE

1. **CLSI.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. CLSI, Wayne, PA.

SUPPLEMENTAL READING

Back, N. A., C. C. Linnemann, M. A. Pfaller, J. L. Staneck, and V. Morthland. 1993. Recurrent epidemics caused by a single strain of erythromycin-resistant *Staphylococcus aureus:* the importance of molecular epidemiology. *JAMA* **270:**1329–1333.

Pfaller, M. A. 1999. Molecular epidemiology in the care of patients. *Arch. Pathol. Lab. Med.* **123:**1007–1010.

Pfaller, M. A., D. S. Wakefield, R. Hollis, M. Fredrickson, E. Evans, and R. M. Massanari. 1991. The clinical microbiology laboratory as an aid in infection control: the application of molecular techniques in epidemiologic studies of methicillin-resistant *Staphylococcus aureus. Diagn. Microbiol. Infect. Dis.* **14:**209–217.

Pfaller, M. A., and L. A. Herwaldt. 1997. The clinical microbiology laboratory and infection control: emerging pathogens, antimicrobial resistance, and new technology. *Clin. Infect. Dis.* **25:**858–870.

12.4.4 Method for Ribotyping by Using a Chemiluminescent Probe

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Ribotyping was first described in 1986 by Grimont and Grimont (2), and since that time it has emerged as one of the most powerful tools of molecular epidemiology. Ribotyping involves the use of Southern blot analysis to detect polymorphisms in the chromosomal regions containing the rRNA genes. Classic ribotyping uses labeled probes containing *Escherichia coli* 16S and 23S rRNA sequences. Both the nature of the probe and the mode of labeling, however, may vary. Ribotyping has three main advantages: (i) the genes coding for rRNA are highly conserved, allowing for the use of a single probe to subtype all eubacteria; (ii) because most bacteria contain multiple rRNA genes, a reasonable number of bands are obtained after probing; and (iii) all strains have rRNA genes. The availability of chemiluminescent labeling techniques and the recent development of an automated ribotyping system (4–6) make ribotyping more feasible for the investigation of nosocomial outbreaks by the clinical laboratory.

◩ **NOTE:** This procedure has been adapted from that of Gustaferro (3) and Stull and LiPuma (8). The method described is optimized for *Staphylococcus aureus* but can also be used for ribotyping other gram-positive and gram-negative bacteria (2–8).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Most organisms grown on a nonselective agar medium for 18 to 24 h will yield chromosomal DNA for this procedure.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

◩ **NOTE:** Unless otherwise stated, all reagents are available from Sigma Chemical Co., St. Louis, MO.

1. Lysostaphin (500 U/ml)
2. 10% Bleach
3. Denhardt's solution
4. Chondroitin sulfate A
5. Bromophenol blue
6. Proteinase K (20 mg/ml)
7. RNasin RNase inhibitor
8. High-pressure liquid chromatography (HPLC) water
9. Enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL)
10. 16S and 23S rRNA from *E. coli* MRE600
11. Restriction endonucleases (individualized for each organism; available from New England BioLabs Inc., Beverly, MA)
12. BHI broth or other appropriate liquid medium
13. TES buffer: 0.01 M Tris (pH 8), 0.1 M NaCl, 0.001 M EDTA
14. 50× TAE buffer: 242.2 g of Tris base, 57.15 ml of 17.5 M glacial acetic acid, 100 ml of 0.5 M EDTA, water to make up 1 liter (pH 8.5)
15. Denaturation solution: 1.5 M NaCl, 0.5 M NaOH
16. Depurination solution: 250 mM HCl
17. Neutralization solution: 1 M Tris-HCl (pH 8.0), 1.5 M NaCl
18. Phenol
19. Chloroform
20. Isoamyl alcohol

III. MATERIALS *(continued)*

21. Prehybridization buffer: 25 mM KH_2PO_4 (pH 7.4), 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 50 μg of salmon sperm DNA per ml, 6 M urea, diethyl pyrocarbonate (DEPC)-treated water
22. Hybridization buffer: 6 M urea, 1.1% sodium dodecyl sulfate (SDS), 0.5× SSC, DEPC-treated water
23. Secondary wash buffer: 2× SSC, DEPC-treated water
24. Electrophoresis loading buffer: 27 ml of glycerol, 3 ml of 10× TAE buffer, 0.1% (wt/vol) SDS, 10 ml of 0.5 M EDTA (pH 8.0), 10 mg of bromophenol blue, water to make up 100 ml
25. Glutaraldehyde
26. Agarose
27. Ethidium bromide
28. 5 M NaCl
29. 70 and 100% ethanol

B. Supplies

1. Cellophane (any brand)
2. Nitrocellulose membrane
3. Gel blot paper
4. Nylon mesh
5. X-ray film
6. Scissors
7. Forceps
8. Eppendorf tubes (1.5 ml)

C. Equipment

1. Agarose gel electrophoresis apparatus
2. Power supply
3. UV transilluminator and photodocumentation setup
4. Autoradiograph cassettes and intensifying screens
5. Vacuum desiccator
6. Vacuum oven
7. Microcentrifuge
8. Block heater (60°C)

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Verify that all materials and reagents meet expiration date and QC parameters per CLSI document M22-A3 (1).

B. A standard control strain of the same species, if available, should be prepared along with the test strains.

C. The use of molecular typing tests to identify related strains is most effective if a group of appropriate epidemiologically unrelated control organisms can be prepared and compared with the isolates in question. Appropriate control strains include the following.

1. Identical species isolated in the same unit on different dates
2. Identical species isolated on the same date in different units

V. PROCEDURE

Observe standard precautions.

A. DNA preparation

1. Methods for extraction of genomic DNA from *S. aureus* are outlined below.
2. Inoculate one colony into 4 ml of broth, and incubate overnight at 35°C.
3. Pipette 1 ml of the sample into each of three 1.5-ml Eppendorf tubes, and centrifuge for 5 min at 14,000 × *g* in a microcentrifuge.
4. Wash pellet once with TES buffer, vortex, and pellet for 5 min at 14,000 × *g*. Discard supernatant into 10% bleach. Suspend pellet in 200 μl of TES buffer by vortexing.
5. Add 10 μl of lysostaphin to the cell suspension, vortex, and incubate at 37°C for 30 min.
6. Add 20 μl of SDS and 1.2 μl of proteinase K, and incubate in a 60°C block heater for 1 to 2 h.
7. Extract genomic DNA with two phenol-chloroform-isoamyl alcohol (25:24:1) extractions (230 μl) followed by one chloroform-isoamyl alcohol extraction (230 μl).
8. Precipitate the DNA by adding 1/25 volume of 5 M NaCl and 2.5 volumes of cold 100% ethanol to the aqueous layer of the final extraction. Mix by inverting the tube four or five times. Store at −20°C for 30 min.

V. PROCEDURE *(continued)*

9. Centrifuge at 14,000 × *g* for 10 min, wash the pellet with 70% ethanol, and centrifuge again (14,000 × *g* for 10 min).
10. Dry the pellet in a vacuum oven at 60°C. Resuspend it in 20 μl of HPLC-grade water. After resuspension, consolidate all three samples from the same isolate.

B. DNA restriction

1. Restriction enzymes must be individualized for each organism. The enzymes *Xba*I, *Hin*dIII, *Kpn*I, and *Eco*RI have been used successfully to identify strains of *S. aureus.*
2. Using a UV spectrophotometer, determine the A_{260} and A_{280} of the extracted DNA. Calculate the A_{260}/A_{280} ratio to determine the purity of the DNA sample. This ratio should be 1:8 or greater. If it is less than 1:8, the DNA should be reextracted and precipitated.
3. Determine the amount of DNA present by using the A_{260}.
4. Dilute 4 μg of test and control DNA to a total volume of 18 μl. Add 2 μl of 10× restriction buffer. Add 2 to 10 U of restriction enzyme, mix, and digest as recommended by the manufacturer.
5. Prepare a 1% agarose gel in 1× TAE buffer. Electrophoretically separate the restricted DNA in TAE buffer for 4 h at 100 V.
6. Stain the gel with ethidium bromide, and visualize the restriction products by using a UV-light box.
7. Depurinate, denature, and neutralize the gel by soaking with gentle shaking in depurination and denaturation buffer for 15 min each and neutralization buffer for 30 min. Rinse the gel with distilled water twice between each buffer. Transfer the DNA to a nitrocellulose membrane by Southern blotting overnight.
8. Soak the nitrocellulose membrane (blot) in 6× SSC for 5 min. Allow it to air dry. Sandwich it between sheets of fresh Whatman 3MM paper, and bake it at 80°C under vacuum for 2 h.

C. Labeling of probe

◩ **NOTE:** The ECL kit is designed to label DNA. To label RNA used as a probe in this typing method, the following protocol should be followed in place of the kit instructions.

1. Dilute 1 μl of 16S–23S rRNA from *E. coli* MRE600 (4 μg/ml) with 399 μl of kit water in a 1.5-ml microcentrifuge tube (final concentration, 10 ng/μl).
2. Boil the mixture for 2 min in a water bath, and then snap-cool it in an ice-water bath for 5 min. Centrifuge briefly to settle contents to bottom.
3. Add 400 μl of DNA-labeling reagent and mix gently. Add 400 μl of glutaraldehyde solution and mix gently.
4. Incubate at 37°C for 10 min. Add 30 U of RNasin RNase inhibitor, mix well, and immediately place on ice.
5. Store the labeled probe in 50% glycerol in a 1.5-ml microcentrifuge tube at −20°C (equal volumes of probe and glycerol).

D. Hybridization

◩ **NOTE:** The hybridization buffer supplied in the ECL kit must not be used. All solutions must be prepared with DEPC-treated water.

1. Roll the blot into a nylon mesh with the DNA surface facing inward. Place it in a hybridization tube. Prehybridize the blots at 40°C in prehybridization buffer (0.125 ml per cm^2 of nylon mesh) in a hybridization oven.
2. After 3 h, discard the prehybridization buffer and replace it with hybridization buffer. Add 20 ng of probe per ml of hybridization buffer. Do not pipette the probe directly onto the blot.
3. Hybridize for 5 h at 40°C.
4. Wash the blot twice in primary wash buffer for 20 min each at 40°C.

V. PROCEDURE *(continued)*

5. Remove the blot from the tube and place it in a clean container. Wash it twice in secondary wash buffer for 5 min at room temperature with gentle agitation.

E. Detection

1. Prepare a tray containing the following items: scissors, forceps, blotting paper, detection reagents 1 and 2 (at 4°C and separate until use), washed blot in secondary wash buffer, cassette and X-ray film, cellophane, and timer.
2. Working as quickly as possible in a darkroom, with the lights on, remove the blot from the wash buffer with forceps. Drain off excess reagents. Place the blot on the tray. Mix equal amounts (~10 ml) of each detection reagent, and pour the mixture onto the blot. Incubate for exactly 1 min.
3. Drain off excess reagents, and lay the blot on a piece of blot paper for 10 s to absorb excess moisture. Cover the blot with cellophane and place it in the cassette with the DNA side up. Smooth out all air bubbles.
4. Turn off the lights. Place the film in the cassette (clip off a corner to mark the orientation). Obtain a 1-min exposure.

F. Procedure notes

1. The sensitivity of chemiluminescent ribotyping is directly related to the amount of DNA analyzed in each reaction. Underloaded lanes will require longer exposure times. Luminescence persists for 3 to 7 h.
2. Labeled probe has been used successfully for up to 3 months when stored at −20°C in 50% glycerol.
3. Molecular size standards can also be labeled with the ECL kit to provide convenient determination of molecular weights.
4. The chemiluminescent ribotyping procedure has been completely automated. The instrumentation and ribotyping kits are available commercially from Qualicon, Wilmington, DE.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A report of the results of ribotyping that details which isolates are indistinguishable (clonal), similar (subtypes), or different should be issued to the service that generated the request for typing.

VII. INTERPRETATION

A. Figure 12.4.4–1 shows examples of ribotype patterns of *S. aureus.*

B. Strains with indistinguishable ribotype patterns are considered to be clonal.

C. Strains with one or two band shifts are also considered to be clonally related subtypes.

D. Strains that differ by more than three bands are considered independent strains.

◩ **NOTE:** Isolates with indistinguishable or highly related ribotype patterns (one to three band differences) may require further analysis by pulsed-field gel electrophoresis or some other highly discriminative technique for optimum strain delineation.

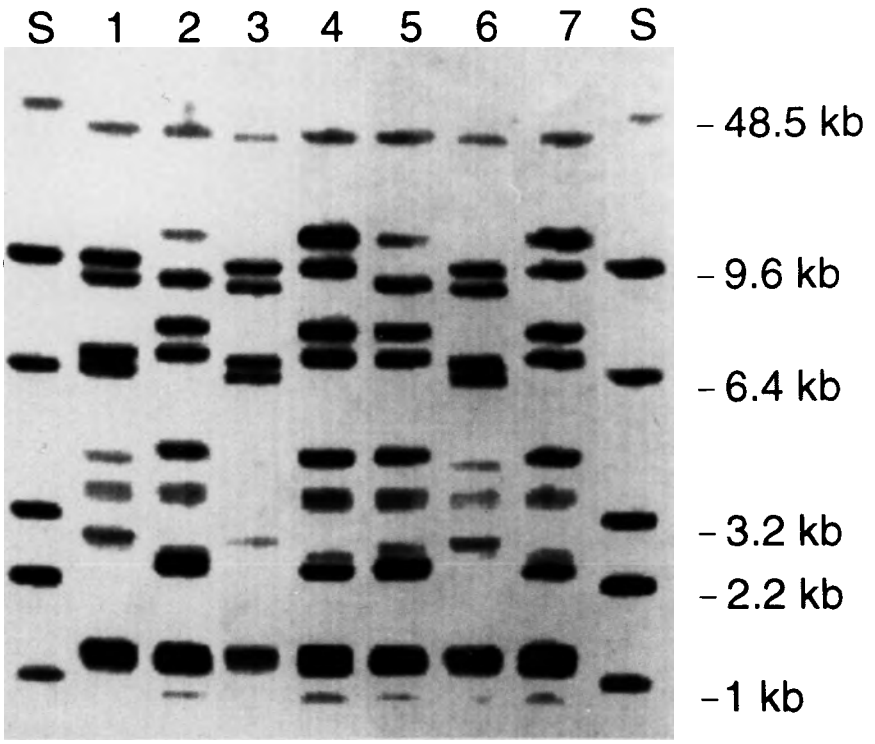

Figure 12.4.4–1 Ribotype profiles of *S. aureus* isolates. Lanes: S, molecular size standards; 1 and 6, ribotype A; 2 and 5, ribotype B; 3, ribotype C; 4 and 7, ribotype D. Figure from **H. D. Isenberg (ed.).** 1998. *Essential Procedures for Clinical Microbiology.* ASM Press, Washington, DC.

REFERENCES

1. **CLSI.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. CLSI, Wayne, PA.
2. **Grimont, F., and P. A. D. Grimont.** 1986. Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Ann. Inst. Pasteur/Microbiol.* (Paris) **137B:**165–175.
3. **Gustaferro, C. A.** 1993. Chemiluminescent ribotyping, p. 584–589. *In* D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), *Diagnostic Molecular Microbiology: Principles and Applications.* American Society for Microbiology, Washington, DC.
4. **Hollis, R. J., J. L. Bruce, S. J. Fritschel, and M. A. Pfaller.** 1999. Comparative evaluation of an automated ribotyping instrument versus pulsed-field gel electrophonesis for epidemiological investigation of clinical isolates of bacteria. *Diagn. Microbiol. Infect. Dis.* **34:**263–268.
5. **Pfaller, M. A., C. Wendt, R. J. Hollis, R. P. Wenzel, S. J. Fritschel, J. J. Neubauer, and L. A. Herwaldt.** 1996. Comparative evaluation of an automated ribotyping system versus pulsed-field gel electrophoresis for epidemiological typing of clinical isolates of *Escherichia coli* and *Pseudomonas aeruginosa* from patients with recurrent gram-negative bacteremia. *Diagn. Microbiol. Infect. Dis.* **25:**1–8.
6. **Pfaller, M. A., J. Acar, R. N. Jones, V. Verhoef, J. Turnidge, and H. S. Sader.** 2001. Integration of molecular characterization of microorganisms in a global antimicrobial resistance program. *Clin. Infect. Dis.* **32**(Suppl. 2)**:**S156–S167.
7. **Stull, T. L., J. J. LiPuma, and T. D. Edlind.** 1988. A broad-spectrum probe for molecular epidemiology of bacteria: ribosomal RNA. *J. Infect. Dis.* **157:**280–286.
8. **Stull, T. L., and J. J. LiPuma.** 1994. Method for ribotyping, p. 10.5.1–10.5.8. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook,* vol. 2, Supplement 1. American Society for Microbiology, Washington, DC.

12.4.5 Chromosomal Restriction Fragment Analysis by Pulsed-Field Gel Electrophoresis: Application to Molecular Epidemiology

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Chromosomal restriction fragment analysis is based on the fact that chromosomes are not static and can undergo both rearrangements and point mutations. Changes in nucleotide sequence are reflected in the restriction endonuclease patterns of chromosomal DNA when the fragments are separated on agarose gels. Physical characterization of bacterial and fungal DNA at the genomic level has been limited by (i) the difficulty in obtaining suitable fragments of chromosomal DNA because of the shearing of large DNA molecules in solution and (ii) the inability of conventional (constant-field) agarose gel electrophoresis to resolve DNA molecules in an appropriate size range (40 to 4,000 kbp). These constraints have been overcome by the development of techniques for (i) preparing unit length chromosomal DNA by the in situ lysis of organisms embedded in agarose and subsequent digestion of the DNA directly in the agarose with restriction enzymes that have infrequent recognition sites and (ii) resolving the resulting large DNA fragments in agarose gels by alternation of electric fields by a technique known as pulsed-field gel electrophoresis (PFGE). Thus, total genomic DNA can be resolved into a limited number of restriction fragments with distinct electrophoretic mobilities. PFGE has been shown to be highly effective in molecular epidemiologic studies of bacterial and yeast isolates.

◩ **NOTE:** This procedure addresses the use of PFGE for typing bacterial isolates and has been adapted from Maslow et al. (2) and Pfaller et al. (3).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Most organisms grown on a nonselective agar or broth medium for 18 to 24 h will yield chromosomal DNA for the procedure.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

◩ **NOTE:** Unless otherwise stated, all reagents are available from Sigma Chemical Co., St. Louis, MO.

1. InCert agarose (low melting temperature) (FMC BioProducts, Rockland, ME)
2. SeaKem agarose (high melting temperature) (FMC BioProducts)
3. Ethidium bromide
4. Restriction endonucleases (individualized for each organism; available from New England BioLabs, Beverly, MA)
5. 10× restriction buffers as provided by manufacturer (New England BioLabs)
6. Molecular size standards, 48.5-kbp lambda ladder molecular size standards (New England BioLabs)
7. Brij 58 (polyoxyethylene 20 cetyl ether)
8. Sodium deoxycholate
9. Sodium lauroyl sarcosine
10. RNase A (molecular biology grade)
11. Lysostaphin
12. Lysozyme
13. Proteinase K
14. PIV buffer: 10 mM Tris (pH 7.6), 1 M NaCl (total volume, 500 ml; autoclave, and store at 4°C)
15. Lysis buffer: 6 mM Tris (pH 7.6), 1 M NaCl, 100 mM EDTA (pH 7.6), 0.5% Brij 58, 0.2% sodium deoxycholate, 0.5% sodium lauroyl sarcosine (total volume, 100 ml; filter sterilize, and store at 4°C)
16. Lysis solution: 20 μg of RNase per ml, 1 mg of lysozyme per ml, 5 U of lysostaphin per ml (add enzymes to lysis buffer just before use)

III. MATERIALS *(continued)*

17. ES buffer: 0.5 M EDTA (pH 8.0), 10% sodium lauroyl sarcosine (total volume, 500 ml; filter sterilize and store at room temperature)
18. ESP solution: 100 µg of proteinase K per ml of ES buffer (total volume, 100 ml; store at 4°C) (20× proteinase stock solution: 100 mg of proteinase K in 50 ml of ES buffer; incubate at 50°C for 1 h and store at 4°C [this solution is highly stable])
19. 1× TE buffer: 10 mM Tris (pH 7.6), 0.1 mM EDTA (pH 7.6) (total volume, 500 ml; autoclave, and store at room temperature)
20. Lysostaphin buffer: 50 mM Tris (pH 7.6), 0.15 M NaCl (total volume, 50 ml; autoclave, and store at room temperature)
21. 10× TBE buffer: Tris base, boric acid, Na_2EDTA

B. Supplies

1. Snap-top tubes (15 ml)
2. Snap-top tubes (5 ml)
3. Round-bottom tubes, screw cap (15 ml)
4. Six-well tissue culture plate
5. Insert mold
6. Glass tray (20 by 30 cm), metal tray (15 by 25 cm)

C. Equipment

1. Orbital shaker
2. Incubators at a variety of temperatures (25, 37, and 50°C)
3. Vacuum line for aspiration of liquids
4. Camera unit for photodocumentation
5. Transilluminator, mid-range UV
6. Test tube roller
7. Heating platform
8. Pulse-field gel box, pump, gel molds, cooling water bath, power supply, pulse wave switches

◩ **NOTE:** Electrophoresis conditions specified in this procedure require the CHEF-DRII system from Bio-Rad with their MiniChiller. However, other forms of PFGE apparatus may be used.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. A standard control strain of the same species, if available, should be prepared along with every preparation as a control for the DNA isolation procedures.

B. The use of molecular typing tests to identify related strains is most effective if a group of appropriate epidemiologically unrelated control organisms can be prepared and compared to the isolates in question. Appropriate control strains would be as follows

1. Identical species isolated in the same unit on different dates
2. Identical species isolated on the same date in different units

C. The many points to be checked are as follows.

1. All lanes should have electrophoresed more or less straight.
2. The restriction digests should be complete. A complete digest will have few if any dim bands located above or between bright bands in the same lane. Bright and dim are relative terms and apply only within the context of the same region of one lane. An incomplete (or partial) digest will have one or more dim bands located above or between bright bands in the same lane.
3. The DNA in all lanes should have digested and have visible bands. Usually, all strains of the same species will have about the same number of bands with the same size distribution for any given restriction enzyme and running conditions.
4. If any of the above conditions are not met, then some or all of the samples must be retested. Sometimes the plugs do not digest, and the failed samples need to be prepared again.

D. DNA size standards

1. *Staphylococcus aureus* ATCC 8325 digested with *Sma*I
2. The chromosomes of *Saccharomyces cerevisiae* (Bio-Rad Laboratories, Richmond, CA)
3. Lambda ladder (48.5 kbp)

IV. QUALITY CONTROL *(continued)*

E. Verify that all materials and reagents meet expiration date and QC parameters per CLSI document M22-A3 (1).

V. PROCEDURE

Observe standard precautions.

A. Sample preparation

1. For each strain, inoculate a single colony into 0.5 ml of broth (TSB).
2. Grow for 2 h or until turbid.
3. Streak out onto an agar plate, and incubate overnight (ON).
4. Pick a single colony, inoculate 5 ml of broth, and incubate ON.
5. Prepare the plugs on day 1.
- **a.** Mix InCert agarose (1.3% [wt/vol]) in PIV buffer in a 15-ml tube. Prepare 1 ml per strain (plus 1 ml extra), vortex, and place into a beaker of boiling water to dissolve. Revortex prior to aliquoting.
- **b.** For each strain, label one 15-ml snap-top tube, one 5-ml snap-top tube, and one 15-ml round-bottom tube.
- **c.** For each strain, label one plug mold. Then tape the bottom of the plug molds, place them in a metal tray, put this tray in a glass tray filled with ice, and place it in the refrigerator for at least 30 min.
- **d.** Put 5 ml of cold PIV buffer into each 15-ml snap-top tube and place on ice.
- **e.** Carefully dispense 1 ml of dissolved agarose into each 5-ml snap-top tube, and keep the tubes in a 50°C heat block.
- **f.** Dispense 1.5 ml of the ON culture into each 15-ml snap-top tube with cold PIV buffer.
- **g.** Centrifuge the 15-ml snap-top tubes at 1,100 × *g* for 15 min at 4°C.
- **h.** Decant the PIV buffer from the cell pellet, suspend the cells thoroughly in 1.5 ml of cold PIV buffer, and place on ice.
- **i.** Add 1 ml of cells in PIV buffer to the 5-ml snap-top tube with 1 ml of molten InCert agarose, and vortex the tube lightly.
- **j.** Working quickly, dispense 105 μl of the mixture into each well of the plug molds.

  ◩ **NOTE:** The final concentration of organisms is ~10^9 CFU/ml. Since 1 CFU contains ~10^{-14} g of DNA, each plug has ~1 μg of DNA.
- **k.** Keep the molds in the tray on ice, and place at 4°C for 30 min to solidify.

6. Lyse the plugs on days 2, 3, and 4.
- **a.** Make fresh lysis solution by adding RNase and lysozyme (for gram-negative bacteria) or lysostaphin and lysozyme (for gram-positive bacteria) to stock lysis buffer.
  - (1) RNase stock solution: dissolve RNase at 10 mg/ml in sterile water, heat in boiling water for 20 to 30 min, dispense 100 μl per microcentrifuge tube, and store at −20°C.
  - (2) Lysozyme stock solution: dissolve 0.5 g of lysozyme in 10 ml of sterile water (final concentration, 50 mg/ml), dispense 200 μl per microcentrifuge tube, and store at −20°C.
  - (3) Lysostaphin stock solution: dissolve 5,000 U of lysostaphin in 10 ml of lysostaphin buffer, dispense 200 μl per microcentrifuge tube, and store at −20°C.
- **b.** Dispense 4 ml of lysis solution into each labeled 15-ml round-bottom tube.
- **c.** For each strain, push out the 12 plugs into the tube with lysis solution.
- **d.** Incubate ON at 37°C with gentle shaking.
- **e.** Chill the tubes on ice for at least 15 min to harden the plugs.
- **f.** Carefully aspirate the lysis solution.

V. PROCEDURE *(continued)*

g. Dispense 4 ml of ESP solution into each tube, and incubate ON at 50°C, shaking gently.

h. Chill the tubes. Change to fresh ESP solution, and again incubate ON at 50°C.

i. The plugs can now be used directly for restriction enzyme digestion as outlined below or can be placed in fresh ESP solution and stored at 4°C (stable for up to 2 years).

B. Restriction enzyme digestion

◩ Perform steps V.B.1 and 2 on day 1 of restriction enzyme digestion.

1. Turn on a water bath at the temperature appropriate for the restriction enzyme.

2. Wash the plugs in 10 ml of 1 × TE buffer at 37°C on a roller. Four washes are required, at least two of which should be a minimum of 2 h; the others should be at least 1 h. For convenience, one of the washes can continue ON.

◩ Perform steps V.B.3 through 6 on day 2 of restriction enzyme digestion.

3. Complete the TE buffer washes, if necessary.

4. For each plug, prepare a labeled microcentrifuge tube containing 10× restriction enzyme buffer and bovine serum albumin (final concentration, 100 μg/ml); add water to a final volume of 250 μl.

5. Add a washed plug to the tube.

6. Add restriction enzyme (2 to 10 U, per manufacturer), mix gently, and incubate ON at the appropriate temperature. See Table 12.4.5–1 for restriction endonucleases appropriate for some species.

◩ Perform steps V.B.7 through 11 on day 3.

7. Chill the tubes before handling the inserts.

8. Pour out a plug into a labeled well of a six-well tissue culture plate. Save the plate.

9. Add 1 ml of ES buffer to a microcentrifuge tube and put the plugs into the tube.

10. Incubate the tube at 50°C for 2 h.

11. Pour out the plug into the same tissue culture plate well as above (step V.B.8). Add 300 μl of ESP solution to each tube. Replace the plug into the tube and store at 4°C.

C. Gel preparation, loading, and electrophoresis

1. Make the gel with SeaKem high-melting-temperature agarose (1% [wt/vol]) dissolved in 0.5× TBE buffer. Wash the gel ON in 0.5× TBE buffer.

2. By using a glass coverslip or surgical blade, cut a slice ~1 mm thick off the end of the plug, and load it into a well of the gel.

3. Fill all the wells with agarose, and place the gel in a PFGE box with 0.5× TBE buffer.

4. Run the gel in accordance with the manufacturer's recommendations for the equipment being used. See Table 12.4.5–2 for CHEF-DRII running conditions.

5. Store the remainder of the plug in ESP solution at 4°C in a microcentrifuge tube.

D. Gel visualization

1. Stain the gel for 30 min with ethidium bromide solution (0.5 μg/ml in water).

2. Rinse the gel with deionized water, and destain in water for at least 2 h.

3. Photograph under UV illumination.

Table 12.4.5–1 Suggested enzymes and running conditions for some species

Organism(s)	Enzyme	Fragment size range (kb)[a]	Running conditions[b]
Acinetobacter spp.	*Sma*I	50–250	C
Citrobacter spp.	*Spe*I	50–300	B/C
Escherichia coli	*Spe*I	50–300	B/C
	*Xba*I	50–400	B
Enterobacter spp.	*Spe*I	50–500	B
	*Not*I	100–700	A
Enterococcus spp.	*Sma*I	<50–250	C
Klebsiella spp.	*Spe*I	50–400	B
Legionella pneumophila	*Sfi*I	100–500	B
	*Asc*I	100–600	A
Moraxella catarrhalis	*Bgl*II	<50–100	C
	*Pme*I	50–250	C
Neisseria meningitidis	*Spe*I	50–250	C
	*Sfi*I	100–350	B/C
Providencia spp.	*Sfi*I	50–450	B
Pseudomonas spp.	*Spe*I	50–500	A
	*Dra*I	50–300	B/C
	*Xba*I	50–250	C
Serratia spp.	*Spe*I	50–450	B
	*Dra*I	<50–100	C
Staphylococcus spp.	*Sma*I	50–400	B
	*Eag*I	50–400	B
Stenotrophomonas spp.	*Xba*I	50–500	B
	*Spe*I	50–400	B
Candida spp.	*Sfi*I	300–800	A
	*BssH*II	100–600	A
	*Eag*I	50–500	A
	Karyotype (uncut)	500–2,000	D

[a] Size range includes the majority of fragments created after digestion with the corresponding enzyme. Some species will have several fragments under 48.5 kbp; however, it is difficult to analyze them when the 48.5-kbp lambda ladder molecular size standard is used. In order to analyze those fragments, use *Hin*d III-cut lambda as a molecular size standard and/or a lower switch time, such as 5 to 15 s.

[b] See Table 12.4.5–2.

Table 12.4.5–2 Examples of running conditions[a]

Running condition	Agarose concn (%)	Switch time(s)		Time (h)	Run (V/cm)	Range of band sizes best separated (kb)	Largest band resolved (kb)
		Initial	Final				
A	1.0	10.0	90	24	6.0	100–650	776.0
B	1.0	5.0	60	23	6.0	50–500	630.5
C	1.0	5.0	30	23	6.0	<50–350	436.5
D	0.7	120	280	48	4.5	500–2,000	2,000

[a] All electrophoretic conditions are at 13°C.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A report of the results of PFGE typing that details which isolates are indistinguishable (clonal), similar (subtypes), or different should be issued to the service that generated the request for typing.

VII. INTERPRETATION

A. Examples of chromosomal-DNA patterns of *S. aureus* and *Stenotrophomonas maltophilia* are shown in Fig. 12.4.5–1 and 12.4.5–2.

B. Strains with identical PFGE patterns are considered to be clonal.

C. Strains with one to three band shifts consistent with a single genetic event (e.g., a point mutation resulting in the loss or gain of a restriction site, an insertion, a deletion, or a chromosomal inversion) are also considered to be clonally related subtypes (Fig. 12.4.5–2).

D. Strains that differ at four or more bands are considered independent strains, although strains with multiple similarities may have a common ancestry.

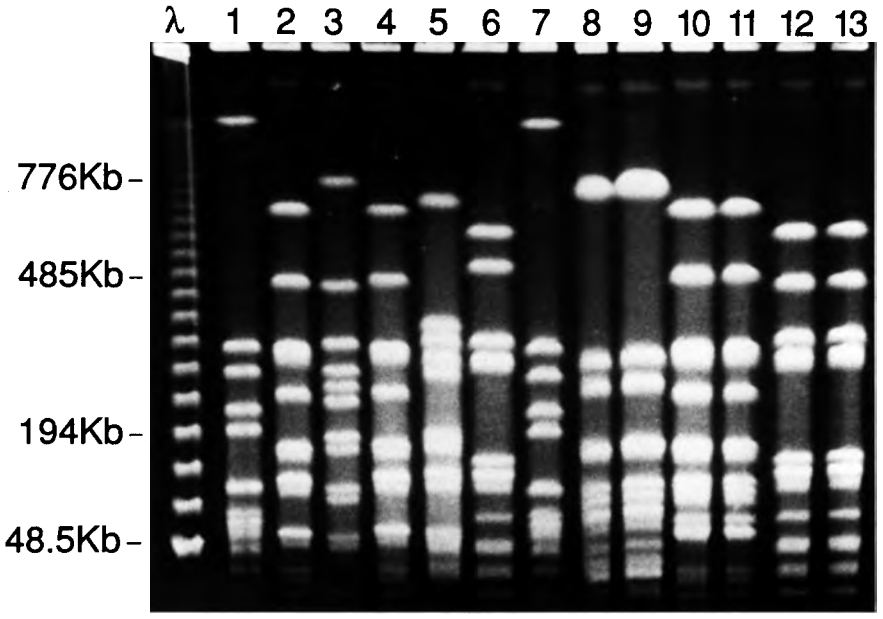

Figure 12.4.5–1 Restriction fragment length polymorphism of *S. aureus* chromosomal DNA digested with *Sma*I. Switch time ramped from 10 to 90 s (running condition A [Table 12.4.5–2]). Lanes 1 through 7 show different patterns. Lanes 8 and 9, 10 and 11, and 12 and 13 represent three pairs of indistinguishable patterns. This photo was kindly provided by Andreas Widmer.

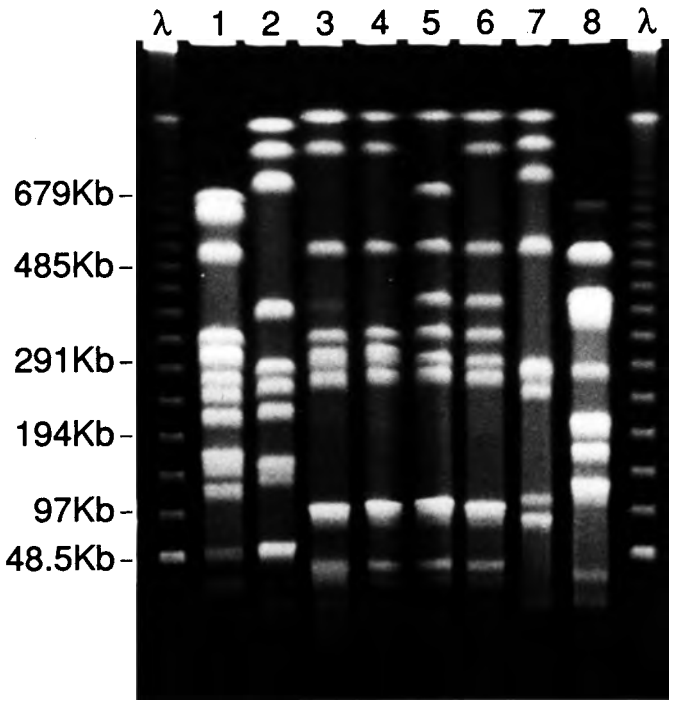

Figure 12.4.5–2 Restriction fragment length polymorphism of *S. maltophilia* chromosomal DNA digested with *Spe*I (running condition A [Table 12.4.5–2). Lanes 3 and 4 show identical patterns, while the patterns in lanes 5 and 6 are similar but not identical and represent subtypes of a single strain.

REFERENCES

1. **CLSI.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. CLSI, Wayne, PA.
2. **Maslow, J. N., A. M. Slutsky, and R. D. Arbeit.** 1993. The application of pulsed-field gel electrophoresis to molecular epidemiology, p. 563–572. *In* D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), *Diagnostic Molecular Microbiology: Principles and Applications.* American Society for Microbiology, Washington, DC.
3. **Pfaller, M. A., R. J. Hollis, and H. S. Sader.** 1994. Chromosomal restriction fragment analysis by pulsed-field gel electrophoresis, p. 10.5.C.1–10.5.C.12. *In*, H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook*, Vol. 2, Supplement 1. American Society for Microbiology, Washington, DC.

SUPPLEMENTAL READING

Birren, B., and E. Lai (ed.). 1993. *Pulsed-Field Gel Electrophoresis: a Practical Guide.* Academic Press, Inc., San Diego, CA.

Goering, R. V. 1993. Molecular epidemiology of nosocomial infection: analysis of chromosomal restriction fragment patterns by pulsed-field gel electrophoresis. *Infect. Control Hosp. Epidemiol.* **14:**595–600.

Maslow, J. N., M. E. Mulligan, and R. D. Arbeit. 1993. Molecular epidemiology: the application of contemporary techniques to typing bacteria. *Clin. Infect. Dis.* **17:**153–164.

Sader, H. S., R. J. Hollis, and M. A. Pfaller. 1995. The use of molecular techniques in the epidemiology and control of infectious diseases. *Clin. Lab. Med.* **15:**407–431.

Soll, D. R., C. Pujol, and S. R. Lockhart. 2007. Laboratory procedures for the epidemiological analysis of microorganisms, p. 129–151. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.

Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* **33:**2233–2239.

12.4.6 Characterization of Pathogenic Microorganisms by Genomic Fingerprinting with Arbitrarily Primed PCR

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Genomic fingerprinting by arbitrarily primed PCR (AP-PCR) is a method for sampling homologous sequences of related pathogens in such a way that small sequence differences are observed. Primers are chosen arbitrarily and annealed (hybridized) to the genome at low stringency (low temperature and high salt concentration). Subsequent PCR results in the amplification of sequences bounded by these low-stringency annealing events. The products are then arrayed by electrophoresis to yield "fingerprints" which differ depending on the relatedness of the genomic templates. The more closely related two genomic templates are, the more similar the resultant fingerprints will be. For very closely related but distinct templates, additional arbitrary primers can be used until a distinction is revealed.

◩ **NOTE:** This procedure is adapted from Welsh and McClelland (2).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Most organisms grown on a nonselective agar or broth medium for 18 to 24 h will yield chromosomal DNA for this procedure.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagents

◩ **NOTE:** Unless otherwise stated, all reagents are available from Sigma Chemical Co., St. Louis, MO.

1. TE buffer: 10 mM Tris (pH 8.0), 1 mM EDTA
2. PK solution: 1 mg of proteinase K per ml, 0.2 M EDTA, 1% *N*-lauroyl sarcosine, 0.1 M Tris (pH 8.0)
3. 10× *Taq* (Mg) buffer: 100 mM Tris (pH 8.3), 500 mM KCl, 40 mM $MgCl_2$
4. AP-PCR master: 500 μl of 10× *Taq* (Mg) buffer, 20 μl of 200 μM oligonucleotide primer, 200 μl of deoxyribonucleoside triphosphates (dNTP) (a 5 mM concentration of each dNTP), 3,730 μl of water, 25 μl of AmpliTaq (5 U/ml) (total volume, 4,500 μl) (New England BioLabs, Beverly, MA).
5. Oligonucleotide primers. The MB universal sequencing and reverse sequencing primers work well, but almost any primer ranging from 10 to 50 nucleotides in length will work (New England BioLabs).
6. Formamide dye: 10 ml of 98% deionized formamide, 10 mg of xylene cyanol, 10 mg of bromophenol blue, 200 μl of 0.5 M EDTA (pH 8.0)
7. Agarose (FMC BioProducts)
8. Ethidium bromide (10 mg/ml)

B. Equipment

1. Power supply
2. Electrophoresis chamber
3. UV transilluminator and photodocumentation equipment
4. 96-well thermal cycler with disposable tubes and racks
5. Multichannel Pipetman

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. A standard control strain of the same species, if available, should be prepared along with the test strains as a control for the DNA isolation and amplification procedures.

B. The use of molecular typing tests to identify related strains is most effective if a group of appropriate epidemiologically unrelated control organisms can be prepared and compared to the isolates in question. Appropriate control strains are as follows.

1. Identical species isolated in the same unit on different dates

2. Identical species isolated on the same date in different units

C. Verify that all materials and reagents meet expiration date and QC parameters per CLSI document M22-A3 (1).

V. PROCEDURE

A. Specimen

Use a fresh colony of the organism to be typed grown on suitable agar medium.

B. DNA sample preparation

1. Grow a 1- to 5-ml culture to late log phase or saturation in a 10-ml culture tube.

2. Pellet cells by low-speed centrifugation (3,000 $\times$ *g* for 10 min).

3. Suspend the cells in 5 ml of TE buffer, and repellet them.

4. Resuspend the cells in 0.2 ml of TE buffer and add 0.2 ml of PK solution.

5. Incubate the mixture at 50°C for 1 h to overnight, until the solution is clear.

6. Add 3 ml of TE buffer, and transfer 0.4 ml to a 1.5-ml centrifuge tube. Add 0.4 ml of phenol, vortex, and centrifuge briefly to separate the phases. Recover and save the aqueous phase. Add 0.4 ml of chloroform, vortex, and centrifuge briefly to separate phases. Recover and save the aqueous phase.

7. Add 1/10 volume of 3 M sodium acetate and 2 volumes of ethanol. Chill the mixture at −20°C. Pellet the DNA by centrifugation in a microcentrifuge.

8. Dry the pellet in a vacuum oven at 60°C, and dissolve the DNA in 100 μl of TE buffer.

9. Check the concentration of DNA by running several twofold serial dilutions on a 1% agarose gel and comparing the ethidium bromide fluorescence of the DNA with a molecular mass standard (New England BioLabs).

C. Genomic fingerprinting

◩ **NOTE:** In order to select the optimum range of DNA concentrations to be used for fingerprinting, an initial experiment with multiple dilutions of the template DNA should be performed. Once the optimum range of DNA concentrations has been determined, as few as two dilutions of each DNA can be used.

1. Array the purified DNAs in a "master" 96-well microtiter tray at about 10 ng/μl in each well.

2. Using a multichannel pipette, perform a series of four fivefold dilutions on a representative subset of DNAs in additional 96-well dishes. The final desired DNA concentration for bacteria in the AP-PCR experiment is generally about 1 ng/μl, but this can vary depending on the DNA quality. For this reason, it is usually wise to perform the initial amplification on more than one dilution at a time.

3. In the 96-well array, combine 45 μl of AP-PCR mixture with 5 μl of DNA dilution stocks of 10, 2, 0.4, 0.08, and 0.016 ng/μl from step V.C.2.

4. Perform the amplification as follows: two cycles of 5 min at 94°C for template denaturation, 5 min at 40°C for primer annealing, and 5 min at 72°C for primer extension, followed by 40 cycles of 1 min at 94°C for template

V. PROCEDURE *(continued)*

denaturation, 1 min at 40°C for primer annealing, and 2 min at 72°C for primer extension.

5. Separate the amplification products by electrophoresis in 1.5% agarose at 100 V for 4 h. Stain the gel with ethidium bromide (0.5 μg/ml in water) and visualize under UV light.
6. After this initial experiment, choose two dilutions that give the most robust and informative fingerprints and use them for all subsequent experiments, allowing 48 strains to be examined at one time.

D. Procedure notes

1. In AP-PCR even slight variations in the pH or ionic strength of the buffers used, the temperature of the reaction, or the source of the DNA polymerase may result in wide variations in the intensities of the individual amplicons.
2. The reproducibility and discriminatory power of each primer and amplification protocol must be validated by analyzing sets of isolates that have been previously well defined by epidemiologic data and/or independent typing studies.
3. The most reliable epidemiologic results are obtained when a set of isolates is tested in a single amplification reaction and analyzed on a single electrophoretic gel.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A report of the results of AP-PCR typing that details which isolates are indistinguishable (clonal), similar (subtypes), or different should be issued to the service that generated the request for typing.

VII. INTERPRETATION

A. Standard guidelines for interpretation of AP-PCR are not yet available, and the interpretation of fingerprinting results obtained with this technique is empirical.

B. Under conditions where variability of amplicon sizes can be demonstrated among epidemiologically unrelated isolates, those showing either no differences or changes only in band intensity can be considered epidemiologically related.

C. AP-PCR patterns with three or more fragment (amplicon) differences may be considered to represent different strains.

D. AP-PCR patterns differing by one or two bands remain difficult to interpret but may be considered to represent isolates which are subtypes of one another and possibly epidemiologically related.

REFERENCES

1. **CLSI.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. CLSI, Wayne, PA.
2. **Welsh, J., and M. McClelland.** 1993. Characterization of pathogenic microorganisms by genomic fingerprinting using arbitrarily primed PCR, p. 595–602. *In* D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), *Diagnostic Molecular Microbiology: Principles and Applications.* American Society for Microbiology, Washington, DC.

SUPPLEMENTAL READING

van Belkum, A. 1994. DNA fingerprinting of medically important microorganisms by use of PCR. *Clin. Microbiol. Rev.* **7:**174–184.

Welsh, J., and M. McClelland. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **18:**7213–7218.

12.4.7 Genotyping of Hepatitis C Virus by INNO-LiPA HCV II

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The INNO-LiPA HCV II is a line probe assay, for in vitro use, for the genotyping of hepatitis C virus (HCV) in human serum and plasma. This test allows the genotyping of the six major HCV types and their most common subtypes. The assay is based on variations found in the 5′ untranslated regions (5′ UR) of the different HCV genotypes. Type-specific probes are tailed with a poly(T) tail by terminal deoxynucleotidyl transferase and attached to nitrocellulose membranes.

Biotin-labeled amplified products are reversibly hybridized to the probes on the strips. The biotin group is incorporated by employing a 5′-biotinylated primer during amplification. The labeled product obtained from the 5′ UR will hybridize only to a probe (or line) that gives a perfect sequence match, allowing stringent discrimination at the subtype level.

After hybridization, streptavidin labeled with alkaline phosphatase is added and bound to any biotinylated hybrid previously formed. Incubation with BCIP-NBT (bromochloroindolylphosphate-nitroblue tetrazolium) chromogen results in a purple-brown precipitate. A line will occur only when there is a perfect match between the probe and the biotinylated PCR products.

Mutations occurring within the quasispecies pool of sequences from one isolate do not affect the outcome of the genotype.

Because of the sensitivity of the PCR method, it is necessary to limit the potential for contamination by performing each step of the process in a separate area of the laboratory and to dedicate equipment to each of the areas.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

A. Specimen

Amplicons from either the Roche HCV Amplicor (qualitative) or Roche HCV Monitor (quantitative) assay may be used.

B. Specimen storage and handling

Amplicons must be stored at 2 to 8°C after amplification and must be used within 7 days of amplification.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Reagent preparation

1. The hybridization solution and the stringent wash solution should be prewarmed at 50°C. Make sure that all crystals are dissolved.
2. Dilute concentrated rinse solution 1:5 in type I water. Prepare 8.5 ml of diluted solution per strip.
3. Dilute the concentrated conjugate 1:100 with conjugate diluent. Prepare 2 ml per strip plus 2 ml excess. Perform dilution during stringent-wash step.
4. Dilute the concentrated substrate 1:100 in substrate buffer. Prepare 2 ml per strip plus 2 ml excess. Perform dilution during conjugate incubation.

B. Area 3

1. INNO-LiPA HCV II genotyping kit (Innogenetics)
2. Incubation trays (Innogenetics)
3. Water bath, shaking, adjustable to 50 ± 0.5°C
4. Calibrated thermometer
5. Aspiration apparatus
6. Type I water
7. Sterile aerosal barrier pipette tips
8. Plastic tweezers
9. Graduated cylinders

III. MATERIALS *(continued)*

10. Adjustable volume pipette
11. Pipetaide
12. Sterile glass pipettes
13. Timer
14. Vortex mixer
15. Dedicated lab coat
16. Powder-free gloves

C. Reagent storage

1. Kit should be stored isolated from any source of contaminating DNA.
2. Store kit at 2 to 8°C. Do not freeze.
3. Bring all kit components to room temperature before use and return to the refrigerator immediately after use.
4. The vial containing denaturation solution should be closed immediately after use; prolonged exposure of this solution to air leads to a rapid deterioration of the denaturing strength.
5. Conjugate and substrate solutions are stable for 24 h at room temperature when stored in the dark.

D. Warnings and precautions

1. Conjugate, conjugate diluent, substrate buffer, and rinse solution contain sodium azide. These solutions are harmful when ingested. Thoroughly flush drains with water after disposal of solutions containing sodium azides.
2. BCIP-NBT substrate contains dimethylformamide and may harm the fetus. This solution is harmful when inhaled or ingested or when skin contact occurs, and it is irritating to the eyes.
BCIP-NBT solution should be removed by the health protection office as chemical hazardous waste.
3. Denaturing solution contains sodium hydroxide and is irritating to the eyes and skin.

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. It is required to include one negative control each time a test is performed. Use the negative control amplicon from the Roche amplification of the same genotyping specimen run. The negative control should show no apparent signal for any of the lines on the strip, except for the conjugate control line.
B. A positive control should be included with each run to help evaluate the reproducibility of the genotyping runs. Use one of the positive control amplicons from the Roche amplification of the same genotyping specimen run.
C. The uppermost line on the strip is the marker line. This allows correct orientation of the strip.
D. The second line controls the addition of reactive conjugate and substrate solution during the detection procedure. This line should always be positive and should have approximately the same intensity on each strip in the same test run.
E. Color intensities between lines on a strip may differ from one line to the other.

V. PROCEDURE

Observe standard precautions.

A. Hybridization

1. Check that the temperature of the water bath is 50 ± 0.5°C. Keep the lid closed at all times.
2. Fill out an HCV genotyping worksheet. Record lot number and expiration date of kit used. Record date of amplicon amplification and whether it was a qualitative or quantitative run.
3. Prewarm the hybridization solution in the water bath. Mix before use and ensure that all crystals are dissolved.
4. Using a Kimwipe wetted in type II water, wipe the surface of the tray to decrease static electricity.
5. Using tweezers, remove the required number of strips from the tube and with a pencil label each strip with a sequential number above the black strip. Place strips individually into the bottom of a trough on the tray.

V. PROCEDURE *(continued)*

6. Pipette 20 µl of denatured amplicon into the upper corner of the trough corresponding to each control and patient.
7. Mix the prewarmed hybridization solution. Using the Pipetaide and sterile glass pipette, add 2 ml of hybridization solution to each trough with a strip. Be careful not to touch the tip of the pipette to the trough. Gently mix. Be careful not to splash reagents from one trough to another.
8. Ensure that each strip is completely submerged.
9. Place the tray in the metal tray in the 50 ± 0.5°C water bath. Close the lid and start the bath shaking. Incubate for 60 min.
10. Prewarm the stringent wash solution at 50 ± 0.5°C during hybridization.

B. Stringent wash

1. Remove the tray from the water bath.
2. Hold the tray at an angle and aspirate the liquid from each trough with a pipette.
3. Add 2 ml of prewarmed stringent wash solution into each trough.
4. Rinse by rocking the tray for 10 to 20 s at room temperature (20 to 25°C).
5. Aspirate the solution from each trough as in step V.B.2.
6. Repeat the washing step in step V.B.3.
7. Incubate each strip in 2 ml of prewarmed stringent wash solution in the shaking water bath at 50 ± 0.5°C for 30 min.
   Be sure to check temperature in water bath before incubation.
8. Dilute the concentrated rinse solution and conjugate during the stringent wash. See Tables 12.4.7–1 and 12.4.7–2 for volume preparation.

Table 12.4.7–1 Rinse solution preparation

No. of strips	Vol (ml) of:		Total vol (ml)
	Type I water	RS concentrate	
1	6.8	1.7	8.5
2	13.6	3.4	17
3	20.4	5.1	25.5
4	27.2	6.8	34
5	34	8.5	42.5
6	40.8	10.2	51
7	47.6	11.9	59.5
8	54.4	13.6	68
9	61.2	15.33	76.5
10	68	17	85

Table 12.4.7–2 Conjugate and substrate preparation

No. of strips	Vol of:		Total vol (ml)
	SB buffer or conjugate diluent (ml)	Substrate or conjugate (µl)	
1	3.96	40	4
2	5.94	60	6
3	7.92	80	8
4	9.9	100	10
5	11.88	120	12
6	13.86	140	14
7	15.84	160	16
8	17.82	180	18
9	19.8	200	20
10	21.78	220	22

V. PROCEDURE *(continued)*

C. Color development

All subsequent incubations are carried out at 20 to 25°C on the dry half of the shaking water bath. Ensure that the strips move back and forth in the trough for homogenous staining.

1. Remove the tray from the water bath.
2. Hold the tray at an angle and aspirate the liquid from each trough with a pipette.
3. Add 2 ml of dilute rinse solution into each trough.
4. Rinse by rocking the tray for 1 min at room temperature (20 to 25°C).
5. Aspirate the solution from each trough as in step V.B.2.
6. Repeat the washing step in steps V.B.3 to 5.
7. Add 2 ml of the diluted conjugate to each trough and incubate for 30 min while agitating the tray on the shaker. Cover shaker with lid covered in foil.
8. Dilute the substrate for ~10 min prior to the end of the conjugate incubation.
9. Wash each strip as in steps V.B.2 to 6 above.
10. Add 2 ml of substrate buffer to each trough and repeat steps V.B.4 and 5 above.
11. Add 2 ml of the prepared substrate solution to each trough and incubate at room temperature for 30 min while agitating the tray in the shaker. Cover the shaker with the lid covered in aluminum foil.
12. Stop the color development by washing the strips twice in 2 ml of type I water while agitating the tray for at least 3 min. Discard waste in formamide waste bottle.
13. Using tweezers, remove the strips from the troughs and place on absorbent paper. Place strips on HCV genotyping worksheet in proper order, with the uppermost line on the strip placed over the marking strip on the worksheet, and tape into place.
14. Allow the strips to dry completely before reading the results. Store developed strips in the dark.

D. Procedure notes

1. Incubation temperature of 50 ± 0.5°C during hybridization and stringent wash is critical.
2. Always close the lid of the water bath during incubation to avoid false positives. Covering the troughs with microplate sealers may cause cross-contamination.
3. The amplitude of motion generated by the shaker is critical. The amplitude should be as high as possible without spilling (setting 5).
4. Adjust the level of the water in the bath between one-third and one-half of the height of the trough.
5. Aspirate the liquid from the trough with a pipette attached to a vacuum aspirator. Hold the tray at an angle to allow all liquid to flow to one end of the trough.
6. Do not allow strips to dry between the washing steps.
7. Do not damage the surfaces of the strips when aspirating. Aspirate the liquid at the top of the strip above the marker line.
8. Make sure the entire strip is washed by immersion in the solution.
9. Do not touch strips with bare hands; use clean tweezers that are dedicated to this procedure.
10. Make sure test strips are placed in the tray with their coated membrane side up (marked side).
11. Throughout the test, the strips should remain in the same trough.

V. PROCEDURE *(continued)*

12. All hybridization and stringent-wash steps should be performed using the rotating shaker. The shaking of the solution over the strips is important in achieving maximum sensitivity.

13. Developed strips should be stored in the dark at room temperature.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A report of the results of HCV genotyping that specifies the genotype of the infecting virus should be issued to the service that generated the request for typing.

VII. INTERPRETATION

A. The second line on the strip is the control for the addition of reactive conjugate and substrate solution during the detection procedure. This line should always be positive and should have the same intensity on each strip in the same run.

B. If this strip is not positive or there is unequal intensity on the strips, fill out a Quality Control Failure/Problem form and notify the lead scientist.

C. The assay result of each negative control should be no apparent signal for any of the lines on the strip, except for the conjugate control line.

D. If any lines appear positive except for the conjugate control line, fill out a Quality Control Failure/Problem form and notify the lead scientist.

E. The assay result of each positive control should be consistent with the genotype of the control used. The strip pattern should be consistent between runs of the same control lot.

F. Collect all line numbers which are positive on the INNO-LiPA HCV II strip and deduce the genotype by using the interpretation chart.

SUPPLEMENTAL READING

Holland, J., I. Bastian, and R.M. Ratcliff. 1998. Hepatitis C genotyping by direct sequencing of the product from the Roche Amplicon test: methodology and application to a South Australian population. *Pathology* **30:**192–195.

Murphy, D., B. Willems, and G. Delage. 1994. Use of the 5′ noncoding region for genotyping hepatitis C virus. *J. Infect. Dis.* **169:**473–475.

Stuyver, L., A. Wyseur, W. van Arnhem, F. Hernandez, and G. Maertens. 1996. Second-generation line probe assay for hepatitis C virus genotyping. *J. Clin. Microbiol.* **34:**2259–2266.

Zein, N. N. 2000. Clinical significance of hepatitis C virus genotypes. *Clin. Microbiol. Rev.* **13:**223–235.

12.5.1 Introduction

Antimicrobial agent resistance is an increasing problem worldwide, particularly among critically ill hospitalized patients. For this reason, there is a renewed interest in monitoring the development and spread of antimicrobial agent resistance and a recognition of the need for effective interventions to limit the spread of resistance to prolong the therapeutic life of the available antimicrobial agents. Unfortunately, conventional methods to perform antimicrobial agent susceptibility testing may be too slow and insensitive in detecting antimicrobial agent resistance to be of much use clinically. The techniques of molecular biology have been used to characterize resistance at the DNA level and may provide rapid, sensitive, and specific information to the clinician for use in therapeutic decision making (1–4). Genetic material that confers antimicrobial agent resistance may be carried on the bacterial chromosome or on transposons or plasmids and has been detected by probe hybridization or by DNA amplification with PCR (Table 12.1–6). Molecular detection of resistance has potential value for decisions directly related to patient care and is useful for calibration of conventional susceptibility tests and for precise definition of the mechanisms of resistance to selected antimicrobial agents. Molecular techniques have been used to detect genes encoding several different mechanisms of resistance against antimicrobial agents, e.g., β-lactam agents, aminoglycosides, macrolides, and fluoroquinolones, after isolation of a clinical isolate (Table 12.1–6).

REFERENCES

1. **Bergeron, M. G., and M. Oullette.** 1998. Preventing antibiotic resistance using rapid DNA-based diagnostic tests. *Infect. Control Hosp. Epidemiol.* **19:**560–564.
2. **Cockerill, F. R., III.** 1999. Genetic methods for assessing antimicrobial resistance. *Antimicrob. Agents Chemother.* **43:**199–212.
3. **Persing, D. H., D. A. Relman, and F. C. Tenover.** 1996. Genotypic detection of antimicrobial resistance, p. 33–57. *In* D. H. Persing (ed.), *PCR Protocols for Emerging Infectious Diseases.* ASM Press, Washington, DC.
4. **Rasheed, J. K., F. Cockerill, and F. C. Tenover.** 2007. Detection and characterization of antimicrobial resistance genes in pathogenic bacteria, p. 1248–1251. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.

12.5.2 Detection of Enterococcal Vancomycin Resistance by Multiplex PCR

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The emergence of vancomycin resistance among enterococci is considered one of the major antimicrobial resistance threats worldwide. The expression of vancomycin resistance involves the complex interaction of several genes, but classification of resistance is based on the altered ligase (an enzyme involved in the D-alanine branch of peptidoglycan synthesis) which is central to the production of cell wall precursors with decreased affinity for vancomycin (Table 12.5.2–1). Currently, the ligase designations include VanA (*vanA*), VanB (*vanB*), and VanC1 to -3 (*vanC-1, vanC-2, vanC-3*). All five Van types may be encountered clinically, but resistance encoded by *vanA* or *vanB* presents the greatest infection control and therapeutic threat because it is genetically transferable, results in high-level vancomycin resistance (MICs, ≥32 μg/ml), and occurs in the two most common enterococcal species (*Enterococcus faecalis* and *Enterococcus faecium*), both of which are frequently resistant to multiple agents, including penicillins and aminoglycosides. Although presumptive identification of the mechanism of vancomycin resistance may be inferred on the basis of phenotypic criteria, the only definitive method for establishing the mechanism of resistance is detection of the ligase gene(s). PCR provides an ideal molecular tool for definitively establishing the mechanism of vancomycin resistance in clinical isolates of enterococci. In the present protocol, a PCR procedure that uses primers internal to each of the known *van* genes is described. This method may be used to arbitrate equivocal susceptibility results obtained by phenotypic methods, to characterize "outbreak" strains, and to evaluate the accuracy of various phenotypic susceptibility testing methods.

◩ **NOTE:** This procedure is adapted from that of Free and Sahm (3).

Work flow in the laboratory must proceed in a unidirectional manner, beginning in the reagent preparation area and moving to the specimen preparation area and then to the amplification and detection area. Preamplification activities must begin with reagent preparation and proceed to specimen preparation. Reagent preparation activities and specimen preparation activities must be performed in separate, segregated areas. Supplies and equipment must be dedicated to each activity and not used for other activities or moved between areas. Lab coats and gloves must be worn in each area and must be changed before leaving that area. Equipment and supplies used for reagent preparation must not be used for specimen preparation activities or for pipetting or processing amplified DNA or other sources of target DNA. Amplification and detection supplies and equipment must be confined to the amplification and detection area at all times.

Table 12.5.2–1 PCR primers for detection of *vanA, vanB, vanC-1,* and *vanC-2* in enterococci

Target gene[a]	Primer designation	Nucleotide sequence, 5′–3′[b]	Product size (bp)
vanA	VanA1	GCT ATT CAG CTG TAC TC	783
	VanA2	CAG CGG CCA TCA TAC GG	
vanB	VanB1	CAT CGC CGT CCC CGA ATT TCA AA	297
	VanB2	GAT GCG GAA GAT ACC GTG GCT	
vanC-1	VanC1-1	GGT ATC AAG GAA ACC TC	822
	VanC1-2	CTT CCG CCA TCA TAG CT	
vanC-2	VanC2-1	CTC CTA CGA TTC TCT TG	439
	VanC2-2	CGA GCA AGA CCT TTA AG	

[a] GenBank accession numbers: *vanA,* X56895; *vanB,* U00456; *vanC-1,* M75132; *vanC-2,* L29638

[b] Primer sequences for VanC1-1, VanC1-2, VanC2-1, and VanC2-2 from Dutka-Malen et al. (2).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Test growth of *Enterococcus* spp. for which the vancomycin MIC is elevated (≥8.0 μg/ml) or which show growth on an agar screen plate from appropriate solid medium. Select two to four well-isolated colonies from pure culture. The optimal colony age is 18 to 48 h; substantially older colonies have been used successfully.

III. MATERIALS

Include QC information on reagent container and in QC records.

◩ **NOTE:** Unless otherwise stated, all materials are available from Sigma Chemical Co., St. Louis, MO.

A. Reagents

1. Agarose (type 1-A, low electroendosmosis)
2. Autoclaved Tris-borate-EDTA (TBE) buffer (pH 8.3 to 8.5)
3. 6× Gel electrophoresis loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol in H_2O)
4. Sterile distilled H_2O
5. Ethidium bromide (0.5 μg/ml of H_2O)
6. Autoclaved light mineral oil
7. PCR master mix
   - **a.** 10× PCR buffer (100 mM Tris-HCl [pH 9], 500 mM KCl, 1% Triton X-100 [Promega, Madison, WI])
   - **b.** 10 mM concentration of each deoxyribonucleoside triphosphate
   - **c.** 25 μM concentration of each primer (primer designations, primer sequences, and amplicon sizes given in Table 12.5.2–1)
   - **d.** 25 mM $MgCl_2$
   - **e.** 5,000 U of *Taq* (Promega) per ml
8. Size marker, ϕX174 phage DNA *Hae*III digest (Promega), diluted 1:40 with 6× electrophoresis buffer

B. Supplies

1. Sterile 0.5-ml snap-cap PCR thermal reactor tubes
2. Sterile, plugged micropipette tips (10, 200, and 1,000 μl)
3. Sterile bacteriologic transfer needles or sterile toothpicks

C. Equipment

1. Vortex mixer
2. Thermal cycler
3. Electrophoresis gel box
4. Electrophoresis power supply
5. UV transilluminator and photodocumentation equipment

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Positive controls

1. *vanA*, *E. faecium* 228
2. *vanB*, *E. faecalis* V583 or *E. faecalis* ATCC 51299
3. *vanC-1*, *E. gallinarum* AIB 39
4. *vanC-2*, *E. casseliflavus* ATCC 25788

B. Negative control, *E. faecalis* ATCC 29212

C. Run appropriate positive and negative controls with each test procedure.

D. Verify that all materials and reagents meet expiration date and QC parameters per CLSI document M22-A3 (1).

V. PROCEDURE

Observe standard precautions.

A. Preparation of PCR master mix for *vanA* and *vanB* detection (prepare in area 1: reagent preparation area)

1. Volumes based on 99-μl reaction mixture.
   ◩ **NOTE:** Overestimating the final required volume by one or two reactions allows for volume loss and pipetting errors and is recommended.
2. Using sterile technique add the following volumes of each reaction component to each PCR thermal reactor tube.
   - **a.** 52.5 μl of distilled H_2O
   - **b.** 10 μl of 10× PCR buffer

V. PROCEDURE *(continued)*

c. 2 μl of each of the following
(1) Deoxynucleoside triphosphate stock (10 mM) (total volume, 8 μl)
(2) *vanA-1* primer stock (25 μM)
(3) *vanA-2* primer stock (25 μM)
(4) *vanB-1* primer stock (25 μM)
(5) *vanB-2* primer stock (25 μM)

d. 20 μl of 25 mM $MgCl_2$ stock

e. 0.5 μl of 5,000-U/ml *Taq* stock

◩ **NOTE:** If the *vanC-1* and *vanC-2* genes are of interest, their respective primers would replace those of *vanA* and *vanB* at the same volume and concentration.

3. Use the vortex mixer to gently but thoroughly mix the contents.

4. Transfer 99 μl of mixture to each reaction tube.

B. Sample preparation: preparation of DNA template (prepare in area 2: specimen preparation area)

1. Use a sterile toothpick or inoculating needle to select two to four well-isolated colonies from pure culture (18 to 48 h old).

2. Emulsify colonies in PCR mixture.

3. Using aseptic technique, gently overlay each tube with 75 μl of sterile mineral oil (not necessary if self-sealing PCR tubes are used).

C. PCR amplification (area 3: amplification and detection area)

◩ **NOTE:** Have the thermocycler on so that cycling can begin as soon as the tubes are ready. Amplify the target region of *van* genes in a programmed thermocycler as follows.

1. 1.3-min hot start at 95°C (bacterial lysis and release of target DNA)

2. 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 1 min

◩ **NOTE:** If not able to perform detection of PCR product soon after completion of this cycle, program the machine to hold at 4°C or place reaction mixture in a refrigerator or freezer until needed for electrophoretic analysis.

D. Electrophoretic detection of PCR products (area 3: amplification and detection area)

1. Prepare a 1.5% agarose gel in 1× TBE buffer.

2. Remove 8.3 μl of reaction mixture from below the mineral oil layer, combine with 1.7 μl of 6× loading buffer, and mix by pipetting.

3. Prepare size marker by adding 0.5 μl of marker to 7.5 μl of H_2O and 2 μl of 6× loading buffer.

4. Apply a total of 10 μl of sample to each well, and perform electrophoresis (70 V for 50 min) in 1× TBE buffer containing ethidium bromide (0.5 μg/ml).

5. Visualize the gel under UV light and photograph with Polaroid type 667 film.

◩ **NOTE:** The gel may be destained in H_2O (approximately 30 min) to optimize visualization of results.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

Report the results of the PCR procedure detailing the specific vancomycin resistance genotype detected. If negative, report "vancomycin resistance genes *vanA*, *-B*, and *-C* not detected."

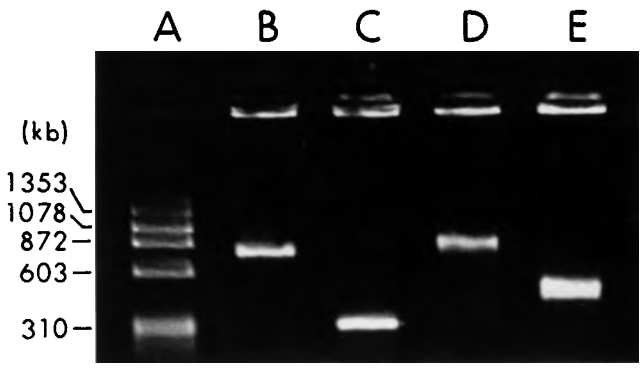

Figure 12.5.2–1 Agarose gel electrophoresis pattern of *van* PCR products. Lanes: A, size marker ϕX174 phage DNA *Hae*III digest; B, *vanA* product (783 bp); C, *vanB* product (297 bp); D, *vanC-1* product (822 bp); E, *vanC-2* product (439 bp). PCR products for *vanA* and *vanB* resulted from the reaction described above; *vanC-1* and *vanC-2* products were obtained with single primer sets per reaction mixture. Figure from Free and Sahm (3).

VII. INTERPRETATION

A. Amplification of a 783- or 297-bp product indicates the presence of *vanA* or *vanB,* respectively (Fig. 12.5.2–1). Either result establishes the presence of acquired and transferable vancomycin resistance and confirms the need to initiate appropriate infection control and patient management measures.

B. If no PCR product is observed with a strain that appeared nonsusceptible by a phenotype-based method and if appropriate results are obtained with control strains (*see* below), the isolate probably contains a *vanC* gene. This can be confirmed by using the *vanC-1* and *vanC-2* primers, which yield 822 and 439-bp amplification products, respectively (Fig. 12.5.2–1).

REFERENCES

1. **CLSI.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 3rd ed. Approved standard M22-A3. CLSI, Wayne, PA.
2. **Dutka-Malen, S., S. Evers, and P. Courvalin.** 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J. Clin. Microbiol.* **33:**24–27.
3. **Free, L., and D. F. Sahm.** 1996. Detection of enterococcal vancomycin resistance by multiplex PCR, p. 150–155. *In* D. H. Persing (ed.), *PCR Protocols for Emerging Infectious Diseases.* ASM Press, Washington, DC.

12.5.3 Detection of Methicillin Resistance in Staphylococci by PCR

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The primary mechanism of resistance to methicillin and related compounds (oxacillin, nafcillin) in both *Staphylococcus aureus* and coagulase-negative staphylococci is the production of a unique penicillin binding protein, termed PBP2a or PBP2′, that has a low affinity for β-lactam antibiotics. The gene encoding PBP2a, known as *mecA*, has been cloned and sequenced. The phenotypic expression of *mecA* is often heterogeneous and difficult to detect in many antimicrobial susceptibility testing systems. An alternative method for identifying methicillin-resistant strains of staphylococci is to detect the *mecA* gene, which is absent in susceptible strains. Detection of *mecA* in staphylococci has been accomplished by both PCR and DNA probe methods. In the present protocol, a PCR procedure that uses oligonucleotide primers to amplify a 533-bp region of *mecA* is described. This method is applicable to both *S. aureus* and coagulase-negative staphylococci and may be used to rapidly identify methicillin-resistant strains for clinical purposes and to aid in the development of phenotypic methods that can more accurately predict methicillin resistance.

Work flow in the laboratory must proceed in a unidirectional manner, beginning in the reagent preparation area and moving to the specimen preparation area and then to the amplification and detection area. Preamplification activities must begin with reagent preparation and proceed to specimen preparation. Reagent preparation activities and specimen preparation activities must be performed in separate, segregated areas. Supplies and equipment must be dedicated to each activity and not used for other activities or moved between areas. Lab coats and gloves must be worn in each area and must be changed before leaving that area. Equipment and supplies used for reagent preparation must not be used for specimen preparation activities or for pipetting or processing amplified DNA or other sources of target DNA. Amplification and detection supplies and equipment must be confined to the amplification and detection area at all times.

◩ **NOTE:** This procedure is adapted from that of Murakami and Minamide (2).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Test growth of *S. aureus* or coagulase-negative staphylococci from Mueller-Hinton agar. Select several colonies from pure culture. The optimal colony age is 18 to 24 h.

III. MATERIALS

Include QC information on reagent container and in QC records.

◩ **NOTE:** Unless otherwise specified, all materials are available from Sigma Chemical Co., St. Louis, MO.

A. Reagents

1. Agarose, standard low M_r (Bio-Rad Laboratories, Richmond, CA)
2. Autoclaved Tris buffer (TE) (10 mM Tris-HCl [pH 8.0], 1 mM EDTA)
3. Electrophoresis loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol in 1× TBE [Tris-borate-EDTA] buffer).
4. Sterile distilled H_2O
5. Ethidium bromide (0.5 μg/ml of H_2O)
6. Autoclaved light mineral oil
7. PCR master mix (GeneAmp PCR reagent kit; Perkin-Elmer Corp., Norwalk, CT)
   - **a.** PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3])
   - **b.** $MgCl_2$ (1.5 mM)
   - **c.** 0.01% Gelatin
   - **d.** Deoxyribonucleoside triphosphates (200 mM each)
   - **e.** AmpliTaq DNA polymerase (0.025 per μl)

III. MATERIALS *(continued)*

f. Primers RSM-2647 and RSM-2648 (each at 0.25 μM [final concentration]) (primer sequences given in Table 12.5.3–1)

g. Size marker, ϕX174 phage DNA *Hae*III digest (Nippon Gene, Toyama, Japan); add 1 volume of electrophoresis loading buffer to 5 volumes of the digest to give a DNA concentration of 40 ng/μl.

8. Achromopeptidase solution: 20,000 U of purified achromopeptidase (Wako BioProducts, Richmond, VA) per ml of TE

B. Supplies

1. Sterile 0.5-ml snap cap PCR thermal reactor tubes

2. Sterile, plugged micropipette tips (10, 200, and 1,000 μl)

3. Sterile bacteriologic transfer needles or sterile toothpicks

4. 0.5 McFarland turbidity standard

5. Microcentrifuge tubes (1.5 ml)

6. Glass test tubes (12 by 75 mm)

C. Equipment

1. Vortex mixer

2. Thermal cycler

3. Electrophoresis gel box

4. Electrophoresis power supply

5. Microcentrifuge

6. Heating block (55 and 100°C)

7. UV transilluminator and photodocumentation equipment

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Positive control, *S. aureus* ATCC 43300

B. Negative control, *S. aureus* ATCC 25923

C. Run positive and negative controls with each test procedure.

D. Verify that all materials and reagents meet expiration date and QC parameters per CLSI document M22-A3 (1).

V. PROCEDURE

Observe standard precautions.

A. Area 2: preparation of template DNA

1. Use a sterile toothpick or inoculating needle to select several well-isolated colonies from pure culture.

2. Emulsify the bacteria in 1 ml of TE by vigorous vortexing to give a turbidity equal to a 0.5 McFarland standard (approximately 1.5×10^8 CFU/ml). Make sure that the bacteria are dispersed well in the TE.

◩ **NOTE:** Too dense a bacterial suspension may give a false-negative result.

3. Transfer 100 μl of the bacterial suspension to a 1.5-ml microcentrifuge tube containing 2 μl of achromopeptidase solution. Vortex and place the tube in a 55°C heating block for 30 min.

4. Add 2.5 μl of 4% sodium dodecyl sulfate (SDS; Polysciences Inc., Warrington, Pa.) to the digest.

◩ **NOTE:** Some brands of SDS inhibit PCR. Vortex and place tube in the 100°C heating block for 5 min. Centrifuge at 10,000 × *g* for 5 min, and use 5 μl of the bacterial lysate for PCR.

B. Area 3: PCR amplification

1. Transfer 95 μl of PCR master mix to a 0.5-ml microcentrifuge tube. Add 5 μl of template DNA to the master mix and mix well by vortexing.

2. Add 2 or 3 drops (about 100 μl) of mineral oil to the reaction mixture to prevent evaporation.

Table 12.5.3–1 PCR primers for detection of *mecA* in staphylococci

Function	Name	Nucleotide sequence, 5′–3′
Primer	RSM-2647	AAA ATC GAT GGT AAA GGT TGG C
Primer	RSM-2648	AGT TCT GCA GTA CCG GAT TTG C

V. PROCEDURE *(continued)*

3. Add a drop of mineral oil to the tube holder of the thermal cycler for efficient heat transmission, and place the tube in the tube holder.
4. Amplify a target region of *mecA* for 40 cycles in a programmed thermal cycler as follows: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min.

C. Area 3: electrophoretic detection of the 533-bp PCR product

1. Prepare a 2% agarose minigel in 1× TBE buffer for electrophoresis.
2. Mix 10 μl of PCR product with 2 μl of loading buffer in a 1.5-ml microcentrifuge tube. Vortex. Apply 6 μl of the sample or size marker to each well, and perform electrophoresis in 1× TBE buffer (100 V for 20 to 30 min). Also apply positive and negative controls to each gel.
3. Stain the gel by gentle shaking in ethidium bromide solution until the 310-bp size marker is visible.
4. Visualize the gel under UV light and photograph with Polaroid type 667 film.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

Report the results of the PCR procedure detailing the presence or absence of the *mecA* gene. Specify resistance to methicillin and all other β-lactam agents if *mecA* positive and susceptibility to methicillin, oxacillin, and nafcillin if *mecA* negative.

VII. INTERPRETATION

A. Amplification of a 533-bp region of *mecA* indicates that the strain tested is methicillin resistant.

B. A few *mecA*-positive strains appear phenotypically susceptible to β-lactam antibiotics because of their inability to express PBP2′. Since these strains have the potential to become truly resistant, they should be regarded as cryptically methicillin-resistant strains.

C. Use of bacterial suspension of too high a density for preparation of template DNA may lead to false-negative results. A bacterial suspension of as few as 2×10^5 CFU/ml for preparation of template DNA is enough to detect *mecA*.

D. Methicillin-resistant strains of coagulase-negative staphylococci can also be detected by this method. Species in which *mecA* has been detected using this procedure include *S. epidermidis, S. haemolyticus, S. sciuri, S. saprophyticus, S. hominis, S. capitis,* and *S. caprae.*

REFERENCES

1. **CLSI.** 2004. *Quality Assurance for Commercially Prepared Microbiological Culture Media*, 3rd ed. Approved standard M22-A3. CLSI, Wayne, PA.
2. **Murakami, K., and W. Minamide.** 1993. PCR identification of methicillin-resistant *Staphylococcus aureus*, p. 539–542. *In* D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), *Diagnostic Molecular Microbiology: Principles and Applications.* American Society for Microbiology, Washington, DC.

SUPPLEMENTAL READING

Kolbert, C. P., J. Arruda, P. Varga-Delmore, X. Zheng, M. Lewis, J. Kolberg, and D. H. Persing. 1998. Branched-DNA assay for detection of the *mecA* gene in oxacillin-resistant and oxacillin-sensitive staphylococci. *J. Clin. Microbiol.* **36:**2640–2644.

Marshall, S. A., W. W. Wilke, M. A. Pfaller, and R. N. Jones. 1998. *Staphylococcus aureus* and coagulase-negative staphylococci from blood stream infections: frequency of occurrence, antimicrobial susceptibility, and molecular (*mecA*) characterization of oxacillin resistance in the SCOPE program. *Diagn. Microbiol. Infect. Dis.* **30:**205–214.

Ramotar, K., M. Bobrowska, P. Jessamine, and B. Toye. 1998. Detection of methicillin resistance in coagulase-negative staphylococci initially reported as methicillin susceptible using automated methods. *Diagn. Microbiol. Infect. Dis.* **30:**267–273.

12.5.4

Detection of the *bla*$_{KPC}$ Gene Encoding *Klebsiella pneumoniae* Carbapenemase by Real-Time PCR

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

In the United States, the most common mechanism of carbapenem resistance is the production of a *Klebsiella pneumoniae* carbapenemase (KPC), an Ambler class A β-lactamase that confers resistance to all β-lactam agents, including carbapenems. KPCs are plasmid mediated and carried on a Tn*4401* transposon element, making their ease of mobility a significant public health concern. Clinical laboratories can implement phenotypic tests, including the modified Hodge test, to screen for carbapenemase-producing isolates. However, these phenotypic tests can be difficult to interpret. PCR can be utilized to detect the presence of specific resistance genes, such as *bla*$_{KPC}$. This method produces clear results and can provide useful epidemiological information. An important limitation of this assay is that isolates may produce carbapenemases other than the KPC enzymes.

This procedure provides instructions for performing real-time PCR detection of *bla*$_{KPC}$ in gram-negative rods using Applied Biosystems' Fast 7500 real-time PCR system (Applied Biosystems, Inc., Foster City, CA). The procedure also utilizes a multiplexed reaction to simultaneously detect the universal bacterial 16S rRNA gene as an endogenous control for validation and subsequent normalization of amplification. This protocol can also be modified as needed to use with other real-time PCR instruments and reagents.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

Use growth of *Enterobacteriaceae* from a trypticase soy agar plate with 5% sheep's blood (TSA). Select several colonies from pure culture. The optimal colony age is 18 to 24 h.

III. MATERIALS

Include QC information on reagent container and in QC records.

A. Supplies and equipment

1. 1-μl Nunc disposable inoculating loops
2. 1,000-, 200-, 20-, and 10-μl pipettes and filter barrier (aerosol-free) pipette tips
3. 0.5- to 0.65-ml microcentrifuge tubes, sterile
4. 0.25-ml microcentrifuge tubes, sterile
5. Fast optical 96-well reaction plate with barcode (0.1 ml), Applied Biosystems P/N 4346906
6. Optical adhesive covers, Applied Biosystems P/N 4311971
7. MicroAmp splash-free 96-well base, Applied Biosystems P/N 4312063
8. Aluminum foil
9. Ice and ice bucket
10. Heating block (needs to reach 99°C for lysis)
11. Desktop microcentrifuge
12. Centrifuge for 96-well plate
13. Applied Biosystems Fast 7500 real-time PCR system

III. MATERIALS *(continued)*

14. Computer with 7500 Fast system software (version 1.4 current)
15. Biological safety cabinet (BSC) and dedicated PCR workstation
16. NanoDrop (UV spectrophotometer) recommended and optional

B. Reagents

1. Sterile reagent-grade type I H_2O
2. QuantiFast probe PCR kit (catalog no. 204254; Qiagen)
$2\times$ QuantiFast probe PCR master mix contains the following.
a. HotStarTaq *Plus* DNA polymerase
b. QuantiFast probe PCR buffer
c. Deoxynucleotide triphosphate mix
d. ROX passive reference dye
3. KPC forward (F) and reverse (R) primers and labeled hydrolysis probe (5′ 6-carboxyfluorescein [FAM] reporter label and 3′ black hole quencher [BHQ]) (Table 12.5.4–1)
4. 16S rRNA F and R primers and labeled hydrolysis probe (5′ Cy5 reporter label and 3′ BHQ) (Table 12.5.4–1)
5. 0.1 N NaOH
6. 0.5 M Tris-HCl, pH 8.0

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL

A. Run controls

At least one positive control, one negative control, and one no-template control (NTC) are included with each run.

1. Positive control: *K. pneumoniae* ATCC BAA-1705
2. Negative control: *K. pneumoniae* ATCC BAA-1706
3. NTC: sterile reagent-grade type I water is used in place of the template DNA

B. Contamination prevention

1. Separate areas for assay setup, specimen preparation and amplification, and detection of amplified products.
2. Follow unidirectional work flow.
3. Use good laboratory techniques and a biosafety cabinet during specimen preparation to avoid contamination between specimens.

V. PROCEDURE

Observe standard precautions.

A. Lysate preparation

1. Perform in extraction room (no primers or probes in this room). Preparation in a biosafety cabinet is ideal. Clean area with freshly prepared 10% bleach. Wipe bleach residue off with 70% alcohol to prevent damage to the surface.
2. Put bottle of sterile reagent-grade type I water on ice to chill. Preheat heating block to 99°C.

Table 12.5.4–1 PCR primers and probe for the detection of KPC

Oligonucleotide designation	Nucleotide sequence, 5′–3′
KPC-F	GGC CGC CGT GCA ATA C
KPC-R	GCC GCC CAA CTC CTT CA
KPC-Probe	FAM-TG ATA ACG CCG CCG CCA ATT TGT-BHQ
16S rRNA-F	TGG AGC ATG TGG TTT AAT TCG A
16S rRNA-R	TGC GGG ACT TAA CCC AAC A
16S rRNA-Probe	Cy5-CA CGA GCT GAC GAC ARC CAT GCA-BHQ[a]

[a] R = A or G.

V. PROCEDURE *(continued)*

3. Pipette 25 μl of sterile reagent-grade type I water into one sterile 0.5-ml microtube for each sample. Label a second 0.5-ml microtube for each sample for use later (will contain supernatant of lysate).
4. Add a loopful (1-μl loop) of organisms from a TSA plate of the test isolates to corresponding labeled microtubes containing 25 μl of sterile water. Also add a loopful (1-μl loop) of positive and negative QC organisms into separate microtubes. Do not add an excessive amount of organisms, since too much DNA could produce a false-negative PCR result.
5. Lightly vortex each tube to homogenize the samples.
6. Add 25 μl of 0.1 N NaOH to each microtube containing the organism and mix well by lightly vortexing.
7. Secure the caps of the microtubes with plastic cap/lid locks; place each tube in a heating block (or water bath) and leave them at 95 to 99°C for 10 min to lyse.
8. After lysing the bacteria, place the tubes directly on ice for 3 to 5 min. Then add 18 μl of 0.5 M Tris-HCl, pH 8.0, solution.
9. Add 400 μl of cold sterile reagent-grade type I water. Mix by inversion three to five times and "flick" tube with finger to mix. DO NOT vortex cell lysates. Centrifuge tubes at 13,000 to 14,000 rpm for 3 min using a desktop microcentrifuge.
10. Remove supernatant (lysate) from each tube and transfer to sterile, labeled 0.5-ml microcentrifuge tubes. Keep on ice until ready to perform the assay, or store at −20°C for longer-term storage.

    ◩ **NOTE:** Ideally, template DNA should not exceed 10 ng/μl. If desired, the concentration of DNA can be measured using a UV spectrophotometer, and appropriate dilutions can then be made.
11. Decontaminate work surfaces with 10% bleach followed by 70% alcohol (ethanol or isopropanol) after use.

B. Master mix preparation

1. Prepare working dilutions of KPC and 16S rRNA primers (10 μM each) and probes (5 μM each) in a PCR workstation. Solutions containing probes are light sensitive and should be wrapped in aluminum foil.
2. Diluted aliquots of primers and probes can be stored at −20°C for up to 1 year. Thaw on ice when ready to perform PCR; leftover reagent aliquots may be refrozen. Keep freeze-thaws to a minimum to prevent reagent degradation.
3. The ABI 7500 Fast instrument and associated computer should be turned on 30 to 60 min before samples are loaded into it.
4. Prepare the required dilutions and master mixes so addition of reagents and specimen lysates to the 96-well plate can be performed in a timely manner. Perform these activities in a PCR clean room, using a PCR workstation containing dedicated equipment.
5. Perform calculations and fill in KPC master P and final master mix worksheets (*see* Appendixes 12.5.4–1 and 12.5.4–2, respectively) and 96-well plate layout for real-time PCR. These worksheets are used to produce two 20-μl reaction mixtures for each sample. Include an additional (phantom) test for every set of 1 to 10 samples to compensate for loss from pipetting.
6. Label a 0.25-ml microtube for each sample.
7. Design the layout of the 96-well plate by filling in each sample, the positive and negative QC templates, and the NTC (each in duplicate). Set up identical plate design on the instrument using the instrument software.
8. Thaw the following reagents and sample lysates by placing on ice (takes about 30 to 60 min)
    a. Primers and probes: KPC primers and probe and 16S rRNA primers and probe

V. PROCEDURE *(continued)*

b. 2× QuantiFast probe PCR master mix*

c. Templates: test lysates and control lysates (positive, negative, and NTC [reagent-grade type I water])

*Store at 2 to 8°C after initial thaw for up to 2 months.

9. In a PCR clean room, prepare the master P mix, including equal parts of each primer and probe as follows.

KPC forward primer (10 μM)	2.5 μl/sample
KPC reverse primer (10 μM)	2.5 μl/sample
KPC probe (5 μM)	2.5 μl/sample
16S rRNA forward primer (10 μM)	2.5 μl/sample
16S rRNA reverse primer (10 μM)	2.5 μl/sample
16S rRNA probe (5 μM)	2.5 μl/sample

Since the probes are light sensitive, any vial containing a probe should be wrapped in aluminum foil.

◩ **NOTE:** It is important to lightly vortex each solution to thoroughly homogenize it before pipetting.

10. In a PCR clean room, prepare the final master mix as follows.

2× QuantiFast probe PCR master mix	25 μl /sample
Master P primer and probe mix	15 μl /sample
Sterile reagent-grade type I H_2O	7.5 μl /sample

Total volume = 47.5 μl, to which 2.5 μl of lysate will be added (this 50-μl mixture will yield two 20-μl PCR mixtures per sample)

◩ **NOTE:** By adding 15 μl of the master P primer and probe mix to each reaction, this is the equivalent of adding 1 μl of each primer (10 μM) and 1 μl of each probe (5 μM) into a single 20-μl PCR mixture. Thus, the end working concentrations of the primers are 500 nM, and those for the probes are 250 nM.

11. Lightly mix the master P solution before adding to the final master mix, but do NOT vortex the 2× QuantiFast probe PCR master mix or the complete PCR mix, as this may inactivate the polymerase. Instead, it is recommended to lightly flick the microcentrifuge tube with your finger or gently invert the tube until the solution is homogenized.

12. Dispense the 47.5 μl of final master mix for each sample in a prelabeled 0.25-ml microcentrifuge tube and then add 2.5 μl of lysate to each (keep tubes in an ice block or ice bath). For the NTC, add 2.5 μl of sterile reagent-grade type I H_2O.

13. Pipette 20 μl from the 50-μl solution (containing lysate and final master mix) for each sample and the three controls into duplicate wells of the 96-well plate corresponding to the positions labeled on the plate layout. Work as efficiently as possible and use minimal lighting to decrease the amount of exposure of the probes to light.

14. When finished pipetting samples, raise the 96-well tray up off the ice block and look at all the wells to verify that liquid is present in the proper well locations.

15. Cover the 96-well tray with an optically clear, adhesive cover and smooth it down using a flat applicator to ensure a tight seal. Remove lateral tabs of plastic adhesive cover by tearing along perforations.

16. If needed, centrifuge the 96-well plate at 1,000 × *g* for approximately 10 to 20 s to get all the liquid into the bottom of the reaction wells (with no bubbles). Be sure you balance the plate with another tray when spinning.

V. PROCEDURE *(continued)*

(If no 96-well tray centrifuge is available, carefully tap the tray to get liquid to bottom of wells.)

17. Load the plate into the ABI 7500 Fast instrument.

18. Place the tray with barcode facing you; A1 position is in upper left corner. Push the tray holder back into the instrument.

C. Software setup instructions for the ABI Fast 7500 real-time PCR assay

1. Create relative quantification (RQ) plate document.
- **a.** Double click on "7500 Fast" icon on the computer for ABI's software.
- **b.** Select Assay.
- **c.** Select Relative Quantification (ddCt) Plate.
- **d.** Accept the default settings for Container and Template (96-well Clear and Blank Document).
- **e.** Run Mode: Fast 7500
- **f.** Enter name in the Plate Name field (e.g., "093008KPC" for date and assay) at bottom of screen.
- **g.** Click Next >.

2. Select detectors to add to the plate document.
- **a.** Click to select detector.
- **b.** Click on detector(s) needed.
- **c.** Click Add >> to add detectors to plate document (need FAM and Cy5).
- **d.** Leave Passive Reference Dye as ROX (already in master mix).
- **e.** Click Next >.

3. Specify the detectors and tasks for each well.
- **a.** Click a well (or group of wells for replicates) (must be contiguous/connecting)
- **b.** Click to select detector(s) for the well.
- **c.** Click to assign detector task (under Task column)
  Two choices to assign per well: Target (T) for KPC (FAM) and Endogenous Control (E) for 16S (Cy5)
- **d.** Click Finish.

4. Enter sample numbers and/or names according to previously filled-out plate layout.
- **a.** On the Plate tab, click and drag to select each sample's wells in duplicate to highlight.
- **b.** Enter the sample name or number.

5. Click on Instrument tab to specify the thermal cycling conditions before starting the run.
- **a.** Select the "Instrument" tab.
- **b.** Set up the cycling conditions as follows.
  - (1) Enzyme activation step: 95°C for 3 min (stage 1)
  - (2) PCR (each cycle): 40 cycles (stage 2)
    Melt: 95°C for 3 s
    Anneal/extend: 60°C for 20 s
    - (a) Verify that the correct thermal cycling conditions have been set and that the correct sample volume and run mode have been entered (i.e., 20 µl and Fast 7500)
    - (b) Expert Mode is an option that allows you to select which filters to enable, thus lowering the overall amount of time for the Fast run. Click the Expert Mode check box; this changes the anneal/extend program in stage 2 from 30 to 20 s. Then click the Select/View New Filters button, and check which filters are to be used for data collection (filter A = FAM, filter E = Cy5, and filter D = ROX). Click OK.
    - (c) Select File > Save As, and then enter a name for the RQ plate document.

V. PROCEDURE *(continued)*

6. Save file. Create a new folder for each day's run (e.g., 101008 DRL) with date and tech initials as the folder name.
7. Press Start. The PCR run will take about 30 to 60 min (depending on if you select to run in Expert Mode and how many samples are being run on the plate).

D. Analyzing the finished run

1. After the software indicates that "The run has been successfully completed," click on the analysis button (green triangle) to analyze the run.
2. Click on Results tab.
3. Choose Amplification Plot.
4. For Detector, choose All. For Line Color, choose Detector Color.
5. Hit Save icon.
6. Choose File.
7. Select New.
8. Choose ddCt (Relative Quantification) Study.
9. Plate Name is the date of the run.
10. Select Next.
11. Click "Add plates" button.
12. Click on displayed default plate file (SPS format).
13. Keep file type as "RQ plate(s)." Leave file name the same.
14. Click on the Amplification Plot tab.
15. For Line Color, choose Detector Color.
16. Select 16S.
17. Click the green arrow to analyze. Select 16S again.
18. Drag the green horizontal line (baseline) to where the fluorescent analysis curves are close to parallel; stay below peak of curve.
19. Click the green arrow to analyze.
20. Select KPC.
21. Drag the green horizontal line to where the fluorescent analysis curves are close to parallel; stay below peak of curve.
22. Click the green arrow to analyze.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. For each specimen, report results as follows.

1. KPC (bla_{KPC} gene) positive
2. KPC (bla_{KPC} gene) negative

B. Report results to physician on record or designee.
Note on the worksheet the name of the person called, date, and time, and initial.

C. Document all testing in hard copy or computerized work card.

D. Include, "This test was developed and its performance characteristics determined by (laboratory name). It has not been cleared or approved by the Food and Drug Administration (FDA)." As recommended by the CAP, add, "The FDA has determined that such clearance or approval is not necessary. This test is used for clinical purposes. It should not be regarded as investigation or for research. This laboratory is certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA-88) as qualified to perform high-complexity clinical laboratory testing." If your laboratory does not participate in proficiency testing related to this method, CLIA requires that two blinded test sets be performed biannually.

E. As applicable, include, "This test is performed pursuant to a license agreement with Roche Molecular Systems, Inc."

VII. INTERPRETATION

A. Results should only be reported if the controls give valid results based on the interpretation table below.

Control	16S rRNA (Cy5) Ct[a] result	KPC (FAM) Ct result	Interpretation
Negative	Ct, 10–30	Undetected[b]	Control is valid
NTC	Ct, >30–40 or undetected	Undetected	Control is valid Note: slight contamination of reagents may show Ct of >30–40 due to manufacturing method
Positive	Ct, 10–30	Ct, 10–30	Control is valid

[a] Ct, cycle threshold.

[b] Undetected, no Ct detected within 40 cycles.

B. If controls are valid, results are interpreted as indicated in the table below.

16S rRNA (Cy5) Ct[a] result	KPC (FAM) Ct result	Action
Ct, 10–30	Ct, 10–30	Report as "KPC positive."
Ct, 10–30	Undetected[b]	Report as "KPC negative."
Ct, 10–30	Ct, >30–40	Repeat procedure with new DNA extract (possible low-level cross-contamination)
Ct, 10–30	Ct, <10	Dilute the template 1:100 and repeat.
Ct, <10 or undetected	Any result	Invalid result[c]: consult supervisor before repeating

[a] Ct, cycle threshold.

[b] Undetected, no Ct detected within 40 cycles.

[c] If the 16S rRNA gene is not detected, the sample result is not valid. Review results to determine the possible problem(s). Bad primers, probes,or reagents or even overinoculation may be the cause. Prepare new lysates or try different reagents, as recommended by the supervisor.

◩ **NOTE:** When finally examining the results of the real-time run, it is important to consider only the amplification between 10 and 30 cycles. Amplification that is lower than 10 cycles means the template concentration needs to be diluted in order to properly assess the well's contents. Amplification over 30 cycles may be due to contamination.

◩ **NOTE:** For reliability of results, it is recommended that samples be tested in duplicate.

VIII. LIMITATIONS OF TESTING

This PCR procedure has been designed for the Fast 7500 real-time PCR system using lysates prepared by combined thermal and chemical lysis for the detection of the KPC and 16S rRNA genes. Different lysis protocols or different thermocycling protocols may change the detection dynamics of a reaction. All changes to the protocol (instruments and or methods) should be validated prior to implementation. If the 16S target has not been detected for an isolate (even if KPC is detected), it is indicative of a problem with the sample/run and the test may need to be repeated.

VIII. LIMITATIONS OF TESTING *(continued)*

Due to the high concentration of target DNA used in the procedure, the potential for cross-contamination of specimens is high. Careful pipetting is essential to ensure integrity of results.

Not all carbapenemases are KPCs, so even if the *Enterobacteriaceae* isolate is determined to be KPC negative by this protocol; it still may produce another carbapenemase.

SUPPLEMENTAL READING

Anderson, K. F., D. R. Lonsway, J. K. Rasheed, J. Biddle, B. Jensen, L. K. McDougal, R. B. Carey, A. Thompson, S. Stocker, B. Limbago, and J. B. Patel. 2007. Evaluation of methods to identify the *Klebsiella pneumoniae* carbapenemase in *Enterobacteriaceae*. *J. Clin. Microbiol.* **45:**2723–2725.

Conrad, S., M. Oethinger, K. Kaifel, G. Klotz, R. Marre, and W. V. Kern. 1996. *gyrA* mutations in high-level fluoroquinolone-resistant clinical isolates of *Escherichia coli*. *J. Antimicrob. Chemother.* **38:**443–455.

Corless, C. E., M. Guiver, R. Borrow, V. Edwards-Jones, E. B. Kacxmarski, and A. J. Fox. 2000. Contamination and sensitivity issues with real-time universal 16S rRNA PCR. *J. Clin. Microbiol.* **38:**1747–1752.

Kaczmarek, F. M., F. Dib-Hajj, W. Shang, and T. D. Gootz. 2006. High-level carbapenem resistance in a *Klebsiella pneumoniae* clinical isolate is due to the combination of bla_{ACT-1} β-lactamase production, porin OmpK35/36 insertional inactivation, and down-regulation of the phosphate transport porin PhoE. *Antimicrob. Agents Chemother.* **50:**3396–3406.

Naas, T., G. Cuzon, M. Villegas, M. Lartigue, J. P. Quinn, and P. Nordmann. 2008. Genetic structures at the origin of acquisition of the β-lactamase bla_{KPC} gene. *Antimicrob. Agents Chemother.* **52:**1257–1263.

Queenan, A. M., and K. Bush. 2007. Carbapenemases: the versatile β-lactamases. *Clin. Microbiol. Rev.* **20:**440–458.

Yang, S., S. Lin, G. D. Kelen, T. C. Quinn, J. D. Dick, C. A. Gaydos, and R. E. Rothman. 2002. Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. *J. Clin. Microbiol.* **40:**3449–3454.

Appendix 12.5.4–1

Master P Worksheet, KPC Detection by Real-Time PCR

	Record lot no.	Starting concn (μM)	Vol per sample (μl)		No. of samples		Final vol (μl)	Concn (nM) in final master mix
KPC F primer		10	2.5	×		=		500
KPC R primer		10	2.5	×		=		500
KPC probe		5	2.5	×		=		250
16S F primer		10	2.5	×		=		500
16S R primer		10	2.5	×		=		500
16S probe		5	2.5	×		=		250
						Total		

Master P = primer and probe mixture for two 20-μl reaction mixtures in the final master mix.

Master P is stable for 1 month at −20°C; it can be freeze-thawed several times, but keep this to a minimum.

For every set of 1 to 10 samples, add 2 extra samples to account for loss of solution due to pipetting.

Appendix 12.5.4–2

Final Master Mix Worksheet, KPC Detection by Real-Time PCR

	Record lot no.	Concn	Vol per sample (μl)		No. of samples		Final vol (μl)
2× QuantiFast probe PCR master mix		10 μM	25	×		=	
Master P			15	×		=	
Sterile reagent-grade type I water			7.5	×		=	
						Total	

Final master mix for two 20-μl reaction mixtures (samples performed in duplicate). Add 2.5 μl of lysate per 47.5 μl of master mix.

For every set of 1 to 10 samples, add 1 extra sample to account for loss of solution due to pipetting (20 μl of the 50-μl mixture is pipetted into each of two reaction wells on the 96-well PCR plate; ~10 μl is left over).

2× QuantiFast PCR master mix is initially kept frozen at −20°C in storage until ready for use the first time. Once opened and thawed, it is kept in a refrigerator at 2 to 8°C until used again; it is stable for 2 months after thawing.

12.6

Appendix 12.6–1—Unidirectional Work Flow and Minimization of Contamination

The extraordinary sensitivity of nucleic acid amplification methods makes them invaluable tools for detecting infectious agents. However, the ability to detect small quantities of nucleic acids also makes these techniques prone to false-positive results due to specimen-to-specimen cross-contamination or carryover contamination from previously amplified products. The risk of contamination can be greatly reduced by maintaining a unidirectional work flow and by strictly adhering to appropriate QC procedures.

Unidirectional work flow refers to the process of performing specified tasks in different areas, with workers moving from one place to the next but never returning to an area in which they previously worked. A typical laboratory setup will employ three separate areas: a reagent preparation area (area 1), a sample preparation area (area 2), and an amplification and detection area (area 3). Work flow will proceed from area 1 to area 2 and then to area 3, and during the course of a day, a technician will not return to an area where he or she previously worked. Each room should have dedicated equipment (pipettes, tips, etc.) and dedicated lab coats and gloves, which should be removed before moving to the next area. No materials that have been in the sample preparation or amplification area (including pens or lab notebooks) should be transferred to the reagent preparation area.

Ideally, a laboratory will be designed to have separate rooms for each area. The reagent preparation area should be positively pressurized to keep amplified products from entering the area, and the sample preparation and amplification areas should be negatively pressurized to minimize the spread of template nucleic acids or amplified products to other areas of the laboratory.

With the advent of real-time PCR technologies, for which tubes containing amplified products are never opened, it may suffice to do sample preparation and amplification in the same areas. However, this can be done only if none of the assays performed in the laboratory involve opening tubes containing amplified product. Also, if separate rooms cannot be created, areas can be spatially separated but unidirectional work flow should remain in place, and rigorous cleaning protocols should be implemented prior to any reagent preparation processes. While having all areas in one room is not optimal, secondary measures can be taken to reduce contamination (such as the use of dUTP and uracil-*N*-glycosylase to degrade carried-over amplicons) and to maximize detection of contamination events by implementing practices such as increasing the number of negative controls utilized.

Sources of more detailed descriptions of procedures used to maintain unidirectional work flow and minimize contamination are listed in the Supplemental Reading section.

SUPPLEMENTAL READING

College of American Pathologists. 2009. Laboratory Accreditation Program, Microbiology Checklist, rev. 06/15/2009. College of American Pathologists, Northfield, IL.

Health Protection Agency. 2006. Good laboratory practice when performing molecular amplification assays. National Standard Method QSOP 38, issue 3. http://www.hpa-standardmethods.org.uk/pdf_sops.asp.

Mitchell, P. S., J. J. Germer, and R. Patel. 2004. Nucleic acid amplification methods: laboratory design and operations, p. 85–93. *In* D. H. Persing, F. C. Tenover, Y.-W. Tang, E. R. Unger, D. A. Relman, and T. J. White (ed.), *Molecular Microbiology: Diagnostic Principles and Practice*. ASM Press, Washington, DC.

Nolte, F. S., F. R. Cockerill, P. J. Dailey, D. Hillyard, S. McDonough, R. F. Meyer, and R. G. Shively. 2006. *Clinical and Laboratory Standards Institute (CLSI). Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline—Second Edition.* Clinical and Laboratory Standards Institute, Wayne, PA.

U.S. Environmental Protection Agency. 2004. *Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples.* U.S. Environmental Protection Agency, Cincinnati, OH. http://www.epa.gov/nerlcwww/qa_qc_pcr10_04.pdf.

SECTION 13

Epidemiologic and Infection Control Microbiology

SECTION EDITORS: *Dan Gregson and Deirdre Church*

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13.1 Introduction

Clinical microbiologists have long been comfortable with the analytical contributions they make to laboratory diagnosis. However, the total testing process for the microbiologist additionally includes preanalytical and postanalytical issues about which a technologist may feel less secure. Clearly, one major area of contribution unique to the clinical microbiologist is the critical data expertly provided to the infection control team of the institution. A sound infection control program of any health care institution is dependent on its relationship with the microbiology laboratory from which most of their critical data come. While accuracy and timeliness of critical microbiology diagnostic reports contribute to positive patient outcomes, the nature of skilled interpretive judgment on the part of trained technologists marks the clinical microbiologist as a uniquely qualified contributor to the data that drive infection control decisions.

Every infection control team must include a member of the microbiology laboratory staff who should be given the opportunity to actively and equally participate as a critical member of that team. The microbiologist will have the knowledge that can link patient care and diagnosis within the institution to community health care issues that impact public health. This linkage, coupled with the expertise of the infection control professional and the attending physicians, provides the strength required to oversee and implement strategies that promote patient safety and health care quality.

This section addresses many issues that may be considered "extra work" for the laboratory and too time-consuming to focus enough expertise to accomplish the requests and needs of the submitter. It is true that relatively few institution-associated infections can be attributed to a microbial challenge originating from the environment. However, the potential contribution of the environment, including water, indwelling medical devices, surfaces, and air, cannot be discounted, particularly in light of the increasing numbers of patients suffering from immunosuppression. We have long known that many factors contribute to infection in humans, including the virulence of the etiologic agent, inoculum size, route of infection, and host factors, among others. In addition, in today's health care environment, more hospitalized patients are sicker than in previous years, and these patient populations require more sophisticated medical manipulation and intervention. Therefore, what may be normal body microbiota or normal environmental organisms can have a negative impact on patient outcome.

The astute microbiologist will be prepared to address these issues with both infection control professionals and attending physicians. Understanding the microbiology of healthy individuals, of hospitalized patients, of special patient populations, and of the environment offers the infection control team a strategic view from which sound strategies for patient safety can arise.

13.2 Laboratory Support for Infection Control: Optimization by Policy and Procedure

The discipline of infection control had its formal beginnings in the 1970s and was refined in the 1980s. This discipline will continue to evolve, becoming part of the health care providers' overall program of continuous quality improvement. Superimposed on the evolution of infection control programs has been the rapid evolution of the health care field, introducing new modalities of care that are prone to high rates of nosocomial infection. Fewer patients are hospitalized, but in general, those who are hospitalized are sicker than similar populations in previous years. The numbers of indwelling lines, implanted devices, and complex procedures have greatly increased, as have the numbers of patients compromised by cancer chemotherapy regimens or transplant immunosuppressive therapy. Transfusion practices have evolved to include a variety of processed subsets of blood, and transplantation now includes many solid organs, bone, bone marrow, corneas, and various connective tissues. In addition, special patient populations such as the elderly, neonates, women, or ethnic minorities are being evaluated for their special health care needs that might require unique services. The microbiology laboratory is increasingly being asked to perform cultures that support the needs of these new programs. The involvement of the laboratory must be optimized by appropriate policies and procedures and the appointment of the chief microbiologist to the Infection Control Committee. With early patient release and with multiple hospitals often served by the same microbiology core laboratory, it is imperative that the laboratory develop methods and communication tools to assist in serving the patients' infection control needs. The Institute of Medicine in 2000 addressed the issue of patient safety, and although it did not specifically focus on health care-associated infections, these are clearly critical areas for improvement and monitoring.

The microbiologist has a complex interface with the infection control team. Routine microbiology laboratory data are employed as one of the major sources of information to suggest incipient problems with health care-associated infections, and laboratorians' expertise must be employed to assist in the investigation of such infections. Adding to the complexity is the problem of pseudoinfections which result from contamination of cultures or culture specimens rather than from patients; such contamination may be introduced in the laboratory during specimen management or by the manufacturer of the collection and transport devices. If found in multiple cultures, such non-patient-related contamination events are termed "pseudoepidemics" and may plague the laboratory facility that does not institute immediate investigation and intervention strategies. The laboratory should play a pivotal role in the investigation of all apparent pseudoepidemics. The initial evaluation effort should focus on the possible contribution of laboratory processing or specimen manipulation in introducing the microorganism. The detection or elimination of the laboratory as a source of the contaminant is usually simple, so it is best to make the laboratory evaluation first. Is there a laboratory diluent or procedure common to all the specimens from which the organism has been grown? In some cases the laboratory is easily eliminated as a source of the contamination, for example, when growth appears in blood culture bottles that have not been sampled after receipt in the laboratory. Once the laboratory is eliminated as the source of the contaminant, the infection control team needs to devise a strategy and put in place a tracking system so that immediate investigation of all new positive culture events can be undertaken. Immediate investigation facilitates the process because it is easier to determine who collected the specimen, and how it was collected, if inquiries are made while responsible parties are still on the service and memories are not dulled by the passage of time. The lot number and type of the antiseptics and/or devices used in the collection can be traced to the opened box in the room where the procedure was performed, and the team can quarantine all supplies of the same lot number pending cultural or epidemiologic resolution of the problem. A brief description of the problem, rationale for the undertaking, the designated responsible persons, and the plan of action should be prepared and distributed to all members of the investigational team, with a copy to the administration's representative on the Infection Control Committee. Most pseudoepidemics are similar enough that a standard type of form can be adapted for use with all of them. Use of a form minimizes the loss of data when investigators make informal notes and subsequently lose them. A sample investigation plan and associated form are supplied in Appendix 13.2–1. Such a formal plan and report form help to optimize the process and clarify the responsibilities. The document can be easily adapted for inclusion in minutes and reports.

The laboratory staff may occasionally be asked to perform inappropriate surveillance cultures that cannot be justified by current practice and that should not be done because the resulting information may be misleading and result in an inappropriate or irrational response. Sometimes well-meaning individuals will ask the laboratory to investigate presumed infectious incidents by culturing food or

pharmaceuticals. Culture protocols for most *clinical* laboratories are insufficient to accommodate the standard methods that these investigations require. Thus, these clinical laboratories should refer such products for culture to public health, commercial reference, or Food and Drug Administration laboratories that have the appropriate methodology in place to produce the results that may be required, should legal proceedings ensue. The microbiology laboratory must limit the scope of its cultural investigations in environmental microbiology and epidemiology to those that are endorsed by the Infection Control Committee.

The laboratory may inadvertently become an innocent victim of inappropriate environmental-culture requests. Specimens may be sent to the laboratory for culture as though they were intended for diagnosis of an infection, when they are really intended to document sterility of a reagent, device, or organ or fluid for administration, implantation, or transplantation. Sterility documentation requires a culture protocol, temperature(s) of incubation, and interpretation different from those that are applicable to diagnostic cultures. Thus, laboratories should institute policies regarding sterility testing and perform such tests only if the laboratory has the proper procedures in place. Moreover, the laboratory should protect itself against inadvertent application of its diagnostic culture test result as though it were a result of a sterility test culture protocol. This type of communication should be accomplished by publishing in the laboratory handbook, and perhaps on all result reports, that the culture result was for diagnosis and cannot be utilized as a documentation of sterility. This is important because it is not always possible for the laboratorian to screen all specimens, reagents, or devices in advance regarding the intent of the culture.

The culture of specimens is just one of many ways in which the laboratory supports the infection control team. The antimicrobial agent susceptibility surveillance results should be published in a timely manner and subjected to careful evaluation for detection of emerging resistance trends. A new emphasis on early detection of emergence of resistance in certain nursing units or from specific body sites suggests that body site-specific antibiograms may be helpful for the intensive care units so that emergence of resistance is not masked by the large denominator of susceptible organisms isolated from outpatient clinics and units in which patients' stays are of short duration. In order to facilitate the interactions in infection control, the Chief of Microbiology should serve on the Infection Control Committee and provide advice regarding appropriate cultural investigation of all epidemics and pseudoepidemics. Often, the ordering of cultures is a reflex reaction to a perceived problem when a little "shoe leather" epidemiology would solve the problem more rapidly and with less expense. Quarantining suspect goods during the investigation of the epidemiology of a problem, pending the determination that cultures must be done, is often superior to reflexively culturing these goods. While the goods are in quarantine, the necessity to culture can be assessed and the appropriate culture technology can be devised. Routine surveillance cultures and ad hoc investigative cultures are often the most overused or misused tests that a laboratory is asked to perform. Their overuse should be controlled by requiring authorization and oversight by officials of the infection control team. Institution of such a requirement is best accomplished by the adoption of a policy for control of such cultures. If adapted hospital wide, the policy will ensure that excessive, inappropriately designed cultural investigations are not launched. Control of excessive or misapplied cultures will allow the resources of the laboratory to be applied where they are needed most.

APPENDIX 13.2–1

Sample Investigation Plan for Infection Control

Cluster of *Burkholderia cepacia* in sputum and urine specimens—strategy for infection control investigation

I. FINDING

There has occurred a cluster of cultures positive for *Burkholderia cepacia* among urine and respiratory specimens that were submitted for patients from various nursing care units. The laboratory does not treat these two types of specimens similarly; thus, it is unlikely that this organism is being introduced into the specimens after their arrival in the laboratory. Thus, a formal investigation of the other potential sources of the organism is recommended as follows.

II. DISCUSSION

Documentation of similar problems from literature

A. Pseudoinfections due to *B. cepacia*

A literature review reveals that contaminated disinfectants and antiseptics (povidone iodine and benzalkonium chloride) have been responsible for pseudoepidemics due to this organism. Other sources have included a contaminated anesthetic solution and a blood gas analyzer. Focus of this investigation should include all water-based, nonsterile fluids to which the patient is exposed.

B. True infections due to *B. cepacia*

1. Cystic fibrosis literature reveals many infections in this patient population, but these would not be applicable to this case.
2. Literature search reveals little documentation of hospital epidemics of true infections due to this organism.

APPENDIX 13.2–1 (*continued*)

III. CONCLUSION

Given the resistant nature of this organism and its potential infectivity, an ongoing investigation should be initiated.

IV. RECOMMENDATIONS/ACTIONS

A. Laboratory responsibility

1. The laboratory will save all isolates of *B. cepacia* for future investigation from the standpoint of molecular epidemiology.
2. When new isolates are identified, the laboratory will immediately notify the nurse epidemiologist who is responsible for the unit in which the new isolates have occurred.

B. Nurse epidemiologist responsibility

The nurse epidemiologist who is in charge of the unit in which the isolate has occurred will initiate an immediate investigation to identify the possible source(s) of the organism, focusing on candidate sources that are most likely with this organism.

1. Specimen collection

   Identify the individual who collected the specimen and determine what antiseptic was utilized in the collection process. If povidone iodine, identify the type (swab stick versus gauze pad versus vial) used and remove from the nursing unit all the lots of the implicated agent. If a collection kit that contains an antiseptic was used, remove samples of the kits. Record the lot number of the implicated kits on the form for investigation (below). Quarantine the implicated materials for possible further testing. This intervention must be done without delay in order to increase the likelihood that the supply on the nursing unit will be similar to that which was there on the day of collection.
2. Patient Chart Review

   Conduct a chart review for possible means of colonization of the implicated patient site, focusing on the patient's recent (past 2 weeks) history. Since the organism is usually associated with water, the investigation should focus on moist or fluid environments but should not be overly restrictive. For example, a contaminated blood gas analyzer in an intensive care unit has been implicated as a source of a pseudoepidemic with this organism. Communicate the results of the investigation immediately to the Director of Clinical Microbiology to devise a strategy to investigate any implicated therapy or environmental source.
3. Notify the attending infectious disease faculty so that they might initiate a clinical consult.
4. Submit a monthly report of the ongoing investigation, summarizing the data from the investigation on the form below. Detail the chart review results on supplemental sheets with a listing of implicated environmental sources or therapies and providing written documentation relative to the action taken regarding any implicated sources.

C. Infectious disease responsibility

1. Consult on individual cases to ensure that therapy is adequate if infection is present.
2. Provide input on changes in recommendations/actions and follow-up throughout the course of the investigation.

V. FOLLOW-UP

Pending results of ongoing investigation, for example, if an implicated source is documented culturally, the source will be removed or cleaned and the monitoring will continue until the organism is no longer a problem.

APPENDIX 13.2–1 (*continued*)

Pseudoepidemic Investigation Report Form

Monthly report cover sheet, nurse epidemiologist_____

A. Cumulative culture results

Specimen type	Patient	Hosp ID #	Ward	Culture accession #	Date specimen collected

B. Specimen collection review

Ward	Antiseptic or other implicated source	Manufacturer	Lot #	Culture result, if indicated

C. Chart review—summary

List common nursing units, health care providers, procedures, devices, and therapies among the colonized or infected patients.

13.3 Policies for Environmental Sampling and Culturing for Infection Control

I. PRINCIPLE

Sampling of the environment should be performed only to answer a specific question, whether to support a defined hypothesis or to confirm an association generated by an epidemiologic investigation. Culturing for infection control purposes should be systematic, consistent, and part of a written infection control plan that is reviewed at least yearly and is specific to each facility. Arbitrary sampling risks generation of irrelevant or uninterpretable information. Worse, such information may be frankly misleading. In each case, inappropriate culturing can result in wasted effort and resources and may even cause harm to patients or personnel. For example, if arbitrary culturing identifies an irrelevant source of an organism, the epidemiologically important source may go undetected, leading to further unnecessary exposures.

Wasted resources may include laboratory time and supplies, infection control service time and effort, unnecessary treatment interventions or cleaning, building materials and worker time, and clinical and administrative efforts. In addition to time and resources, meaningless results may lead to unwarranted fear and concern among workers and patients.

When indicated, environmental sampling may strengthen the results of an epidemiologic investigation. In addition to this confirmatory role, environmental cultures may have utility in measuring the impact of an intervention, e.g., confirming that a contaminated water source identified by an outbreak investigation has been adequately cleaned.

II. GUIDANCE FOR ENVIRONMENTAL SAMPLING

When considering whether to collect or process environmental cultures, the following criteria should be met.

A. The decision to obtain environmental cultures should be based on epidemiologic evidence indicating an association between the environment and patient or personnel health.

B. The decision to obtain environmental cultures must be supported by the infection control service, the hospital epidemiologist, and the microbiology laboratory director.

C. Materials or surfaces to be cultured should be selected based on an epidemiologic association.

D. Culture results should have a direct impact on an intended plan of action. If results will not affect the plan, cultures should not be performed.

III. CONTRAINDICATIONS TO ENVIRONMENTAL SAMPLING

Environmental culturing should never be performed merely to satisfy curiosity. Neither should environmental cultures precede an epidemiologic assessment of an outbreak (4). Though it may seem prudent to collect samples early for fear that they may not be available later, a prompt epidemiologic assessment is preferable and remains the best way to select the best specimens and avoid processing unnecessary, possibly misleading, samples.

IV. GUIDANCE FOR INFECTION CONTROL CULTURES

A. Surveillance cultures

Surveillance cultures are performed prospectively, in contrast to retrospective evaluations of a patient's illness or an outbreak. Surveillance cultures may be of use when describing the local microbial ecology of a facility and the burden of a pathogen therein. However, organisms, sites, and populations for surveillance cultures must be chosen carefully to allow meaningful interpretation of data. Protocols for surveillance cultures should be designed in consultation with the infection control service, the hospital epidemiologist, and the clinical laboratory director. Surveillance protocols should ensure that culture methodology and intensity, population studied, and method of interpretation are all consistent and yield data that will have a meaningful impact on health care practices at the facility. If used, protocols should be instituted for predesignated time periods and reevaluated at least annually to decide whether further surveillance is warranted. Data should be analyzed and shared with appropriate clinical staff members.

B. Occupational health and employee cultures

Reasons for performing diagnostic studies of health care workers include confirmation of an epidemiologic link to an outbreak and evaluation of the infectious status of a worker recovering from a known, transmissible infectious disease, e.g., tuberculosis or influenza (1). States may differ regarding which infections require such testing. Local regulations should be consulted.

Routine screening cultures of employees are not useful and may yield confusing or harmful results. When an epidemiologic association has been demonstrated between an outbreak or cluster of infections and a particular worker, a confirmatory culture demonstrating the presence of related organisms may be helpful. The decision to perform such testing should be made with the infection control service, the hospital epidemiologist, and the clinical laboratory director, with attention to appropriate protection of worker confidentiality.

C. Testing of outbreak sources: pharmaceuticals and food and blood products

Clinical laboratories may receive requests to culture materials associated with outbreaks, e.g., food, pharmaceuticals, or other products that are suspected to be the source of an outbreak. Laboratories that do not routinely perform such testing should store specimens appropriately and refer them to the state or other reference laboratory for evaluation. Foods are stored at 4°C (6), and pharmaceuticals are stored according to manufacturers' instructions. Blood, transplant materials, and other biological products may be tested according to standardized protocols. Sources of protocols include the American Association of Blood Banks, the National Organ Procurement and Transplantation Network, the United Network for Organ Sharing, the American Association of Tissue Banks, and the Eye Bank Association of America.

D. Nonhuman and research materials

Animal specimens and research materials such as cell cultures should not be accepted for routine evaluations in a clinical microbiology laboratory. Appropriate media, methods, and safety precautions are necessary to perform effective cultures and to prevent transmission of animal pathogens to laboratory personnel (2). Research materials that may contain radioisotopes or hazardous chemical agents may pose additional risks. Requests to evaluate such materials should be assessed on a case-by-case basis, including careful discussion with the investigators and the clinical laboratory director.

V. SPECIAL CONSIDERATIONS

A. Medical progress continually generates new populations at increased risk of infectious diseases. Such fragile populations warrant special vigilance, effective surveillance, and appropriate infection control (3). Environmental sampling, however, should still meet the basic criteria outlined above.

V. SPECIAL CONSIDERATIONS (*continued*)

B. There are many organisms in the environment that are of interest and can have an effect on health. Arbitrary examination of the environment as a potential reservoir of such organisms may be a part of a research protocol, e.g., to describe microbial ecology. However, this is distinct from surveillance, infection control, or clinical care.

C. Construction at or near a health care facility has been shown to be an independent risk factor for fungal infections in certain patient groups (7). Responses to construction activity should include protective measures such as controlling airflow to prevent movement of dust and particles from the construction site to patient care areas, prohibiting shared elevators and passages between construction personnel and high-risk patients, and ensuring that appropriate surveillance practices are in place to promptly alert care providers to unusual clusters of illness. Arbitrary environmental cultures are not useful.

D. Concerns about water and air quality at health care facilities may lead to requests for environmental culturing. Sampling should be driven by clinical need and by specific features of individual facilities, e.g., water sources, climate, etc. The decision to perform environmental cultures should be made after careful consideration by the clinical laboratory director, the infection control service, and the hospital epidemiologist. Procedural and interpretive expertise is critical to the correct application of environmental microbiology. Facilities that do not routinely perform such testing should consider the services of a reference laboratory specializing in environmental microbiology.

E. When an investigation reveals an epidemiologic association between a suspected environmental source and clinical infections, health care providers may request environmental cultures, e.g., of surfaces or equipment, to assess the effectiveness of cleaning measures. Such culturing should be performed only after the microbiology laboratory director, the infection control service, and the hospital epidemiologist have agreed upon the details of the process. These details include a statement of what specific organism is to be identified and how the culture results are going to be interpreted, i.e., what the exact parameters for defining adequate or inadequate cleaning will be.

REFERENCES

1. **Centers for Disease Control and Prevention.** 1998. Guideline for infection control in health care personnel. *Infect. Control Hosp. Epidemiol.* **19:**407–463.
2. **Centers for Disease Control and Prevention.** 2007. *CDC and NIH Biosafety in Microbiological and Biomedical Laboratories*, 5th ed. U.S. Government Printing Office, Washington, DC. http://www.cdc.gov/od/ohs/biosfty/bmbl5/BMBL_5th_Edition.pdf.
3. **Centers for Disease Control and Prevention.** 1996. The Hospital Infection Control Practices Advisory Committee (HICPAC): recommendations for isolation precautions in hospitals. *Am. J. Infect. Control* **24:**24–52.
4. **Jarvis, W. R.** 1991. Nosocomial outbreaks, the Centers for Disease Control's Hospital Infections Program experience. *Am. J. Med.* **9:**3B101S–3B106S.
5. **Sehulster, L., R. Y. Chinn, Centers for Disease Control and Prevention, and HICPAC.** 2003. Guidelines for environmental infection control in health-care facilities. Recommendations of CDC and the Healthcare Infection Control Practices Advisory Committee (HICPAC). *MMWR Recommend. Rep.* **52**(RR-10)**:**1–42.
6. **Speck, M. L.** 1984. *Compendium of Methods for the Microbiologic Examination of Foods*, American Public Health Association, Washington, DC.
7. **Weems, J. J., B. J. Davis, O. C. Tablan, L. Kaufman, and W. J. Martone.** 1987. Construction activity: an independent risk factor for invasive aspergillosis and zygomycosis in patients with hematologic malignancy. *Infect. Control* **8:**71–75.

13.4 Outbreak Investigations: Laboratory and Epidemiologic Concepts

I. PRINCIPLE

Epidemiologic investigations may be performed in response to a change in the rate of an event or outcome compared with a community or facility's baseline or to evaluate a rate that is considered unacceptable compared with published rates in similar populations (20). The degree of departure from baseline needed to identify an outbreak is variable, depending on factors such as the population in which it occurs and the organism or agent responsible. A single case of a rare or virulent infection, e.g., West Nile encephalitis, rabies, measles, or cholera, may be enough to herald an outbreak. For more common or endemic organisms, like methicillin-resistant *Staphylococcus aureus* (MRSA), an outbreak may mean a significant increase above baseline rates of clinical isolates or illness. Thus, specific thresholds for concern vary from case to case. If a particularly fragile population is involved, e.g., low-birth-weight infants in the neonatal intensive care unit or patients in a burn unit, the threshold for investigation may be lower. In addition to the pathogen and the population involved, outbreaks may be defined by a change in prevalence of certain organism characteristics, e.g., antimicrobial susceptibility or toxin production. In each of the above cases, meaningful surveillance data against which to measure a change are a critical component of outbreak investigation (12).

Outbreaks in health care facilities may have a single source, such as a contaminated intravenous solution or a malfunctioning sterilization device, or multiple sources of transmission related to practices or behaviors, for example, noncompliance with handwashing (8, 19). The extent and severity of an outbreak are influenced by multiple factors. A change in the population receiving care can give rise to a new, susceptible subgroup, such as a bone marrow transplant population. Organisms or procedures that are not dangerous in the general population may take on new significance when such a vulnerable group is created.

An alteration in the environment, such as a change in airflow characteristics or water delivery, can create new opportunities for transmission of infection (16, 21). Another feature of the environment that can affect infections is the quantity, duration, or type of antimicrobials used in that setting. As has been documented with vancomycin-resistant enterococci and MRSA, antimicrobial use can promote the selection of resistant organisms within a population or facility (6, 11). The use of new materials, equipment, or procedures also may give rise to unexpected opportunities for infection, such as infections associated with synthetic polymers and prosthetic joints. From a microbial perspective, these constitute new, permissive environments.

II. GOAL OF INVESTIGATION

The goal of an outbreak investigation is threefold: to identify the source of a problem or pathogen, to identify factors that may place people at greater risk for an adverse event or infection, and to identify opportunities for prevention (13).

Epidemiologic tools help us to understand the impact of a disease on a population by defining which individuals among a population are affected, the geography of the problem, and its timing (i.e., answering the questions *who*, *what*, and *where*). Both the number of events (infections, isolates, deaths, etc.), the numerator, and the magnitude of opportunity for the event (number of days at risk, number of procedures performed, number of patients admitted), the denominator, are crucial for understanding the epidemiology of a problem. The numerator is described by the selected case definition, i.e., what is chosen to be counted as an event (10). Careful definition of the denominator, the population being assessed, and the use of precise case definitions for the numerator, including microbiologic data, allow useful comparisons that can identify risk factors for a problem or disease (10). Lack of precision in defining either will prevent meaningful comparison of groups. A critical tool in epidemiology is surveillance (the ongoing, systematic collection and analysis

II. GOAL OF INVESTIGATION (*continued*)

of incidence data that are communicated to the persons making decisions) (18). In order to determine whether an outbreak is occurring and to measure its magnitude, it is necessary to have a baseline rate against which to compare a change. Point prevalence measurements are used to assess the burden of disease in a population at a single point in time and to identify risk factors for the disease (9, 15). For both measurements, risk stratification is used to adjust for differences in inherent risk (1). Thus, a population is assessed as a collection of subgroups that share roughly similar traits. For example, two populations may not be useful for comparison if the severity or rates of predisposing illnesses, invasiveness of care, and other factors are very different (e.g., comparing rates of bloodstream infections [BSI] among intensive care unit [ICU] patients and healthy outpatients). To make comparison meaningful, it is best to compare events in similar-risk groups, e.g., BSI among patients in similar types of ICUs at different facilities or at different times (8).

III. PITFALLS IN EPIDEMIOLOGY

As surveillance has become increasingly important for monitoring health care quality, it has become clear that precise denominators must be selected. In the past, whole-hospital surveillance was common, i.e., surveillance in which the number of events, such as BSI, was counted per year for the combined hospital population. With all patients at a hospital comprising the denominator, comparison of two facilities measuring whole-hospital BSI rates is likely to be confounded by differences in the proportions of patients at risk, e.g., the number of ICU patients at each facility. One facility may have a higher rate of BSI, but this may be because it provides care to a larger proportion of very ill patients. Therefore, a more useful denominator for BSI has been the number of ICU patient-days, where one patient-day represents one patient residing on a ward for 1 day; two patients in the ICU for a week generate 14 ICU patient-days (5). In addition to allowing comparisons between facilities, this denominator accommodates comparisons within a single facility over time, where the number of ICU patient-days may vary seasonally. In very small facilities that may not have an ICU or that are unlikely to generate enough specimens in any one subsection of the hospital, whole-hospital numbers may still be used to compare the facility to itself over time (3). If this is done, care must be taken to assess changes in patient census, etc., which would affect results.

A related issue is the problem of imprecise case definitions. Meaningful comparisons, either over time or between facilities, require the use of precise, consistent case definitions, including demographic information, such as age and sex; information on location, such as the ward, service, or city; and a time span during which cases occurred (10).

An increasingly common tool in outbreak investigation, molecular typing techniques (e.g., pulsed-field gel electrophoresis) are a valuable resource that some laboratories may offer. It is essential, however, to recognize that these techniques are not well suited for initial assessments of an outbreak. Misuse of molecular typing techniques often creates misleading or useless information. In an outbreak setting, these tools are used to verify relatedness of organisms after appropriate epidemiologic evaluation has been performed to identify the source of a pathogen (4, 14). If molecular typing is used as a screening tool for outbreaks prior to epidemiologic evaluation, the data generated may be impossible to interpret. Since isolates identical by molecular typing techniques may be epidemiologically unrelated and since related organisms in an outbreak may be of different molecular types, epidemiologic guidance is critical to the accurate interpretation of molecular typing results. Without careful assessment of the associations between cases of illness, one will not know how to respond to isolated typing data. These tests are best suited for confirming epidemiologic associations (17).

III. PITFALLS IN EPIDEMIOLOGY (*continued*)

In addition to outbreak evaluation, molecular typing may play a role in surveillance (identifying unique patterns associated with strains that exist in a facility or population). However, most facilities will not be able to routinely provide this service on site. If used for surveillance purposes, it is necessary to assess the degree of variability of strain types both in the facility and in the community. If variability is minimal, i.e., most isolates in a facility or community share a particular molecular subtype, then the information gained by ongoing typing of surveillance isolates is not likely to be useful.

IV. ROLE OF THE MICROBIOLOGY LABORATORY

The clinical microbiology laboratory has several important roles in surveillance and outbreak assessments. In most settings, both services benefit by a close partnership between the clinical laboratory and the infection control service (7). The former may rely on an infection control professional (ICP) to identify trends or clusters that are clinically significant and to direct efforts away from issues without substantial impact. The ICP, in turn, relies on the microbiologist for early warnings of unusual pathogens, for advice as to the verity of a culture result, and for diagnostic support of epidemiologic assessments. Accurate microbiologic diagnosis, including antimicrobial susceptibility testing, is a critical component of many case definitions for both surveillance and outbreak evaluations. For surveillance, records of isolates from normally sterile sites form a valuable information source. Records of pathogens recovered from specific specimens can be used to describe baseline levels and trends. This can be particularly meaningful when there is epidemiologic information available for association with each isolate, e.g., location of the patient in a hospital, clinical diagnoses, and demographic information. Computerized databases using shared identifiers such as a hospital identification number can facilitate useful linking of microbiology, pharmacy, and patient information sources. Clinical laboratories can also provide early-warning "sentinel" surveillance by flagging organisms of interest and notifying the ICP, particularly when the organism is uncommon (e.g., highly antimicrobial-resistant organisms such as resistant *Acinetobacter* spp. or *S. aureus* with intermediate susceptibility to vancomycin; uncommon organisms such as *Malassezia pachydermatis* or *Bacillus anthracis* from sterile sites; and organisms with strong epidemiologic implications such as *Coccidioides immitis* or *Salmonella enterica* serovar Typhi) (2). In partnership with infection control departments, laboratories performing such sentinel surveillance may identify a problem at a facility much earlier than if identification relied only on overt clusters of clinical cases.

Collaboration between the infection control service and the clinical laboratory can be facilitated by several routine practices. Laboratory records that allow efficient assessment of historical patterns can be very helpful. When choosing data elements for record keeping or routine report generation, it is best to select data that will help answer the epidemiologic questions of interest. Building the anticipated database elements into a laboratory information system is an ideal way to ensure that extraction of useful data will be possible. For example, if isolates of an antimicrobial-resistant strain of an organism are being recorded, collecting appropriate case isolate information (location in the facility, service, date, diagnosis) for all isolates of the organism and denominator information (total number of resistant and susceptible isolates of the organism, where they were from, when they were obtained) will make these records most useful. Any changes in protocol or procedure, such as a change in specimen screening criteria or collection of surveillance cultures, that would affect either numerator or denominator counts should be documented (22). For example, an increase in the incidence of ventilator-associated *Serratia* isolates may reflect a new protocol requiring routine daily respiratory cultures in an ICU, rather than a true rise in infections. Also, by knowing the usual number of

IV. ROLE OF THE MICROBIOLOGY LABORATORY (*continued*)

infections per 1,000 ICU patient-days (the baseline rate) caused by antimicrobial-resistant and -susceptible *Acinetobacter* spp., for example, it is then possible to detect changes either in the number or proportion of such infections. The use of patient-days allows us to control for changes in census that might affect a comparison. The use of ICU patient-days, in particular, risk stratifies the comparison, so patients with similar levels of risk are compared over time. By knowing the rate of antimicrobial-susceptible infections and not just the resistant subset, one can determine whether an increase in resistant organisms causing the infections is due to an overall increase in *Acinetobacter* infections in that population and thus a secondary rise in the resistant subset. The alternate explanation would be that the total number remained the same, while a greater proportion was antimicrobial resistant. This clarifies the clinical problem by determining whether something is causing more infections or something is creating more antimicrobial resistance. One additional source of increases in antimicrobial-resistant isolates is multiple repeated cultures. Often a patient discovered to have an antimicrobial-resistant pathogen will have multiple follow-up cultures. If care is not taken to identify and correct such duplication, laboratory-based surveillance can reflect an artificial increase in incidence.

A strategy for storing saved isolates should be devised based on available resources and the infection control/epidemiology needs of each facility. Although it may not be reasonable to save all isolates of potential interest, a subset of organisms may be important to a facility under specific circumstances. For example, an unusual pathogen isolated from a patient in a high-risk unit might warrant ongoing collection of related isolates until an outbreak can be ruled out or assessed. Guidelines to determine when an isolate should be saved for further evaluation will vary, depending on the population being served.

Lastly, the combined leadership of the clinical laboratory and infection control should clearly list specimens or culture sources that are not accepted without special discussion. For quality assurance and for resource utilization purposes, guidance to prevent the submission of poor-quality or inappropriate specimens is critical (23).

V. RESOURCES AND PARTNERSHIPS

Clinical laboratories have several potential resources for determining their optimal role in specific outbreak investigations or surveillance protocols. The most common sources of input are ICPs and hospital epidemiologists, usually a part of a facility's Infection Control Committee, along with representatives from medical, nursing, and administrative departments. The Infection Control Committee should include a microbiologist. By working closely with these colleagues, and offering sound microbiological advice on the selection and utility of laboratory methods for surveillance or outbreak assessment, laboratories can reduce the likelihood of inappropriate or nonproductive utilization of tests and increase the value of the clinical laboratory to the institution.

VI. SOURCES FOR CONSULTATION

A. State health departments vary in the types of services offered. Contact the state health department to find out which consultative services and diagnostic procedures are available. A listing of contact sites for each state is available at http://www.cdc.gov/other.htm.

B. The CDC Division of Healthcare Quality Promotion at the National Center for Infectious Diseases offers consultative services for epidemiologic assessment and outbreak investigations in health care settings. Reference laboratory services are available by referral through the state public health department and are reviewed on a case-by-case basis.

REFERENCES

1. **Armstrong, B. K., E. White, and R. Saracci.** 1992. *Principles of Exposure Measurement in Epidemiology.* Oxford University Press, New York, NY.
2. **Bates, D. W., A. C. O'Neil, D. Boyle, J. Teich, G. M. Chertow, A. L. Kormarof, and T. A. Brennan.** 1994. Potential identifiability and preventability of adverse events using information systems. *J. Am. Med. Info. Assoc.* **1:**404–411.
3. **Boyce, J. M.** 1995. Hospital epidemiology in smaller hospitals. *Infect. Control Hosp. Epidemiol.* **16:**600–606.
4. **Buffington, J., R. Reporter, B. A. Lasker, M. M. McNeil, J. M. Lanson, L. A. Ross, L. Mascola, and W. R. Jarvis.** 1994. Investigation of an outbreak of invasive aspergillosis: utility of molecular typing with the use of random amplified polymorphic DNA probes. *Pediatr. Infect. Dis. J.* **13:**386–393.
5. **Centers for Disease Control and Prevention.** 1991. Nosocomial infection rates for interhospital comparison: limitations and possible solutions. A report from the National Nosocomial Infections Surveillance System. *Infect. Control Hosp. Epidemiol.* **12:**609–621.
6. **Centers for Disease Control and Prevention.** 1995. Recommendations for preventing the spread of vancomycin resistance. Hospital Infection Control Practices Advisory Committee. *Infect. Control Hosp. Epidemiol.* **16:**105–113.
7. **Emori, T. G., and R. P. Gaynes.** 1993. An overview of nosocomial infections, including the role of the microbiology laboratory. *Clin. Microbiol. Rev.* **6:**428–442.
8. **Freeman, J., and J. E. McGowan, Jr.** 1978. Risk factors for nosocomial infections. *J. Infect. Dis.* **138:**811–819.
9. **French, G. L., A. F. Cheng, S. L. Wong, and S. Donnan.** 1989. Repeated prevalence surveys for monitoring effectiveness of hospital infection control. *Lancet* **ii:**1021–1023.
10. **Garner, J. S., W. R. Jarvis, T. G. Emori, T. C. Horan, and J. M. Hughes.** 1988. CDC definitions for nosocomial infections. *Am. J. Infect. Control* **16:**128–140.
11. **Goldmann, D. A., R. A. Weinstein, R. P. Wenzel, O. C. Tablan, R. J. Duma, R. P. Gaynes, J. Schlosser, and W. J. Martone.** 1996. Strategies to prevent and control the emergence and spread of antimicrobial-resistant microorganisms in hospitals: a challenge to hospital leadership. *JAMA* **275:**234–240.
12. **Haley, R. W., D. H. Culver, J. W. White, W. M. Morgan, T. G. Emori, V. P. Munn, and T. M. Hooten.** 1984. The efficacy of infection surveillance and control programs in preventing nosocomial infections in U.S. hospitals. *Am. J. Epidemiol.* **121:**282–287.
13. **Jarvis, W. R.** 1991. Nosocomial outbreaks, the Centers for Disease Control's Hospital Infections Program experience. *Am. J. Med.* **9:**3B101S–3B106S.
14. **Jarvis, W. R.** 1994. Usefulness of molecular epidemiology for outbreak investigations. *Infect. Control Hosp. Epidemiol.* **15:**500–503.
15. **Rhame, F. S., and W. D. Sudderth.** 1981. Incidence and prevalence as used in the analysis of the occurrence of nosocomial infections. *Am. J. Epidemiol.* **113:**1–11.
16. **Rudnick, J. R., C. M. Beck-Sague, R. L. Anderson, B. Schable, J. M. Miller, and W. R. Jarvis.** 1996. Gram-negative bacteremia in open heart surgery patients traced to probable tap water contamination of pressure monitoring equipment. *Infect. Control Hosp. Epidemiol.* **17:**272–275.
17. **Sadler, H. S., R. H. Hollis, and M. A. Pfaller.** 1995. The use of molecular techniques in the epidemiology and control of infectious diseases. *Clin. Lab. Med.* **15:**407–431.
18. **Scheckler, W. E.** 1992. Continuous quality improvement in a hospital system: implications for hospital epidemiology. *Infect. Control Hosp. Epidemiol.* **13:**288–292.
19. **Simmons, B., J. Bryant, K. Nieman, L. Spencer, and K. Arheart.** 1990. The role of hand washing in prevention of endemic intensive care unit infection. *Infect. Control Hosp. Epidemiol.* **11:**589–594.
20. **Stamm, W. E., R. A. Weinstein, and R. E. Dixon.** 1981. Comparison of endemic and epidemic nosocomial infections. *Am. J. Med.* **70:**393–397.
21. **Weems, J. J., B. J. Davis, O. C. Tablan, L. Kaufman, and W. J. Martone.** 1987. Construction activity: an independent risk factor for invasive aspergillosis and zygomycosis in patients with hematologic malignancy. *Infect. Control* **8:**71–75.
22. **Wenzel, R. P., and S. A. Streed.** 1989. Surveillance and use of computers in hospital infection control. *J. Hosp. Infect.* **13:**217–229.
23. **Wilson, M. L.** 1996. General principles of specimen collection and transport. *Clin. Infect. Dis.* **22:**766–777.

APPENDIX 13.4–1

Websites and Contacts

Division of Healthcare Quality Promotion
http://www.cdc.gov/ncidod/dhqp/about.html

Infection control in health care settings
http://www.cdc.gov/ncidod/dhqp/index.html

Laboratory testing and practices (antimicrobial resistance and other diseases transmitted during health care)
http://www.cdc.gov/ncidod/dhqp/labTesting.html

Other infectious diseases
http://www.cdc.gov/DiseasesConditions/

13.5 Epidemiologic Strain Typing

The diversification of health care settings (e.g., home health care, nursing homes, assisted living, or outpatient dialysis centers) presents new challenges for the clinical microbiologist, hospital epidemiologist, and infection control professional as they work together to detect and investigate disease trends and outbreaks. A valuable tool in epidemiologic investigations is strain typing. This procedure is a quick guide that offers a simplified list of typing techniques of potential utility to clinical microbiologists. Readers seeking in-depth descriptions or detailed protocols should refer to References and Supplemental Reading.

Strain typing may be used (i) to recognize or confirm an outbreak, (ii) to confirm the source of transmission of an organism, (iii) to detect recurrent versus new infections in a patient, and (iv) to measure the impact of intervention strategies or prevention programs or to monitor trends in a microbial population, e.g., increasing proportions of a resistant strain of *Staphylococcus aureus* in a dialysis center (4).

Strain typing techniques are used to compare isolates of the same species to determine whether they share features, e.g., DNA macrorestriction patterns, nucleic acid sequences, or phenotypic traits that would suggest that they share a common origin. A strain, for purposes of this discussion, is represented by a group of isolates that have a common ancestor, and are indistinguishable from one another, but when analyzed by an effective typing method can be distinguished from other isolates of the same species. Organisms causing outbreaks can be related by strain type and by epidemiologic associations (7). Occasionally, an outbreak may include multiple strains.

Typing systems should be selected based on their utility for use with the target organism, discriminatory power, reproducibility, and ease of interpretation and use. The method chosen should be based on a feature of the target organism that is relatively stable and has adequate diversity within the species. Serotyping, for example, is based on antigenic surface determinants and is useful for typing organisms including *Salmonella, Shigella, Legionella pneumophila,* and *Haemophilus influenzae.* There are over 2,000 antisera available for *Salmonella* typing (3). In contrast, serotyping is not useful for *S. aureus* because the organism does not possess adequate antigenic heterogeneity; i.e., epidemiologically unrelated organisms are likely to share a serotype (8). The discriminatory power of a typing method refers to its ability to differentiate among unrelated strains. A typing system should give consistent, reproducible results for the same isolate when tested on multiple occasions and be consistent for isolates over time, since epidemiologic investigations may be conducted over several months or even years (8). Typing methods vary greatly in their species-specific utility, complexity, interpretation, and rapidity (Table 13.5–1).

Typing methodologies are broadly divided into phenotypic (expressed) and genotypic (chromosomal and plasmid DNA, or RNA) categories.

I. PHENOTYPIC

Observe standard precautions.

A. Biotyping

Biotyping was one of the first methods used to investigate hospital outbreaks. The biotype is a profile generated from a battery of biochemical reactions used to identify an organism. Since isolates that vary by one or more reactions are not recognized as related, biotyping, for epidemiologic purposes, is not very discriminating (2, 4).

B. Antimicrobial susceptibility testing (AST)

AST may identify a new or changing antimicrobial susceptibility pattern that can be the first signal of an outbreak or a clinically significant trend. The information is readily available and inexpensive and may be useful in certain situations. The utility of AST for epidemiologic typing, however, is limited by changes in the microbial ecology of health care settings, e.g., the increasing rates of endemic methicillin-resistant *S. aureus* in hospitals. Susceptibility of organisms in an environment can change relatively quickly in response to the

Table 13.5–1 Typing systems for common outbreak isolates[a]

Organism(s)	Primary method	Time (days) to result	Other methods used
S. aureus	PFGE	3	AP-PCR, plasmid profiling, Rep-PCR
Coagulase-negative staphylococci	PFGE	3	Plasmid profiling
Enterococci	PFGE	3	AP-PCR
Streptococcus pneumoniae	PFGE	3	Serotyping
Listeria monocytogenes	PFGE	3	
Burkholderia, Stenotrophomonas, Acinetobacter	PFGE	3	
E. coli, Citrobacter, Proteus, Providencia	PFGE	3	Plasmid analysis, Rep-PCR, AP-PCR
Klebsiella, Enterobacter, Serratia	PFGE	3	Plasmid analysis
Salmonella, Shigella	Serotyping	1	PFGE
Pseudomonas aeruginosa	PFGE	3	
C. difficile	Rep-PCR, AP-PCR	1	PFGE
Mycobacterium tuberculosis	IS*6110* RFLP	1	Rep-PCR
Other mycobacteria	PFGE	3	

[a] Compilation of data from references 1, 4, 6, and 8. AP-PCR, arbitrarily primed PCR.

I. PHENOTYPIC (*continued*)

selective pressure of antimicrobial use and can affect the utility of AST for epidemiologic typing (2).

C. Serotyping

Serotyping is based on antigenic determinants of bacterial cell components such as outer membranes, flagella, or capsules. Serotyping is used for *Salmonella, Shigella, Klebsiella, Streptococcus pneumoniae,* and other organisms but is not very discriminating for many other organisms.

D. Polyacrylamide gel electrophoresis

PAGE detects differences in cellular proteins by molecular size. Proteins are separated electrophoretically into visible bands within an acrylamide gel matrix. The protein bands can be visualized by staining or, rarely, detected radioactively (1).

E. Immunoblotting

Immunoblotting (Western blotting) is performed as a way to extract more information from PAGE. Following electrophoretic separation, proteins are transferred to a nitrocellulose membrane that is then exposed to specific antibodies, a labeled antiserum, and a detection substrate for visualization (1).

F. Bacteriophage typing

Bacteriophage typing identifies strains by their patterns of susceptibility to a specific set of phages (viruses that infect bacterial cells and cause lysis). This technique has been used primarily for *S. aureus*; however, DNA techniques have become the preferred method for *S. aureus* in the United States (2).

G. Multilocus enzyme electrophoresis (MLEE)

MLEE uses electrophoretic separation to distinguish among variants of neutral proteins, e.g., metabolic enzymes, of the organism. Variations are analyzed using mathematic algorithms (4).

II. GENOTYPIC

Observe standard precautions.

A. Plasmid profile analysis (PPA)

PPA (also known as plasmid fingerprinting) is one of the oldest genotypic methods of typing for epidemiologic support. Testing can be performed on many species, provides results in one day, is useful for high-volume testing, and is fairly inexpensive. PPA also is useful when serotyping of rough-phenotype colonies is not possible (5). However, plasmids can be acquired or deleted. For organisms that have few or no plasmids, this technique cannot be used. Plasmids are collected from the organism and assessed using restriction endonuclease digestion to yield enzyme-specific arrays of fragment sizes. This typing method has utility for assessing staphylococci, *Klebsiella, Enterobacter,* and *Serratia* (4, 8).

B. Restriction endonuclease analysis (REA)

Chromosomal REA uses restriction endonucleases that cut frequently to yield enzyme-specific fragments of the entire chromosome. This results in hundreds of bands. The variable presence of plasmid DNA, which is not reliably eliminated in this process, can affect the resulting patterns. This method has been used successfully for *Clostridium difficile* (7).

C. Southern blotting

Southern blotting utilizes the polymorphic nature of the locations of restriction enzyme target sequences in chromosomal DNA. Digested fragments are separated by gel electrophoresis, transferred to nitrocellulose or nylon membranes, and treated with radioactive, colorimetric, or chemiluminescently labeled DNA probes that bind to homologous chromosomal fragments. This method is more time-consuming and laborious than other methods available for routine epidemiologic typing (8).

D. Ribotyping

Ribotyping is an adaptation of the Southern blot-restriction fragment length polymorphism (RFLP) method. Probes from the 16S and 23S rRNA genes of *Escherichia coli* are used, reducing the complexity of patterns seen with simple restriction digestion while retaining enough variability to allow discrimination of strains. This method has been used for epidemiologic studies of pathogens including *E. coli* and *S. aureus* (7, 8).

E. Pulsed-field gel electrophoresis (PFGE)

PFGE was developed in 1984 by Schwartz and Cantor, originally for eukaryotic cells, and adapted for use in bacteria (6). Organisms are embedded in agarose, wherein cells are lysed and chromosomal DNA is released and digested with restriction enzymes. The DNA fragments are separated by electrophoresis through the agarose matrix using a pulsed current, oriented diagonally, allowing separation of very large DNA fragments. This method is highly reproducible and discriminatory (4).

F. PCR-based locus-specific restriction fragment length polymorphism (RFLP) typing

PCR-based locus-specific RFLP typing amplifies a gene locus using specific primers and then analyzes the product by electrophoresis. PCR is based on the amplification of a minute quantity of DNA by repetitive replication cycles. Heat-stable polymerase enzymes are used, and incubation in heating devices with automated temperature controls (thermocyclers) allows repetitive association and dissociation between a DNA target sequence and a selected set of replication primers. The amplified DNA sequence is cut by restriction endonuclease digestion, and fragments are separated electrophoretically in an agarose or polyacrylamide gel and visualized by staining with ethidium bromide (4). PCR can detect DNA from organisms that might not be retrievable by conventional culture methods, including nonviable organisms. However, PCR techniques are extremely susceptible to contamination and cross-contamination, since any amount

II. GENOTYPIC (*continued*)

of contaminating DNA can be amplified and result in the appearance of spurious bands on gel electrophoresis.

G. Randomly amplified polymorphic DNA (RAPD) analysis

RAPD analysis is performed using an arbitrary sequence for the primer. A short primer that hybridizes at random chromosomal sites results in amplification of an array of sequences of various lengths. The resultant sequences are analyzed by gel electrophoresis (5).

H. Repetitive element (Rep)-PCR

Rep-PCR amplifies repetitive sequences in bacterial genomes, which result in strain-specific patterns. Enterobacterial repetitive intergenic consensus (ERIC) is another set of elements that are used for typing. ERIC patterns are not as complex as Rep patterns. When used in conjunction, both Rep and ERIC amplifications enhance the discriminatory ability of PCR. Rep-PCR can be automated and data from multiple outbreaks can be compared (5).

I. Cleavase fragment length polymorphism (CFLP)

CFLP uses a thermostable endonuclease, cleavase 1, which recognizes and cuts DNA at the sites of secondary structures formed only at higher temperatures (5).

J. Amplified fragment length polymorphism (AFLP)

AFLP amplifies DNA fragments created by restriction enzyme digestion. The most common procedure uses two enzymes to make fragments with differing end sequences. The cut DNA fragments are linked to fragments containing the sequence for binding the PCR primer. PCR is performed, amplified fragments are separated electrophoretically, and the patterns are examined using radioactive or fluorescent labels or ethidium bromide staining (5).

K. Nucleic acid sequencing

Nucleic acid sequencing can be performed on amplified sequences of DNA or RNA. Sequencing reactions incorporating labeled nucleotides are examined by gel electrophoresis. The sequence is read using differential fluorescent labeling of nucleotides. Resulting data can be stored on computers for sequence comparison. This method is the "gold standard" for viral typing (5). The utility of single-gene sequencing of bacteria in epidemiologic investigations varies with the bacteria and gene sequence selected. For example, sequencing the *emm* gene of *S. pyogenes* provides similar data to M serotyping. Selecting genes with high variation provides more discrimination than selecting stable genes. Similarly, sequencing multiple genes (see below) also provides better discrimination.

L. Multilocus sequence typing (MLST)

Nucleic acid sequencing of multiple housekeeping genes has emerged as a major tool in the typing of many species of organisms. The utilization of more stable portions of the genome is very effective in identifying strains of common lineage (1). While MLST is helpful for understanding global epidemiology and evolutionary changes over time, PFGE has greater discriminatory power.

III. RESEARCH VERSUS CLINICAL TOOLS

This brief overview of strain typing methods demonstrates the wide range of techniques available. The list is not exhaustive, and even among those methods included, many are too costly or are unavailable for clinical use, or they generate results that are not useful for purposes of epidemiology. Nucleic acid amplification technology has resulted in powerful methods that raise the possibility of identifying and characterizing organisms directly from clinical specimens, instead of awaiting pure cultures. These methods now allow identification of organisms that we remain unable to culture, e.g., hepatitis C virus and the organism causing Whipple's disease, *Tropheryma whipplei* (4). New organisms are now being classified using 16S rRNA sequencing.

Table 13.5–2 Comparison of strain typing methods[a]

Typing method	Range of utility	Reproducibility	Discrimination	Ease of use	Ease of interpretation
Phenotypic methods					
AST	√√√√	–	–	√√√√	√√√√
Biotyping	√√√√	√	–	√√√√	√√√√
Serotyping	√√√	√√	√	√	√√
Phage typing	√√√	√	√	–	√
MLEE	√√√√	√√√√	√√	√√	√√√√
Genotypic methods					
Plasmid profiling	√√√	√√	√√	√√	√√
Chromosomal REA	√√√√	√	√	√√	√
Ribotyping	√√√√	√√√√	√√	√√	√√
PFGE	√√√√	√√√√	√√√√	√√	√√
RFLP	√√√√	√√√√	√√√	–	√√
Arbitrarily primed PCR	√√√√	√√	√√	√√	√√
Sequencing	√√	√√√√	√√	√√√	√√√
MLST	√√√	√√√	√√√	√	√√√

[a] –, poor; √, fair; √√, good; √√√, very good; √√√√, excellent.

III. RESEARCH VERSUS CLINICAL TOOLS (*continued*)

Techniques currently felt to be clinically useful, adaptable, and readily available for strain typing to support epidemiologic investigations are compared above (Table 13.5–2).

IV. RELATIVE COST

Strain typing costs are determined by the same parameters as other laboratory tests. Instrumentation costs vary greatly; for RFLP, RAPD analysis, and CFLP, equipment costs range from $8,000 to $12,000; PFGE equipment costs range from $10,000 to $20,000; and AFLP and DNA sequencing equipment costs range between $45,000 and $130,000. The relative cost per test has been estimated at $11 for RAPD analysis, $14 for RFLP, $20 for AFLP, $22 for PFGE, $40 for CFLP, and $40 for DNA sequencing (5).

REFERENCES

1. **Maslow, J., and M. E. Mulligan.** 1996. Epidemiologic typing systems. *Infect. Control Hosp. Epidemiol.* **17:**595–604.
2. **Mayhall, C. G. (ed.).** 1999. *Hospital Epidemiology and Infection Control,* 2nd ed., p. 1426–1435. Lippincott Williams & Wilkins, Philadelphia, PA.
3. **McWhorter-Murlin, A. C., and F. W. Hickman-Brenner.** 1994. *Identification and Serotyping of Salmonella and an Update of the Kauffmann-White Scheme*. U.S. Department of Health and Human Services, Washington, DC.
4. **Murray, P. R., E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.).** 2007. *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
5. **Olive, D. M., and P. Bean.** 1999. Principles and applications of methods for DNA-based typing of microbial organisms. *J. Clin. Microbiol.* **37:**1661–1669.
6. **Schwartz, D. C., and C. R. Cantor.** 1984. Segregation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell* **37:**67–75.
7. **Tenover, F., R. Arbeit, R. Goering, P. Mickelsen, B. Murray, D. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* **33:**2233–2239.
8. **Tenover, F., R. Arbeit, R. Goering, and the Molecular Typing Working Group of the Society for Healthcare Epidemiology of America.** 1997. How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. *Infect. Control Hosp. Epidemiol.* **18:**426–439.

SUPPLEMENTAL READING

Maiden, M. C. 2006. Multilocus sequence typing of bacteria. *Annu. Rev. Microbiol.* **60:**561–588.

Persing, D. H., F. C. Tenover, J. Versalovic, Y.-W. Tang, E. R. Unger, D. A. Relman, and T. J. White. 2003. *Molecular Microbiology: Diagnostic Principles and Practice.* American Society for Microbiology, Washington, DC.

13.6 Culture of Hospital Water for *Legionellaceae*

I. PRINCIPLE

A. Ecology and transmission

1. *Legionella* spp. are ubiquitous in the aquatic environment. They are commonly found in a variety of natural and manufactured water sources, including drinking water systems, cooling towers, evaporative condensers, hot-water heaters, and respiratory therapy equipment.

2. The epidemiologic reservoir for nosocomial infections caused by *Legionella* spp. has been shown to be hospital water distribution systems, especially those for hot water. These organisms have been shown to be part of the microbiota that normally populates these systems (11).

3. Acquisition of legionellosis occurs primarily after inhalation of aerosols containing *Legionella* organisms (2). It has not been possible to document the environmental source of all *Legionella* infections of humans, particularly when sporadic cases occur, making "common-source" investigation irrelevant.

4. Certain people are more susceptible to *Legionella* infections than others. The more susceptible include the elderly, those with a history of smoking, those with underlying diseases such as reduced immune response, and surgical patients (9).

B. Source confirmation and isolation criteria

1. Recovery of *Legionella* spp. from an environmental source does not constitute proof that this is the source of the infecting agent. Legionellae are frequently isolated from water sources unrelated to human disease (2). Routine testing of water sources is generally not indicated unless there have been clinical cases of legionellosis or it is required by local regulations.

2. Four criteria must be considered when investigating the source of a *Legionella* outbreak.

a. Association between exposure and a potential source

b. Identification of the mechanism of aerosol production

c. Clear documentation of *Legionella* etiology

d. Isolation of the same *Legionella* species from patients and implicated environmental source. In settings where a species of *Legionella* is ubiquitous, subtyping may be necessary for adequate association of the disease with an environmental source.

II. REAGENTS, MEDIA, AND SUPPLIES

A. Collection containers and swabs

1. Widemouthed glass or plastic bottles; for example, Nalgene polypropylene 4- and 32-oz. (1 oz. = 29.573 ml) bottles (*see* Appendix 13.6–1 for addresses of suppliers)

2. Sterile cotton or polyester applicator; for example, S/P sterile Dacron-tipped applicator

B. Filters and filter assemblies

1. 0.2-µm-pore-size polycarbonate membrane filters (Nuclepore)

2. Plastic filter funnel, 47 mm; for example, Gelman 47-mm magnetic plastic filter funnel

3. Filter funnel holder; for example, Gelman three-port filter funnel manifold

4. Stainless steel forceps; for example, Nalgene stainless steel filter forceps

C. Media

1. The most widely used medium is an ACES (*N*-2-acetamido-2-aminoethanesulfonic acid)-buffered (pH 6.9) charcoal yeast extract agar supplemented with cysteine, ferric pyrophosphate, and, optimally, α-ketoglutarate ($BCYE_\alpha$) (*see* Appendix 13.6–2 for formulation).

2. For environmental sites, selective media may also be necessary to prevent overgrowth of non-*Legionella* organisms. The two most commonly used selective media are GPVA medium ($BCYE_\alpha$ supple-

II. REAGENTS, MEDIA, AND SUPPLIES (*continued*)

mented with glycine, polymyxin B, vancomycin, and anisomycin) and DGVP (BCYE$_\alpha$ supplemented with dyes, glycine, vancomycin, and polymyxin B) (*see* Appendix 13.6–2 for formulation).

3. Other *Legionella* selective media have been described and are also satisfactory. However, different manufacturers may have slight variations in antibiotic supplement formulations.

D. Reagents

1. Acid treatment reagent, pH 2.0
   Acid treatment of water is used to isolate *Legionella* spp. by suppressing the growth of other organisms not able to withstand exposure to acid (pH 2.0) (*see* Appendix 13.6–2).
2. Membrane-filtered reagents
   Membrane filtration of all direct-fluorescence antibody reagents is recommended to eliminate *Legionella* spp. or cross-reacting organisms that have been found in these reagents and that cause false-positive direct-fluorescence assay reactions. Steam (or other) sterilization techniques are inadequate because they render organisms nonviable but do not remove them from the reagents, where they can be visually detected (*see* Appendix 13.6–2).
   a. Filter-sterilized phosphate-buffered saline, pH 7.6
   b. Filter-sterilized deionized or distilled water
3. Chlorine neutralizer for water collection container: sodium thiosulfate (Sigma Chemical Co.)
4. Buffered-glycerol mounting fluid, pH 9.0 (*see* Appendix 13.6–2).
5. *Legionella* direct-fluorescence assay polyclonal reagent (commercially available); for example, SciMedx polyvalent polyclonal direct-fluorescence assay conjugate for *Legionella pneumophila*, serogroups 1 to 6.

E. Microscope and supplies

1. Fluorescence microscope with a filter system for fluorescein isothiocyanate detection and filters that produce a maximum excitation wavelength of 490 nm and a mean emission wavelength of 520 nm; for example (other microscopes and assemblies may give comparable results), Leitz Dialux 20 fluorescence microscope with HBO-100 mercury incident light source, Leitz I cube filter system (2 × KP490 and 1-mm GG455 primary filter, TK 510 dichroic beam-splitting mirror, and K515 secondary filter), 10× to 40× dry objective, 63× to 100× oil objective, and 6.3× to 10× eyepiece
2. Stereoscopic microscope; for example (other microscopes and assemblies may give comparable results), Reichert-Jung Series 40 Student stereoscopic microscope with either 15× fixed or 10× to 20× Magni-Changer magnification with 10× wide-field eyepiece
3. Illuminator (for oblique lighting); for example, Reichert-Jung Stereostar/200M Starlite illuminator with fixed, prefocused condenser
4. Fluorescence microscope slides
   Slide has two etched 10-mm circles and is frosted on one side of one end; for example, S/P fluorescent slide

PREANALYTICAL CONSIDERATIONS

III. SPECIMENS

Observe standard precautions.

A. Site and sample selection

1. Select sampling sites based on epidemiologic data suggesting an association between patients and potential sources of water.
2. Sources of water that have been shown to contain *Legionella* spp. (2) are listed below as a guide for selecting samples.
   a. General potable-water system of hospital
      (1) Hot-water heater (water and sediment)
      (2) Holding tank
   b. Potable-water final-distribution outlets (patient contact areas)
      (1) Shower outlet
      (2) Hot- and cold-water faucets
      (3) Hemodialysis water (incoming)

III. SPECIMENS (*continued*)

c. Equipment
(1) Respiratory therapy
(2) Humidifiers
(3) Ice maker and ice
(4) Whirlpools (water and sediment)

B. Sample collection

The two primary types of samples that should be collected in a potable-water system are a swab and a 1-liter water sample at each site. Larger volumes of water (1 to 10 liters) may be needed for detection of legionellae in water having very low counts. Swabs are collected to address the issue of localization of the organisms in the distal termini of the water distribution system, from various points within plumbing systems, or from surfaces of spas or cooling towers. If necessary, hot-water storage tank samples can be collected as outlined below.

1. Swab samples
a. After removal of showerheads or aerators, insert sterile swabs into shower and sink outlets and rotate them against the interior surface to dislodge residue (approximately four times while moving swab upwards into the opening).
b. Plate swabs directly onto $BCYE_{\alpha}$ and $BCYE_{\alpha}$ selective media by streaking them down the middle of the agar surface and cross-streaking with a fresh sterile swab or loop (*see* Appendix 13.6–2 for medium formulations).
c. To keep swabs moist in case further processing is necessary, place swabs in a small volume of original water taken at the same time. Store refrigerated.

2. Water samples
a. After the swab sample has been taken, collect a 1-liter sample in an appropriate sterile water collection bottle. Use aseptic technique.
b. Sample containers should be sterile, preferably widemouthed, and either plastic or glass. If water is chlorinated, include sodium thiosulfate in the sterile container as a chlorine neutralizer. Add 0.83 ml of 0.1 N sodium thiosulfate solution to each liter sample container prior to sterilization. This concentration of sodium thiosulfate will neutralize approximately 5 mg of free chlorine per liter (1).
c. Transport samples to the laboratory in insulated containers to protect samples from extremes in temperature.
d. If samples cannot be processed the same day they are collected, refrigerate them at 4°C to minimize decline of *Legionella* spp. and to minimize overgrowth of competing microbiota in sample. Process samples in the laboratory as soon as possible, preferably within 48 h of collection.

3. Hot-water storage tanks
a. Immediately collect the first 10 to 50 ml of the water into a suitable sterile container. This sample reflects the stagnant water in the supply pipe.
b. Allow the water to flush for approximately 30 s, and then collect another 10 to 50 ml in a suitable sterile container. This water reflects the tank.
c. Process both samples with direct-plating technique (*see* item V.B).

ANALYTICAL CONSIDERATIONS

IV. QUALITY CONTROL AND QUALITY ASSURANCE

A. Commercially prepared, ready-to-use media

The CLSI subcommittee on medium QC recommends that media not be retested by the user as long as the media are obtained from commercial sources that employ the QC criteria listed in the CLSI standard for commercially prepared microbiological culture media (5).

IV. QUALITY CONTROL AND QUALITY ASSURANCE (*continued*)

B. Media prepared in-house

Each batch of media must be tested for pH and the ability to support the growth of legionellae (5, 7).

1. Measure pH.

a. The pH of the medium is critical. It must be 6.9 ± 0.05.

b. To check solid media, use a surface electrode (if available), or emulsify the agar from one plate in distilled water and measure the pH of the emulsion.

c. If given lots of ingredients do not conform to the acceptable pH range, adjust the pH of the completed liquid medium by adding sterile 1 N HCl or 1 N KOH.

2. Check for support of *Legionella* growth.

a. Prepare a standardized inoculum by emulsifying actively growing (in logarithmic stage) *L. pneumophila* in sterile distilled or deionized water to a turbidity equivalent of a 0.5 McFarland standard. The CLSI subcommittee on medium QC recommends using *L. pneumophila* ATCC 33152 and *Legionella bozemanii* ATCC 33217 (5).

b. Dilute the cell suspension 1:100 in sterile phosphate-buffered saline, and inoculate each test plate with a 10-μl calibrated loop of the diluted suspension to provide 10^3 to 10^4 CFU per plate. Streak the plates for isolated colonies, and incubate at 35°C in a humidified atmosphere (>50%). If this does not provide isolated colonies on the media, use a 10-fold-lighter inoculum (10).

c. Examine the media daily. On agar plates, growth should be present in the heavily inoculated area after 1 day. Isolated colonies should be macroscopically visible in 2 to 3 days. If cells in standard inoculum have been refrigerated or frozen, growth will be slower.

3. Selective capability of selective medium

Since the CLSI subcommittee on medium QC has not listed an ATCC organism with which to test the inhibitory capacity of *Legionella*-selective media and since there is no current literature on this subject, a standard procedure cannot be recommended (5). If possible, selective media should be checked using an environmental water sample known to contain legionellae and other bacteria.

4. Sterility check

Incubate one uninoculated plate to check for sterility.

C. QC of reagents

Fluorescent-antibody reagent

1. Each day the test is performed, use a positive and a negative control to verify that the staining agent is reactive and that all reagents are *Legionella* free.

2. Process a positive control smear separately from sample smears to avoid possible carryover.

D. QA

Report all isolates of *Legionella* to the Infection Control Committee for review of QA.

V. SAMPLE PREPARATION

A. Concentration by filtration (potable water)

Samples such as potable ("drinking") water have very low bacterial counts and usually require concentration by filtration for successful recovery of *Legionella* (Fig. 13.6–1).

1. Filter samples though sterile 47-mm filter funnel assemblies containing 0.2-μm-pore-size filters. Capillary pore membranes are preferred (i.e., Nuclepore polycarbonate) because of their uniform, smooth-bore pores.

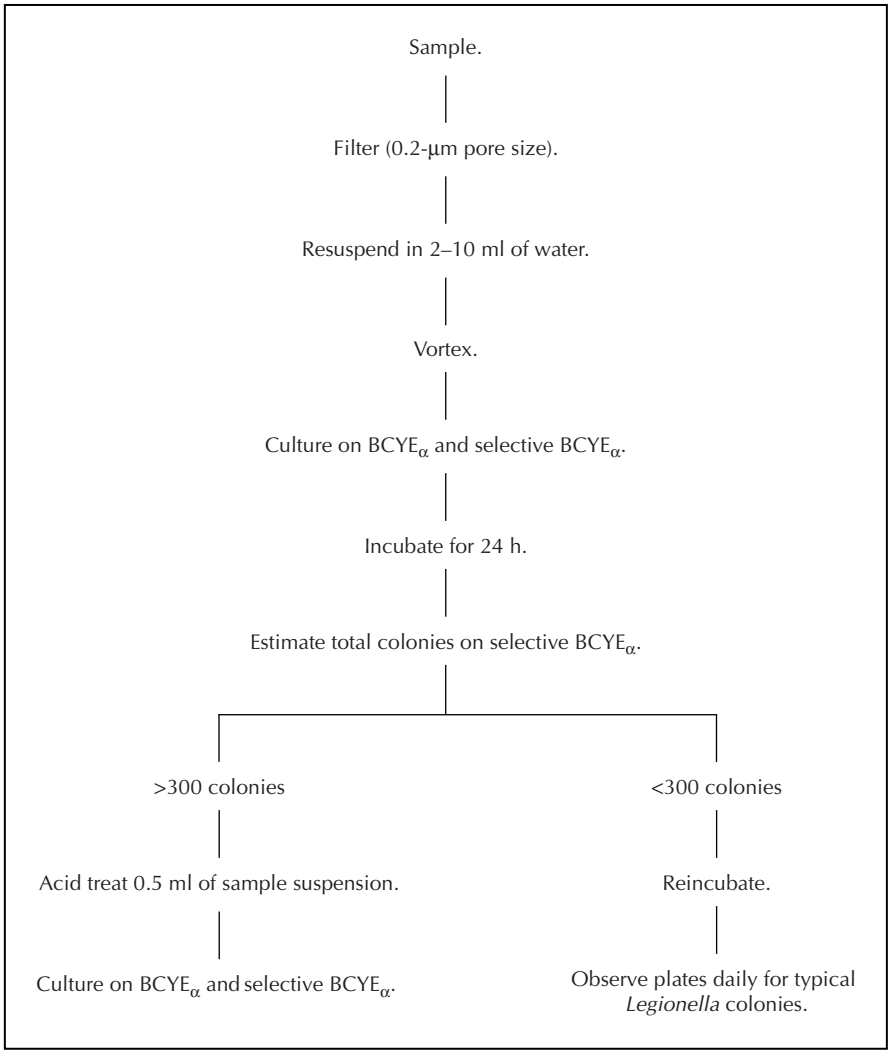
Sample.
Filter (0.2-μm pore size).
Resuspend in 2–10 ml of water.
Vortex.
Culture on BCYE$_\alpha$ and selective BCYE$_\alpha$.
Incubate for 24 h.
Estimate total colonies on selective BCYE$_\alpha$.
>300 colonies
<300 colonies
Acid treat 0.5 ml of sample suspension.
Reincubate.
Culture on BCYE$_\alpha$ and selective BCYE$_\alpha$.
Observe plates daily for typical *Legionella* colonies.

Figure 13.6–1 Flowchart for concentration by filtration.

V. SAMPLE PREPARATION (*continued*)

2. Immediately remove the filter with sterile forceps, and place it soiled side down or folded to the outside in a 50-ml sterile centrifuge tube or similar-size vessel containing 2 to 10 ml of original sample (record volume). The smaller amount of original sample will yield a lower detection limit.
3. If more than one filter is required to concentrate a sample, combine all filters into one tube.

B. Direct plating (nonpotable water and sediment)

Samples other than drinking water, such as nonsterile humidifier water, need not be concentrated (Fig. 13.6–2). Place a 10-ml sample in a 50-ml centrifuge tube and process directly.

C. Sample dispersion for both concentration and direct-plating samples

Tighten caps on sample centrifuge tubes. Vortex for 30 s to disperse organisms from filter or break up aggregates in water (3, 7).

D. Acid treatment for concentration by filtration (7)

1. Samples that are found to have high concentrations of bacteria after plating will require acid (pH 2.0) treatment to improve recovery of *Legionella* spp.

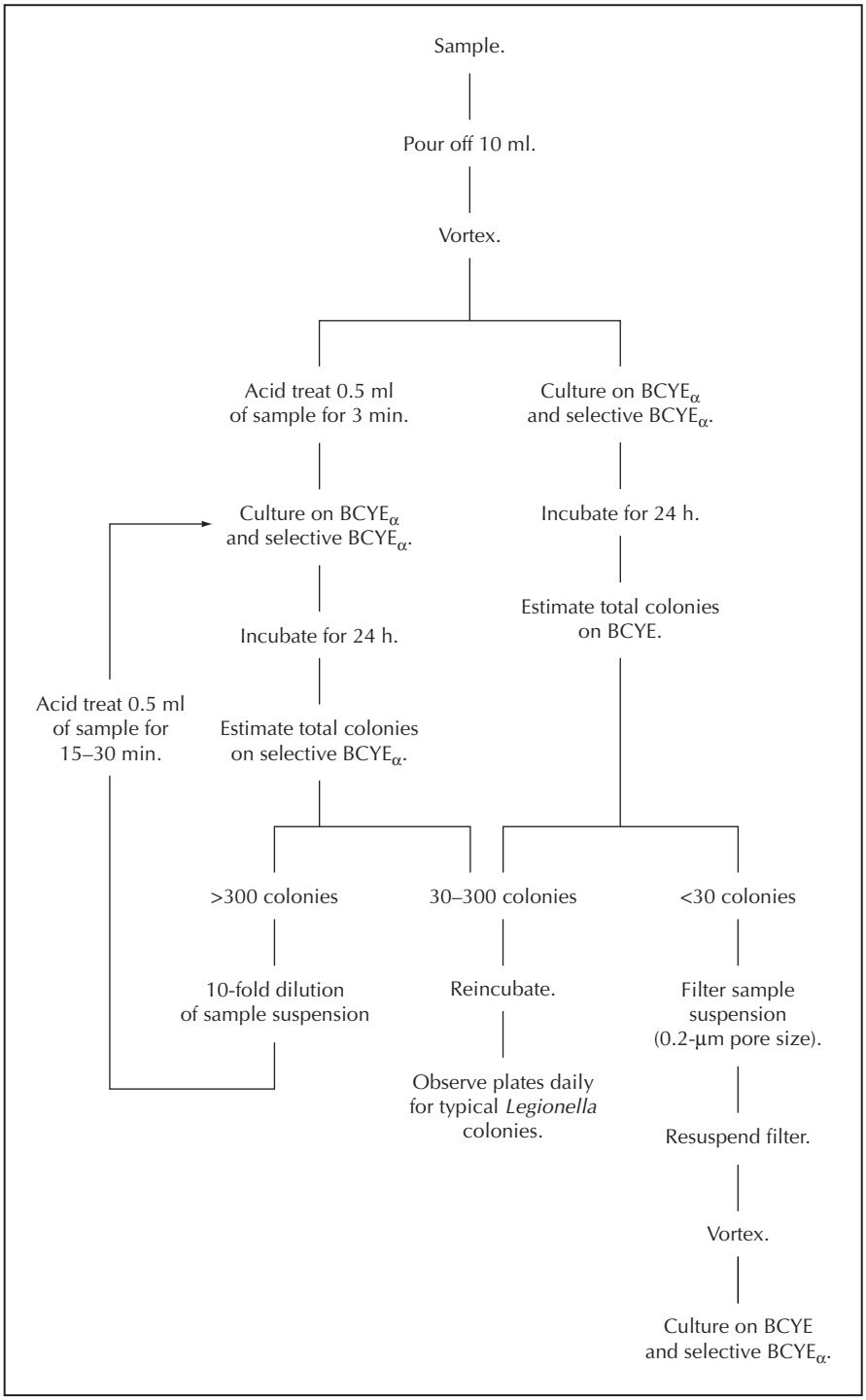
Sample.
Pour off 10 ml.
Vortex.
Acid treat 0.5 ml of sample for 3 min.
Culture on BCYE$_\alpha$ and selective BCYE$_\alpha$.
Culture on BCYE$_\alpha$ and selective BCYE$_\alpha$.
Incubate for 24 h.
Incubate for 24 h.
Estimate total colonies on BCYE.
Acid treat 0.5 ml of sample for 15–30 min.
Estimate total colonies on selective BCYE$_\alpha$.
>300 colonies
30–300 colonies
<30 colonies
10-fold dilution of sample suspension
Reincubate.
Filter sample suspension (0.2-µm pore size).
Observe plates daily for typical *Legionella* colonies.
Resuspend filter.
Vortex.
Culture on BCYE and selective BCYE$_\alpha$.

Figure 13.6–2 Flowchart for direct plating.

V. SAMPLE PREPARATION (*continued*)

2. Place 0.5 ml of the vortexed suspension into a sterile screw-cap tube (13 by 100 mm) containing 0.5 ml of acid treatment reagent, and mix. (The final pH of the mixture should be approximately 2.2.)
3. Let the acidified suspension stand for 3 min at room temperature. Acid treatment can be extended to 15 to 30 min if plates are overgrown after the initial 3-min treatment period (12).

VI. SPECIMEN PLATING

A. Concentration-by-filtration samples
Plate specimens without acid treatment.

B. Direct-plating specimens
Plate both acid-treated and untreated suspensions.

C. Procedure

1. Inoculate 0.1 ml of suspension onto duplicate plates of $BCYE_\alpha$ and selective $BCYE_\alpha$, such as GPVA or DGPV. A BAP may also be utilized for each sample to indicate the presence of organisms with colony morphologies similar to those of legionellae. If species other than *L. pneumophila* are expected, include duplicate $BCYE_\alpha$ plates that contain 1.0% albumin ($ABCYE_\alpha$). *Legionella micdadei* and *L. bozemanii* have shown a preference for this medium (9).

2. Spread inoculum with a sterile glass (or disposable plastic) rod.

D. Storage of sample suspensions
Store sample suspensions at 4°C in case further dilutions, concentrations, or acid treatment is necessary.

VII. INCUBATION

Incubate all plates at 35°C in a humidified atmosphere (>50%) for up to 10 days.

◩ **NOTE:** A candle jar or humidified CO_2 incubator (2 to 5% CO_2) is acceptable.

VIII. CULTURE EVALUATION

A. Total-bacterial-count examination
After 24 h of incubation, examine plates to determine the adequacy of processing for each sample set. Ideally, there should be a plate with 30 to 300 colonies (Fig. 13.6–1 and 13.6–2).

1. Original direct-plating samples

a. If the total count of non-*Legionella* organisms in an acid-treated sample exceeds 300 colonies on $BCYE_\alpha$ selective medium, make a further 10-fold dilution of the refrigerated sample suspension. Repeat acid treatment (for 15 to 30 min) and plating (*see* item V.D above).

b. If the total count in a non-acid-treated sample is fewer than 30 colonies on $BCYE_\alpha$ agar, concentrate the original sample water as previously described (*see* item V.A above).

2. Original concentration-by-filtration sample

a. If the total count in a sample exceeds 300 colonies on $BCYE_\alpha$ selective medium, treat the refrigerated sample suspension with acid as previously described for direct-plating samples (*see* item V.D above).

b. If the total count in a sample is fewer than 300 colonies on $BCYE_\alpha$ agar, reincubate plates.

B. Examination of cultures for legionellae

1. After the initial 24 h of incubation, examine all cultures daily with the aid of a dissecting microscope for the appearance of gray-white bacterial colonies that have a "ground-glass" appearance.

2. Using a bacteriological needle, aseptically pick each suspect colony onto BCYE agar and a $BCYE_\alpha$ agar plate prepared without L-cysteine. If $BCYE_\alpha$ agar without L-cysteine is not available, a BAP can be used with the following precaution: some *Legionella* strains adapt to grow on blood agar in the absence of L-cysteine but require cysteine when first isolated (6).

3. Streak the inoculated portion of each plate with a sterile loop to provide areas of heavy growth, and incubate for up to 48 h. One plate can be divided, and several suspect colonies can be evaluated for growth requirements.

VIII. CULTURE EVALUATION (*continued*)

C. Presumptive identification of *Legionella* (Fig. 13.6–3)

1. Colony characteristics

a. Macroscopic: slow-growing (3 to 5 days) colonies on $BCYE_{\alpha}$ agar that are gray-white, glistening, convex, 1 to 4 mm in diameter, round with entire edges; absence of growth on $BCYE_{\alpha}$ without cysteine or on sheep blood agar; early in growth, show a pink or blue-green iridescence; older colonies become creamy white and often lose their iridescence.

b. Magnified with dissecting microscope with high oblique illumination: speckled opalescence resembling cut or ground glass.

c. Tenacity: colonies stringy, difficult to pick off, do not streak uniformly on subculture.

d. Colony fluorescence

Place the $BCYE_{\alpha}$ agar plate with suspect *Legionella* colonies under long-wave (366-nm) UV light. The colonies of some species (Fig. 13.6–3) autofluoresce a brilliant blue-white or bright red.

◩ **NOTE:** *L. pneumophila*, the most commonly isolated *Legionella* sp., does not autofluoresce.

2. Gram stain reaction

a. Gram stain suspected *Legionella* colonies, preferably with carbol fuchsin instead of safranin counterstain, because safranin produces very faintly pink cells.

b. *Legionella* cells are small, gram-negative bacilli, 0.5 μm wide by 1 to 2 μm long. Depending on their physiologic state, some cells may be pleomorphic, with lengths up to 20 μm.

IX. CONFIRMATORY IDENTIFICATION OF LEGIONELLAE

Confirm presumptive *Legionella* spp. by immunofluorescence or nucleic acid analysis. When these confirmatory techniques are not available in the laboratory, proceed with the biochemical tests listed in Fig. 13.6–3 for presumptive identification, and send subcultures of the presumptive legionellae to a reference public health laboratory for further identification and subtyping. Because there are many subtypes in some species, especially *L. pneumophila*, subtyping of patient and environmental isolates may be necessary to confirm the source of the outbreak (2).

Immunofluorescence procedure

A. Direct water testing: smear preparation

When there is strong epidemiological evidence of a waterborne outbreak of legionellosis but water culture results are negative, an immunofluorescence procedure can be performed on the water to detect viable, nonculturable *Legionella* organisms, as reported in the literature (8). See Supplemental Reading for general references on immunofluorescence. Since there are specific problems associated with these techniques in the detection of *Legionella* spp., a specific step-by-step procedure is given here.

Caution: *General directions are given here, but more-specific instructions are given by the manufacturers of the reagents chosen.*

1. Using sample suspension selected from those in item V above, fill the two 10-mm circles on a two-well fluorescence microscope slide.

a. If concentration of original material is necessary, centrifuge 100 ml at 3,500 × *g* for 30 min at room temperature.

b. Reconstitute the sediment with 1 to 2 ml of filter-sterilized deionized or distilled water or phosphate-buffered saline (pH 7.6).

2. Air dry sample smears.

3. Gently heat fix by rapidly passing the slide through a flame.

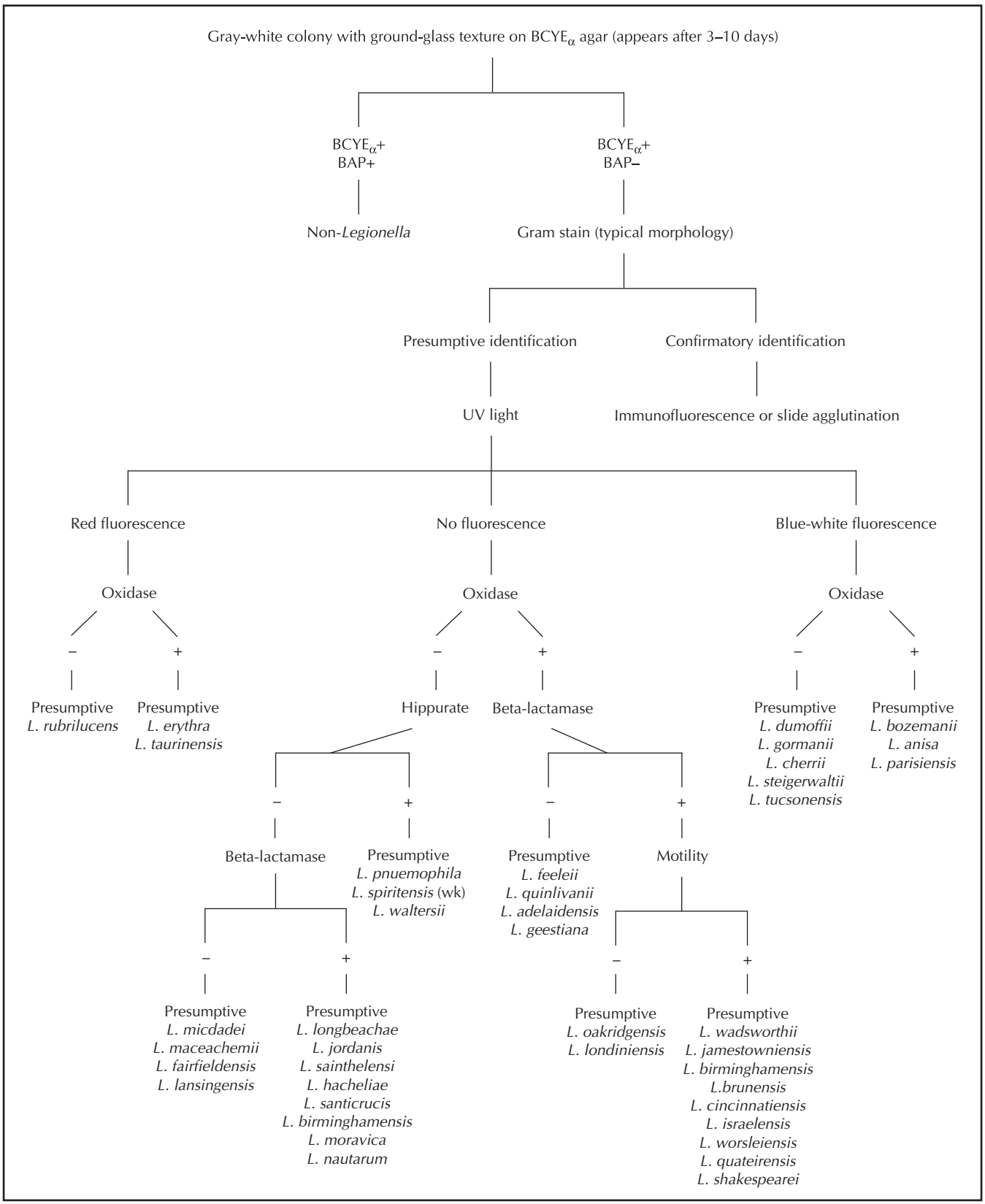
Gray-white colony with ground-glass texture on BCYE$_\alpha$ agar (appears after 3–10 days)
BCYE$_\alpha$+ BAP+
BCYE$_\alpha$+ BAP–
Non-*Legionella*
Gram stain (typical morphology)
Presumptive identification
Confirmatory identification
UV light
Immunofluorescence or slide agglutination
Red fluorescence
No fluorescence
Blue-white fluorescence
Oxidase
Oxidase
Oxidase
Presumptive *L. rubrilucens*
Presumptive *L. erythra* *L. taurinensis*
Hippurate
Beta-lactamase
Presumptive *L. dumoffii* *L. gormanii* *L. cherrii* *L. steigerwaltii* *L. tucsonensis*
Presumptive *L. bozemanii* *L. anisa* *L. parisiensis*
Beta-lactamase
Presumptive *L. pnuemophila* *L. spiritensis* (wk) *L. waltersii*
Presumptive *L. feeleii* *L. quinlivanii* *L. adelaidensis* *L. geestiana*
Motility
Presumptive *L. micdadei* *L. maceachemii* *L. fairfieldensis* *L. lansingensis*
Presumptive *L. longbeachae* *L. jordanis* *L. sainthelensi* *L. hacheliae* *L. santicrucis* *L. birminghamensis* *L. moravica* *L. nautarum*
Presumptive *L. oakridgensis* *L. londiniensis*
Presumptive *L. wadsworthii* *L. jamestowniensis* *L. birminghamensis* *L.brunensis* *L. cincinnatiensis* *L. israelensis* *L. worsleiensis* *L. quateirensis* *L. shakespearei*

Figure 13.6–3 Flowchart for identification of the family *Legionellaceae.* wk, weak.

IX. CONFIRMATORY IDENTIFICATION OF LEGIONELLAE (*continued*)

4. Treat slide with filter-sterilized 1% formalin for 10 min.
5. Rinse with filter-sterilized phosphate-buffered saline (pH 7.6).

B. Culture confirmation: smear preparation

1. Using aseptic technique, remove a distinct colony of suspect *Legionella* from the culture plate, and suspend it in a test tube containing 0.5 to 1 ml of filter-sterilized 1% formalin in saline to the turbidity of a McFarland no. 0.5 standard. Avoid suspensions above a no. 0.5 McFarland standard, because fluorescence may be quenched by a prozone effect (too many organisms).
2. Using a Pasteur pipette, apply 1 or 2 drops of the suspension to a well on the slide, and then remove with the same Pasteur pipette. A thin film will remain on the well.
3. Air dry the smears.
4. Heat fix by rapidly passing the slide through a flame.

C. Fluorescence staining procedure

1. Apply enough *Legionella* fluorescent-antibody reagent to cover the specimen. It is best to use a polyclonal conjugate, because failures of some monoclonal reagents with environmental *Legionella* spp. have been reported (13).
2. Follow instructions on incubation time, temperature, and rinse given by the manufacturer, making sure all rinse water is filter sterilized (0.2-μm-pore-size filter).
3. Air dry slides.
4. Add 2 or 3 drops of mounting medium to slide. Apply coverslip.

D. Examination of stained slides

1. Observe slides with a fluorescence microscope with a filter system for fluorescein isothiocyanate detection.
2. Use a 10× to 40× objective to screen slides for fluorescent rods.
3. Use a 63× to 100× oil objective to confirm cellular morphology.
4. Typical *Legionella* fluorescent-antibody morphology: short coccobacillary rods (direct water samples) or long filamentous rods (culture samples) showing bright (3 to 4+) apple-green cell wall fluorescence with darker centers.
5. Organisms that stain with ≤2+ fluorescence intensity or that do not show typical *Legionella* morphology are reported as fluorescent-antibody negative.

E. Follow-up tests

If organisms of appropriate morphology are stained by a polyvalent conjugate, stain additional smears with serogroup- or species-specific reagents (whichever is appropriate) to further identify *Legionella* organisms (4). An organism that gives positive fluorescent-antibody results with only one serogroup-specific conjugate can be reported as belonging to that serogroup.

POSTANALYTICAL CONSIDERATIONS

X. REPORTING RESULTS

A. Qualitative

1. Negative results should be reported as "*Legionella* species not found."
2. Positive results should be reported as "presumptive" or "confirmed" (whichever is appropriate). Give the *Legionella* species and serogroup (if known) and method performed; for example, *Legionella pneumophila* serogroup 1 found (confirmed by direct-fluorescence antibody and culture).

B. Quantitative

1. Swab sample: report as CFU per swab; for example, *Legionella pneumophila* found: 20 CFU per swab.
2. Water sample: report as CFU per milliliter or CFU per 100 ml; for example, if 1,000 ml of sample was filtered, the filter was placed in 2 ml of original

X. REPORTING RESULTS (*continued*)

sample, and 0.1 ml was plated on two $BCYE_{\alpha}$ plates, the detection limit would be <1 CFU per 100 ml or <0.01 CFU/ml.

a. Negative results would be reported as "*Legionella* species not found: <detection limit" (i.e., <0.01 CFU/ml or <1 CFU/100 ml with the example above).

b. Positive results should be reported as the *Legionella* species and serogroup, method performed, and quantitation (number of colonies on each plate multiplied by detection limit); for example, *Legionella pneumophila* serogroup 1 found: 100 CFU/100 ml or 1 CFU/ml (confirmed by direct-fluorescence antibody and culture).

REFERENCES

1. **American Public Health Association.** 1998. *Standard Methods for the Examination of Water and Wastewater*, 20th ed. American Public Health Association, Washington, DC.
2. **Barbaree, J. M., G. W. Gorman, W. T. Martin, B. S. Fields, and W. E. Morrill.** 1987. Protocol for sampling environmental sites for legionellae. *Appl. Environ. Microbiol.* **53:**1454–1458.
3. **Barbaree, J. M., W. E. Morrill, B. S. Fields, W. T. Martin, and G.N. Sanden.** 1988. Detection and recovery of *Legionella* in water. *Toxicity Assessment* **3:**479–490.
4. **Brenner, D. J., A. G. Steigerwalt, G. W. Gorman, H. W. Wilkinson, W. F. Bibb, M. Hackel, R. L. Tyndall, J. Campbell, J. C. Feeley, W. L. Thacker, P. Skaliy, W. T. Martin, B. J. Brake, B. S. Fields, H. W. McEachern, and L. K. Corcoran.** 1985. Ten new species of *Legionella. Int. J. Syst. Bacteriol.* **35:**50–59.
5. **CLSI.** 2004. Quality control for commercially prepared microbiological culture media, 3rd ed. Approved standard M22-A3. CLSI, Wayne, PA.
6. **Edelstein, P. H.** 1982. Comparative study of selective media for isolation of *Legionella pneumophila* from potable water. *J. Clin. Microbiol.* **16:**697–699.
7. **Gorman, G. W., J. M. Barbaree, and J. C. Feeley.** 1983. *Procedures for the Recovery of Legionella from Water*. Developmental manual. Centers for Disease Control, Atlanta, GA.
8. **Hussong, D., R. R. Colwell, M. O'Brien, E. Weiss, A. D. Pearson, R. M. Weiner, and W. D. Burge.** 1987. Viable *Legionella pneumophila* not detectable by cultures on agar media. *Bio/Technology* **5:**947–950.
9. **Korvick, J. A., and V. L. Yu.** 1987. Legionnaires' disease: an emerging surgical problem. *Ann. Thor. Surg.* **43:**341–347.
10. **Morrill, W. E., J. M. Barbaree, B. S. Fields, G. N. Sanden, and W. T. Martin.** 1990. Increased recovery of *Legionella micdadei* and *Legionella bozemanii* on buffered charcoal yeast extract agar supplemented with albumin. *J. Clin. Microbiol.* **28:**616–618.
11. **Stout, J. E., M. G. Best, and V. L. Yu.** 1986. Susceptibility of members of the family *Legionellaceae* to thermal stress: implications for heat eradication methods in water distribution systems. *Appl. Environ. Microbiol.* **52:**396–399.
12. **Ta, A. C., J. E. Stout, V. L. Yu, and M. M. Wagener.** 1995. Comparison of culture methods for monitoring species in hospital potable water systems and recommendations for standardization of such methods. *J. Clin. Microbiol.* **33:**2115–2123.
13. **Vickers, R. M., J. M. Stout, and V. L. Yu.** 1990. Failure of a diagnostic monoclonal immunofluorescent reagent to detect *Legionella pneumophila* in environmental samples. *Appl. Environ. Microbiol.* **56:**2912–2914.

SUPPLEMENTAL READING

Cherry, W. B., and R. M. McKinney. 1979. Detection of Legionnaires' disease bacteria in clinical specimens by direct immunofluorescence, p. 91–103. *In* G. L. Jones and G. A. Hebert (ed.), *Legionnaires': the Disease, the Bacterium and Methodology*. Centers for Disease Control, Atlanta, GA.

Sehulster, L. M., R. Y. W. Chinn, M. J. Arduino, J. Carpenter, R. Donlan, D. Ashford, R. Besser, B. Fields, M. M. McNeil, C. Whitney, S. Wong, D. Juranek, and J. Cleveland. 2004. *Guidelines for Environmental Infection Control in Health-Care Facilities. Recommendations from CDC and the Healthcare Infection Control Practices Advisory Committee (HICPAC).* American Society for Healthcare Engineering/American Hospital Association, Chicago, IL. Available at http://www.cdc.gov/ncidod/dhqp/gl_environinfection.html.

Wilkinson, H. W. 1988. *Hospital-Laboratory Diagnosis of Legionella Infections*, p. 13–15. Centers for Disease Control, Atlanta, GA.

APPENDIX 13.6–1

Addresses of Manufacturers and Suppliers

Gelman Sciences
600 South Wagner
Ann Arbor, MI 48106-1448

Gen-Probe
9620 Chesapeake Dr.
San Diego, CA 92123

Nalge Co.
Division of Sybron Corp.
Box 20365
Rochester, NY 14602-0365

Nuclepore Corp.
7035 Commerce Cir.
Pleasanton, CA 94566

Leica Inc. (Reichert-Jung)
111 Deerlake Rd.
Deerfield, IL 60015

SciMedX
400 Ford Rd., Bldg. 100
Denville, NJ 07834

Sigma Chemical Co.
P.O. Box 14508
St. Louis, MO 63178

APPENDIX 13.6–2

Reagents and Media

A. Reagent formulations

1. Acid treatment reagent, pH 2.0 (0.2 M KCl or HCl) (2)
   a. Solution A: 0.2 M KCl (14.9 g of KCl per liter of deionized or distilled water)
   b. Solution B: 0.2 M HCl (16.7 ml of 10 N HCl per liter of deionized or distilled water)
   c.

0.2 M HCl	3.9 ml
0.2 M KCl	25.0 ml

   Mix these solutions, and measure pH (should be approximately pH 2). Dispense into screw-cap tubes in 1.0-ml volumes, and sterilize by autoclaving.
2. Filter-sterilized phosphate-buffered saline, pH 7.6 (0.01 M buffer, 0.85% NaCl)
   a. Concentrated stock solution

Na_2HPO_4	12.36 g
$NaH_2PO_4 \cdot H_2O$	1.80 g
NaCl	85.00 g
deionized or distilled water to make final volume of	1,000 ml

   b. Working solution

concentrated stock solution	100 ml
deionized distilled water to make final volume of	1,000 ml

   Filter sterilize working solution through 0.22-μm-pore-size filter, place in sterile container, and store in refrigerator.
3. Filter-sterilized deionized or distilled water
   Filter sterilize distilled or deionized water through 0.22-μm-pore-size filter, place in sterile container, and store in refrigerator.
4. Filter-sterilized formalin, 1%

formalin (commercial formaldehyde)	1 ml
filter-sterilized laboratory pure water	99 ml

5. Buffered-glycerol mounting fluid, pH 9.0

glycerol, reagent grade	90 ml
0.2 M Na_2HPO_4	10 ml

6. McFarland nephelometer density standard no. 0.5 (1)
   Add 0.5 ml of 0.048 M $BaCl_2$ (1.175% [wt/vol] $BaCl_2 \cdot 2H_2O$) to 99.5 ml of 0.18 M (0.36 N) H_2SO_4 (1% [vol/vol]). Distribute 4 to 6 ml into screw-cap tubes of the same size as those used in growing or diluting the broth culture inoculum. Tightly seal these tubes to prevent any evaporation, label them, and store them in the dark at room temperature. Vigorously agitate this standard on a mechanical vortex mixer just before use.

APPENDIX 13.6–2 (*continued*)

B. Media

Individual ingredients listed below can be purchased from medium manufacturers or from Sigma Chemical Co.

1. $BCYE_\alpha$ base (2)

activated charcoal (Norit SG)	2.0 g
yeast extract	10.0 g
ACES buffer	10.0 g
ferric pyrophosphate, soluble	0.25 g
L-cysteine $HCl \cdot H_2O$	0.4 g
agar	17.0 g
potassium α-ketoglutarate	1.0 g
deionized or distilled water	1.0 liter

Dissolve yeast extract, agar, charcoal, and α-ketoglutarate in approximately 850 ml of water, and boil. Dissolve 10 g of ACES buffer in 100 ml of warm water, adjust the pH to 6.9 ± 0.05 with 1 N KOH, and mix. Autoclave for 15 min at 121°C. Cool to 50°C in a water bath. Dissolve 0.4 g of cysteine and 0.25 g of ferric pyrophosphate in 10 ml of water each, and filter sterilize separately (0.22-μm-pore-size filter). After base has cooled to 50°C, add the filtered cysteine and ferric pyrophosphate, in that order. Adjust the pH to 6.9 at room temperature with sterile 1 N KOH, and bring to a final volume of 1.0 liter with distilled water. Swirl the medium to maintain the charcoal in suspension between pouring plates (15 by 100 mm) of 20 ml each.

Appropriately label batch with name of medium, date prepared, pH, lot number, and technician initials. Expiration date is 2 months if the medium is stored bagged and at 4°C.

2. GPVA medium (2)

glycine	0.3%
polymyxin B	100 U/ml
vancomycin	5 μg/ml
anisomycin	80 μg/ml

To cooled BCYE base with glycine (50°C), add filter-sterilized antibiotics (0.22-μm-pore-size filter), and mix. Adjust pH to 6.9 + 0.05 with sterile 1 N KOH, and bring to a final volume of 1.0 liter with distilled water. Swirl the medium to maintain the charcoal in suspension between pouring plates (15 by 100 mm) of 20 ml each. Appropriately label batch with name of medium, date, prepared, pH, lot number, and technician initials. Expiration date is 1 month if the medium is stored bagged and at 4°C.

3. DGVP medium (4)

glycine	0.3%
dyes:	
bromocresol purple	10 μg/ml
bromothymol blue	10 μg/ml
vancomycin	1.0 μg/ml
polymyxin B	50 μg/ml

To cooled $BCYE_\alpha$ base with glycine (50°C), add filter-sterilized antibiotics and dyes (0.22-μm-pore-size filter), and mix. Prepare 1% concentration of dyes by dissolving in 0.1 N KOH. Add 1 ml of respective dye per liter of medium. Adjust pH to 6.9 ± 0.05 with sterile 1 N KOH, and bring to a final volume of 1.0 liter with distilled water. Swirl the medium to maintain the charcoal in suspension between pouring plates (15 by 100 mm) of 20 ml each. Appropriately label batch with name of medium, date, prepared, pH, lot number, and technician initials. Expiration date is 1 month if the medium is stored bagged and at 4°C.

4. $ABCYE_\alpha$ medium (3)

To $BCYE_\alpha$ base described above, add 10 g of bovine serum albumin (fraction V) per liter.

APPENDIX 13.6–2 (*continued*)

References

1. **CLSI.** 2004. Quality control for commercially prepared microbiological culture media, 3rd ed. Approved standard M22-A3. CLSI, Wayne, PA.
2. **Gorman, G. W., J. M. Barbaree, and J. C. Feeley.** 1983. *Procedures for the Recovery of Legionella from Water*. Developmental manual. Centers for Disease Control, Atlanta, GA.
3. **Morrill, W. E., J. M. Barbaree, B. S. Fields, G. N. Sanden, and W. T. Martin.** 1990. Increased recovery of *Legionella micdadei* and *Legionella bozemanii* on buffered charcoal yeast extract agar supplemented with albumin. *J. Clin. Microbiol.* **28:**616–618.
4. **Ta, A. C., J. E. Stout, V. L. Yu, and M. M. Wagener.** 1995. Comparison of culture methods for monitoring species in hospital potable water systems and recommendations for standardization of such methods. *J. Clin. Microbiol.* **33:**2115–2123.

13.7 Culture and Endotoxin Assay of Hemodialysis Fluids

I. PRINCIPLE

Patients undergoing hemodialysis are at risk for the development of endotoxin-mediated pyrogenic reactions, gram-negative bacteremia with sepsis, and chronic inflammatory response syndrome. These events may be related to excessive levels of gram-negative bacillary contamination of the water and dialysate used for hemodialysis applications. This cause-and-effect relationship has led to the establishment of microbiological guidelines pertaining to the allowable levels of contamination in both the dialysate bath and dialysis water. These recommendations include frequency of monitoring and a list of methods considered acceptable for the performance of quantitative testing. Endotoxin testing is discussed in the appendixes at the end of this procedure because it is not routinely done in the clinical setting and is performed usually by renal reference laboratories.

PREANALYTICAL CONSIDERATIONS

II. TEST FREQUENCY AND TIMING

A. Routine (no contamination or infection problem indicated)

Collect samples for monitoring purposes at least monthly.

B. Repeat

Collect repeat cultures when bacterial counts exceed the allowable level. If culture growth exceeds permissible standards, reculture the water system and dialysis machines weekly until acceptable results are obtained.

C. Ad hoc

Collect samples when clinical indications suggest pyrogenic reaction and/or septicemic complications following a specific request by the clinician and/or the infection control practitioner.

D. Timing

Samples should always be collected before sanitization/disinfection of the water treatment system and dialysis machines. If the system has just been disinfected (e.g., formaldehyde, hydrogen peroxide, chlorine, peracetic acid), flush the system completely before collecting samples. Drain and flush storage tanks and distribution system until disinfectant residual is no longer detected before collecting samples. Culture water and dialysis fluid weekly for new systems until an established pattern has been determined. For established systems, culture monthly unless a greater frequency is dictated by historical data at a given institution.

III. SPECIMENS

A. Dialysis water

1. Sample at a point immediately past the water treatment system (e.g., reverse-osmosis unit, deionization units, etc.).

III. SPECIMENS (*continued*)

2. If storage tanks are employed, sample as water exits the storage tank.
3. Sample just before the water enters the dialysis machine or central proportioner.
4. If the unit reprocesses hemodialyzers for reuse on the same patient, sample the water that is used to rinse the dialyzers and prepare the dialyzer disinfectant.

B. Dialysate

1. Following dialysis, collect dialysis fluid from the effluent dialysate port on the dialyzer.
2. With some newer dialysis machines, dialysate flow stops when the effluent line is disconnected from the port. In these instances the machines are equipped with dialysate sampling ports that can be accessed using a syringe.

C. Volume

Collect a minimum of 50 ml from each sampling point into sterile endotoxin-free containers. Most commercially available sterile plastic centrifuge tubes are actually endotoxin free.

IV. SUPPLIES FOR HETEROTROPHIC PLATE COUNT

A. TSA (soybean casein digest agar in 100-mm-diameter plates); other acceptable media include standard methods agar and plate count agar. Blood agar and CHOC are not appropriate for this testing.
B. 95% ethanol
C. Glass rod spreader (bent in the shape of a hockey stick) or commercially available sterile spreaders
D. 35°C incubator
E. Sterile 1-ml and 100- and 10-μl pipettes

ANALYTICAL CONSIDERATIONS

V. PROCEDURE

Observe standard precautions.

A. Mix water samples by swirling or vortexing the sample container. Plate the following volumes onto the center of agar plates: 0.1 and 1 ml. For dialysate samples, add a third plate and inoculate with 10 μl of sample. Spread inocula with a cool alcohol-flamed glass rod spreader or a sterile, plastic disposable spreader.
B. Incubate plates in ambient air at 35°C for 48 h.
C. Calculate the total colony count as follows: colony count × volume of sample plated. Use plates with colony counts between 30 and 300.
D. Identification of organisms is not required but can be done using standard procedures (in some cases identification may be difficult). Retain a plate containing representative colonies for 1 week in case bacteriologic follow-up is necessary.
E. Report total CFU per milliliter.
F. *Note:* Membrane filtration is the recommended method for testing hemodialysis fluids (1, 3). Commercially produced samplers (2) are also available for testing within the facilities but should not be used in the laboratory as an alternative to either membrane filtration or the spread plate method.

POSTANALYTICAL CONSIDERATIONS

VI. INTERPRETATION

A. Dialysate

Favero and coworkers (5) and Dawids and Vejlsgaard (4) showed that the attack rate for pyrogenic reactions among patients undergoing renal dialysis was directly related to the number of organisms in the patients' dialysate bath. These studies provided the basis for the guideline of <2,000 CFU/ml for dialysate. These dialysate standards will be lowered over the next 5 years.

VI. INTERPRETATION (*continued*)

B. Water used for hemodialysis applications
The standard for bacteria in water was set to 1 $\log_{01}$ less than that of the dialysate bath (<200 CFU/ml). For levels greater than 50 CFU/ml in the product water, corrective action should be taken to reduce these levels (3).

C. *Note:* All water used for hemodialysis applications (i.e., preparing concentrates and dialysates, reprocessing dialyzers) must also be tested for the presence of endotoxin. In addition, there will most likely be an endotoxin requirement for the dialysate bath as well.

VII. CULTURE REPORTING

Report colony count per milliter. When colony counts exceed 200 CFU/ml (water) and 2,000 CFU/ml (dialysate), call the medical director of the dialysis unit and the infection control practitioner immediately. For colony counts exceeding 50 CFU/ml in water, notify the technical staff responsible for maintaining the water treatment system.

VIII. QUALITY CONTROL AND QUALITY ASSURANCE

Report the results of all tests to the Infection Control Committee and to the nurse manager of the dialysis unit responsible for QA oversight.

REFERENCES

1. **American Public Health Association.** 1998. *Standard Methods for the Examination of Water and Wastewater,* 20th ed. American Public Health Association, Washington, DC.
2. **Arduino, M. J., L. A. Bland, S. M. Aguero, L. A. Carson, M. Ridgeway, and M. S. Favero.** 1991. Comparison of microbiologic assay methods for hemodialysis fluids. *J. Clin. Microbiol.* **29:**592–594.
3. **Association for the Advancement of Medical Instrumentation.** 2006. *American National Standard: Dialysate for Hemodialysis.* ANSI/AAMI RD52:2004. Association for the Advancement of Medical Instrumentation, Arlington, VA.
4. **Dawids, S. G., and R. Vejlesgaard.** 1976. Bacteriological and clinical evaluation of different dialysate delivery systems. *Acta Med. Scand.* **199:**151–155.
5. **Favero, M. S., N. J. Peterson, L. A. Carson, W. W. Bond, and S. H. Hindman.** 1975. Gram-negative water bacteria in hemodialysis systems. *Health Lab. Sci.* **12:**321–334.

SUPPLEMENTAL READING

Arduino, M. J., and M. S. Favero. 1998. Microbiologic aspects of hemodialysis. *Water Quality for Hemodialysis.* AAMI monograph WQD-1998. Association for the Advancement of Medical Instrumentation, Arlington, VA.

Association for the Advancement of Medical Instrumentation. 2006. *American National Standard: Water Treatment Equipment for Hemodialysis Applications.* ANSI/AAMI RD62:2006. Association for the Advancement of Medical Instrumentation, Arlington, VA.

Association for the Advancement of Medical Instrumentation. 2001. *American National Standard: Water Treatment Equipment for Hemodialysis Applications.* ANSI/AAMI RD62-2001. Association for the Advancement of Medical Instrumentation, Arlington, VA.

Bland, L. A., M. S. Favero, and M. J. Arduino. 1993. Hemodialysis fluid—should it be sterile? *Semin. Dial.* **6:**34–36.

Favero, M. S., and N. J. Peterson. 1977. Microbiologic guidelines for monitoring hemodialysis systems. *Dial. Transplant.* **6:**34–36.

Favero, M. S., M. J. Alter, J. I. Tokars, and M. J. Arduino. 1998. Dialysis-associated infections and their control, p. 357–380. *In* J. V. Bennet and P. Brachman (ed.), *Hospital Infections,* 4th ed. Lippincott-Raven Publishers, Philadelphia, PA.

Gordon, S. M., C. W. Oettinger, L. A. Bland, J. C. Oliver, M. Arduino, M. Favero, and W. R. Jarvis. 1992. Pyrogenic reactions in patients receiving conventional, high-efficiency, or high-flux hemodialysis treatments with bicarbonate dialysate containing high concentrations of bacteria and endotoxin. *J. Am. Soc. Nephrol.* **2:**1436–1444.

Oettinger, C. W., M. J. Arduino, J. C. Oliver, and L. A. Bland. 1994. The clinical relevance of dialysate sterility. *Semin. Dial.* **7:**263–267.

Pegues, D. A., C. W. Oettinger, L. A. Bland, J. C. Oliver, M. J. Arduino, S. M. Aguero, S. K. McAllister, S. M. Gordon, M. S. Favero, and W. R. Jarvis. 1992. Prospective study of pyrogenic reactions in patients receiving conventional, high efficiency, or high flux hemodialysis treatments with ultrafiltered bicarbonate dialysis fluids. *J. Am. Soc. Nephrol.* **3:**1002–1007.

APPENDIX 13.7–1

Bacterial Endotoxin Test (*Limulus* Amebocyte Lysate)

I. PRINCIPLE

Endotoxins are lipopolysaccharide (LPS) components of the gram-negative bacterial outer membrane. The toxic component of LPS resides in the lipid portion, lipid A. The polysaccharide subunit contributes to the toxicity of the molecule by promoting its water solubility. In humans, endotoxin may cause fever and leukopenia or, in more severe cases, shock. The threshold pyrogenic response is 5 endotoxin units (EU)/kg of body weight/h (intravenous administration). Endotoxin in hemodialysis fluids may contribute to a phenomenon now described as the chronic inflammatory response syndrome.

The use of *Limulus* amebocyte lysate for the detection of endotoxin evolved from the observation that gram-negative bacterial infections of the horseshoe crab, *Limulus polyphemus*, resulted in fatal intravascular coagulation. This is now known to be due to an enzyme reaction catalyzed by endotoxin. This phenomenon has led to the development of a series of tests that can be used for the detection of endotoxin in materials (e.g., devices, drugs, biologicals) used for human therapy.

Several test formats are commercially available, from a simple tube gel clot test to kinetic detection methods (e.g., colorimetric and turbidimetric assays).

PREANALYTICAL CONSIDERATIONS

II. SPECIMEN

A. Any liquid may be tested for endotoxin activity. Some compounds or solutions, because they may contain metals or surfactants or have high ionic strength or osmolarity, may give false-positive or false-negative results, produce enhancement, or inhibit the reactions. Testing methods and interpretation of results should be carefully reviewed. Some clinical laboratories may prefer to contract a commercial laboratory for this testing.

B. The pH of the sample should be 6.0 to 7.5.

C. Collect samples as in specimen for the culture of water and dialysate as described in item III of this procedure. If glass containers are used, they should be made endotoxin free by baking in an oven at 180°C for 4 h.

D. Samples can be stored at 2 to 8°C for 24 h. For longer storage or shipping to a reference laboratory, samples can be frozen at −20°C.

III. REAGENTS AND SUPPLIES

Several commercial sources can supply endotoxin reagents and standards, testing systems, and test kits. Some of the leading U.S. vendors are listed below. This list is intended for informational purposes only and may not include every vendor or manufacturer selling these products in the United States.

A. Associates of Cape Cod, Falmouth, MA (endotoxin standards, gel clot reagents, multitest and single-tube tests, colorimetric and turbidimetric assays, contract testing services), http://www.acciusa.com/

B. Bio-Whittaker, Walkersville, MD (endotoxin standards, gel clot reagents, turbidimetric reagents), http://www.cambrex.com/operating_companies/biowhittaker/

C. Charles River Laboratories, Charleston, SC (endotoxin standards, traditional gel clot reagents, colorimetric and turbidimetric assays, contract testing services), http://www.criver.com/products/endotoxin/index.html

D. DiaPharm, West Chester, OH (chromogenic *Limulus* amebocyte lysate [LAL] assay), http://www.diapharma.com/

ANALYTICAL CONSIDERATIONS

Observe standard precautions.

IV. PROCEDURE

Follow the manufacturer's instructions in the package labeling or package insert for the test kit or in the procedure manual for the LAL kinetic assay systems.

V. QUALITY CONTROL

A. The test sample must be run in parallel with dilutions of the endotoxin standard that bracket the end-point sensitivity of the kit.

APPENDIX 13.7–1 (*continued*)

B. The endotoxin control standard end point can be 0.001 to 5 EU/ml depending on whether a kinetic qualitative assay (e.g., turbidimetric, colorimetric) or a semiquantitative assay (gel clot, colorimetric) is used. If the control end point is less than the required standard sensitivity of the kit, notify the supervisor or director for instructions on how to restart.

C. The sample inhibition control (sample plus an endotoxin spike containing two to four times the sensitivity of the LAL reagent) must be positive for each sample or the test is invalid.

POSTANALYTICAL CONSIDERATIONS

VI. REPORTING RESULTS

A. If the test result of the sample is negative, report each negative sample as below the detectable limit of the test utilized. This will depend on the sensitivity of the reagents used, e.g., <0.03 EU/ml, <0.06 EU/ml, etc.

B. Reporting of positive results will vary depending on whether one is using a quantitative kinetic assay, gel clot assay, or single-tube test. For the kinetic assays, results will be reported as EU per milliliter; for the gel clot test the EU per milliliter of the last tube to clot and the breakpoint will be reported. The first tube not to gel would be the range between these two points. For single-tube tests the result would be reported as greater than or equal to the sensitivity of the single-tube test, e.g., ≥ 0.06 EU/ml, ≥ 0.125 EU/ml.

VII. LIMITATIONS

A. The LAL reaction is enzyme mediated and has an optimal pH and specified salt and divalent-cation requirements. Occasionally, test samples may have to have pH adjusted or diluted to bring samples into optimal test conditions. Negative results with samples that inhibit the LAL test do not necessarily mean that endotoxin is not present in the sample.

B. In some instances reactions may be enhanced. In these instances the sample may contain other contaminants, i.e., β-glucans such as cellulosic material (i.e., cupraammonium rayon, cellulose, cellulose acetate, or cellulose di- or triacetate), which may react with the LAL reagents. These materials are LAL-reactive materials and do not necessarily indicate the presence or absence of endotoxin.

C. Standards for dialysates are set forth in the *American National Standard: Dialysate for Hemodialysis* (1). The CDC recommendations mirror this standard (2). For conventional dialysis water these set the colony count at ≤ 200 CFU/ml and the bacterial lipopolysaccharide activity at <2 EU/ml. Corrective action is required for fluids with ≥ 50 CFU/ml or ≥ 1 EU ml. Ultrapure dialysates have limits of ≤ 0.1 CFU/ml and <0.03 EU/ml.

References

1. **Arduino, M. J.** 1998. How should dialyzers be reprocessed? *Semin. Dial.* **11:**282–284.
2. **Arduino, M. J., and M. S. Favero.** 1998. Microbiologic aspects of hemodialysis. *Water Quality for Hemodialysis.* AAMI monograph WQD-1998. Association for the Advancement of Medical Instrumentation, Arlington, VA.
3. **Association for the Advancement of Medical Instrumentation.** 1992. *American National Standard: Hemodialysis Systems.* ANSI/AAMI RD5-1992. Association for the Advancement of Medical Instrumentation, Arlington, VA.
4. **Association for the Advancement of Medical Instrumentation.** 1993. *American National Standard for Hemodialyzer Reuse.* ANSI/AAMI RD47-1993. Association for the Advancement of Medical Instrumentation, Arlington, VA.
5. **Association for the Advancement of Medical Instrumentation.** 2001. *American National Standard: Water Treatment Equipment for Hemodialysis Applications.* ANSI/AAMI RD62-2001. Association for the Advancement of Medical Instrumentation, Arlington, VA.

APPENDIX 13.7–1 (*continued*)

Supplemental Reading

Baz, M., C. Durand, A. Ragon, K. Jaber, D. Andrieu, T. Merzouk, R. Purgus, M. Oliver, J. Reynier, and Y. Borland. 1991. Using ultrapure water in hemodialysis delays carpal tunnel syndrome. *Int. J. Artif. Organs* **14:**681–685.

Bland, L. A. 1995. Microbiological and endotoxin assays of hemodialysis fluids. *Adv. Renal Replace. Ther.* **2:**70–79.

Bland, L. A., J. C. Oliver, M. J. Arduino, C. W. Oettinger, S. K. McAllister, and M. S. Favero. 1995. Potency of endotoxin from bicarbonate dialysate compared with endotoxins from *Escherichia coli* and *Shigella flexneri*. *J. Am. Soc. Nephrol.* **5:**1634–1637.

Mangram, A. J., L. K. Archibald, M. Hupert, J. I. Tokars, L. C. Silver, P. Brenman, M. Arduino, S. Peterson, A. Raymond, M. McCullough, M. Jones, A. Wasserstein, S. Korbin, and W. R. Jarvis. 1998. Outbreak of sterile peritonitis among cycling continuous peritoneal dialysis patients. *Kidney Int.* **54:**1367–1371.

Outschoorn, A. S. 1982. The USP bacterial endotoxins test. *Prog. Clin. Biol. Res.* **93:**33–38.

Temporada-Cookson, S., J. J. Nora, J. A. Kithas, M. J. Arduino, W. W. Bond, P. H. Miller, J. Monoahen, R. E. Hoffman, B. M. Groves, and W. R. Jarvis. 1997. Pyrogenic reactions in patients undergoing cardiac catheterization associated with contaminated glass medicine cups. *Cathet. Cardiovasc. Diagn.* **42:**12–18.

APPENDIX 13.7–2

Bacterial Contaminant Standards for hemodialysis (AAMI Standard RD 52)

Fluid	CFU/ml		EU/ml	
	Maximum	Action limit	Maximum	Action limit
Prepare dialysate or reprocessing hemodialyzers	200	20	2	1
Ultrapure dialysate	0.1	NA[a]	0.03	NA
Dialysate for infusion (e.g., hemofiltration)	10^{-6}	NA	0.03	NA

[a] NA, not applicable.

13.8 Culture of Peritoneal Dialysis Fluid

I. PRINCIPLE

A. Patient population

Patients with end-stage renal disease (ESRD) can receive transplants or be treated by either hemodialysis or peritoneal dialysis. In 1998, of the 346,453 patients in the United States with ESRD, 29% had a functioning graft, 61% were being treated by maintenance hemodialysis, and 7.3% were being treated by some form of peritoneal dialysis (15). The predominant types of peritoneal dialysis performed in the United States are continuous ambulatory peritoneal dialysis (CAPD) and continuous cycling peritoneal dialysis (CCPD).

B. Method

Dialysis fluid is infused into the peritoneum through a permanent catheter (Tenckhoff, Ash, Swan Neck, Toronto Western Hospital). This treatment can be done at home, which allows more freedom and flexibility. In ambulatory peritoneal dialysis (APD), there is 2 liters of dialysis fluid in the peritoneal cavity all the time, so the blood is being cleaned constantly. The fluid is changed regularly throughout the day.

There are two kinds of APD: CAPD and CCPD.

In CAPD, the dialysis fluid is exchanged four times a day. An exchange is done by draining the old fluid and refilling the peritoneal cavity with fresh fluid. This takes about 45 min. The exchanges are spaced throughout the day.

In CCPD, the patient is attached to an automatic cycler machine during the night and exchanges are done during sleep. In the morning, when the patient is coming off the machine, about 2 liters of dialysis fluid is left in the peritoneal cavity for the day. In the evening, this fluid is drained out when the patient is reconnected to the automatic cycler for the night.

Intermittent peritoneal dialysis (IPD) is done on an inpatient basis. Most people need about 36 to 44 h of IPD weekly, so it means spending 2 or 3 days away from home every week. In IPD, the dialysis fluid is left in the peritoneal cavity for a short time and then is drained out. One complete cycle is called an exchange, and an IPD exchange takes about 1 h to complete.

C. Precautions

All bag manipulations are performed using aseptic techniques to reduce the danger of external contamination.

D. Outcome

Even with aseptic techniques infections are not uncommon, and incidence rates vary from center to center. Approximately one episode of peritonitis in 24 patient-treatment months has been observed. Some centers have even reported one episode in 60 patient-treatment months (5). See Appendix 13.8–1 for further discussion.

PREANALYTICAL CONSIDERATIONS

II. SPECIMEN

A. Collection and transport

1. Safety precautions

Enclose dialysate bag in a larger plastic bag. Place this bag into a disposable plastic or other leakproof container and transport it to the laboratory.

2. Obtain the first cloudy effluent for culture (greatest probability of obtaining a positive culture). Patients who receive automated peritoneal dialysis (e.g., CCPD) may have effluent that appears clear; if this is the case, a second exchange is performed with a dwell time of at least 1 h and the appearance of the fluid is reevaluated.

Approximately 6% of adults with culture-positive peritonitis have effluent that is clear and experience abdominal pain (3, 5, 6, 10).

II. SPECIMEN (*continued*)

3. Conditions
   - **a.** Deliver to the laboratory immediately, transport at room temperature.
   - **b.** If delivery will take more than 1 h after collection, refrigerate but do not freeze.

B. Processing

1. Mix contents of the bag by inverting the bag 10 to 20 times.
2. Disinfect the entry port with povidone-iodine (allow to air dry), and withdraw 100 ml of fluid and place in sterile 50-ml screw-cap centrifuge tubes.
3. Perform a cell count with differential. If there are >50 WBCs/mm^3, culture may be indicated (7). An elevated dialysate WBC count, >100 WBC/mm^3, of which at least 50% are PMNs, is supportive of a diagnosis of microbially induced peritonitis (5, 6, 10). Cell count and differential may be performed by hematology or another appropriate laboratory if the microbiology laboratory is not equipped to do it.
4. Centrifuge samples for 15 min at 3,000 × *g* and carefully decant the supernatant from each tube.
5. Perform Gram stain from the sediment.
6. Vortex and suspend the sediment with 5 ml of sterile saline.
7. Use resuspended sediment from step II.B.6 above to incoculate media (*see* item IV.B below).

III. MATERIALS

Media, supplies, and instruments are those commonly utilized in the laboratory and are employed selectively, as dictated by the choice of culture protocol. In general, the following may be employed: blood agar plate, CHOC plate, EMB or MacConkey agar, inhibitory mold agar plate, BHI agar with blood or Sabouraud's dextrose agar, and blood culture media (e.g., BacT/Alert FAN blood bottle, Septi-Chek, or BACTEC bottle).

ANALYTICAL CONSIDERATIONS

IV. PROCEDURE

Observe standard precautions.

A. Microscopic examination

1. Gram stain
   - **a.** Concentrate specimen by centrifugation as described above.
   - **b.** Prepare a Gram-stained smear, and review microscopically.
   - **c.** If organisms are detected by microscopic examination, report these immediately.
   - **d.** A Gram stain is positive in 9 to 40% of peritonitis episodes, and when positive, it is predictive of culture results in approximately 85% of the cases (5). Even though the Gram stain is positive in less than 50% of the cases, it is still clinically useful in promoting early therapy.
2. Acid-fast smear

   The clinical utility of acid-fast stains is extremely limited. Leukocyte differential and acid-fast smears often fail to aid in the diagnosis of peritonitis associated with *Mycobacterium* spp. Tuberculous peritonitis is a rare complication of peritoneal dialysis (5, 17). Nontuberculous mycobacteria (*Mycobacterium fortuitum, Mycobacterium kansasii, Mycobacterium gordonae*) have been associated with peritonitis. Clinically, these organisms should be considered as etiologic agents in cases of sterile peritonitis.

B. Culture

1. Bacteria
   - **a.** Inoculate aerobic culture plates and broth (blood culture bottles) with concentrated specimen. Using a Pasteur pipette, aspirate the sediment and place 1 drop on bacteriological culture plates (blood agar, CHOC, EMB,

IV. PROCEDURE (*continued*)

or MacConkey agar). Place 5 ml of sample into a blood culture bottle. Incubate plates in 5% CO_2 at 35°C for 48 h and the blood culture system (lysis centrifugation, BACTEC, BacT/Alert, Septi-Chek) for 5 to 7 days (1, 2, 8, 11–14, 16, 18–21).

b. Anaerobic bacteria in CAPD, CCPD, or IPD peritonitis are rare. Anaerobic bacteria are associated with severe peritonitis, and it may be useful to include them in a workup. The International Society for Peritoneal Dialysis considers culturing for anaerobes an optional procedure (5).

(1) Inoculate nonselective anaerobic culture media (*see* section 4 of this handbook), and incubate them at 35°C under an anaerobic atmosphere for 7 days.

(2) A broth should be incubated for an additional week for delayed appearance of slowly growing anaerobes.

2. Mycobacteria and fungi

Cultures can be performed either in parallel or in tandem.

a. Parallel cultures

(1) Concentrated dialysate is inoculated onto both mycobacterial and fungal media on the same day.

(2) This is indicated if acid-fast smear, calcofluor white stain, or KOH preparation is positive.

b. Tandem cultures

(1) Concentrated dialysate is reserved in the refrigerator for 5 days.

(2) If the bacterial cultures are negative and the patient is not responding to therapy, fungal and mycobacterial cultures are set up. The extended incubation period will allow for increased yield of bacteria or rapidly growing yeasts and mycobacteria. This will reduce the need for supplemental cultures for acid-fast bacteria or fungi.

V. LIMITATIONS OF THE PROCEDURE

A. Supplemental biopsy

Biopsy may be indicated when bacterial cultures are negative and tuberculous peritonitis is suspected.

B. Other supplemental methods

Limulus amebocyte lysate and other tests for the detection of infectious agents may not be sufficiently sensitive or specific to be of value unless employed rigidly for specific therapeutic protocols (4, 9).

POSTANALYTICAL CONSIDERATIONS

VI. INTERPRETATION

A. Contamination

Patient skin microorganisms (*Staphylococcus aureus* and other *Staphylococcus* species) are the predominant cause of peritonitis associated with ambulatory and automated dialysis. Dialysis-associated peritonitis is characterized by low levels of these organisms; thus, the primary difficulty in interpretation of culture results lies in distinguishing exogenous contaminants from those actively producing infection. Here are some simple caveats.

1. Consider the following positive.

a. One or more colonies or colony types, regardless of the identity of the organism, that grow on streak lines or in inoculated areas

b. One or more colonies or colony types that grow in the inoculum or on streaked lines that differ in appearance from those that grow outside of the streaked lines or inoculum

VI. INTERPRETATION (*continued*)

2. Consider the following contaminants.
   a. Colonies found exclusively outside both the inoculum and streak lines
   b. Colonies of the same appearance that grow on streak lines as those outside the streak lines or inoculum

B. Quantitation

Small numbers of organisms are considered potentially significant, but confounding organisms are frequently isolated from patients without peritonitis. Use clinical judgment.

C. Clinical correlation

1. Cell count
   a. Normal peritoneal fluid (6-h dwell time for CAPD)
      (1) Contains 50 to 100 WBCs/mm^3, mostly mononuclear
      (2) Visibly clear
   b. Peritoneal dialysis fluid
      (1) Contains >100 WBCs/mm^3
      (2) Visibly cloudy
2. Differential
   a. Bacterial peritonitis: PMNs predominate (>50%) (5, 6, 10)
   b. Mycobacterial peritonitis
      (1) Neutrophils are sometimes associated with mycobacterial peritonitis (17).
      (2) Mononuclear cells are often associated with mycobacterial peritonitis (5, 17).
   c. Allergic peritonitis: eosinophils comprise up to 10% of the WBC count (5, 6).
3. Signs and symptoms
   a. Patients with cloudy fluid and/or abdominal pain and/or fever (>4-h dwell time, CAPD)
   b. Patients with cloudy fluid and/or abdominal pain, and/or fever at any point in the automated peritoneal dialysis treatment cycle (CCPD). In equivocal cases, i.e., abdominal pain and/or fever and clear dialysate, add a second exchange with a dwell time of at least 2 h (5).
4. Clinical response

   For patients who fail to demonstrate clinical improvement in 48 to 96 h, a reevaluation is essential. Specifically, cell counts, Gram stain, and cultures should be repeated with the aid of antimicrobial removal devices. Among clinical concerns in patients with persistent symptoms is the presence of intra-abdominal or gynecological pathology requiring surgical intervention, or the presence of mycobacteria, fungi, or other fastidious organisms (5, 6, 10).

VII. QUALITY ASSURANCE

A. QC

1. Routine QC of media and reagents as described elsewhere in this handbook
2. Parallel culture of sterile saline or distilled water used to resuspend the cells in step II.B.6.

B. QA

Report all positive cultures both to Infection Control and to the referring dialysis unit. Upon establishing that there is an increased infection rate, a repetitive microorganism, or an apparent pseudoinfection, Infection Control and the dialysis facility should initiate an investigation and consider the institution of appropriate intervention or monitoring strategies.

REFERENCES

1. **Alfa, M. J., P. Degagne, N. Olson, and G. K. Harding.** 1997. Improved detection of bacterial growth in continuous ambulatory peritoneal dialysis effluent by use of BacT/Alert FAN bottles. *J. Clin. Microbiol.* **35:**862–866.
2. **Forbes, B. A., P. A. Frymore, R. T. Kopecky, J. M. Wojtaszek, and D. J. Pettit.** 1988. Evaluation of lysis-centrifugation system for culturing dialysates from continuous ambulatory peritoneal dialysis patients with peritonitis. *Am. J. Kidney Dis.* **11:**176–179.
3. **Fried, L., and B. Piraino.** 2000. Peritonitis, p. 545–564. *In* R. Gokal, R. Khanna, R. Krediet, and K. Nolph (ed.), *Textbook of Peritoneal Dialysis*, 2nd ed. Kluwer Academic Publishers, Boston, MA.
4. **Hausmann, M. J., R. Yulzari, E. Lewis, Y. Saisky, and A. Douvdevani.** 2000. Gel-clot LAL assay in the initial management of peritoneal dialysis patients with peritonitis: a retrospective study. *Nephrol. Dial. Transplant.* **15:**680–683.
5. **Keane, W. F., G. R. Bailie, E. Boeschoten, R. Gokal, T. A. Golper, C. J. Holmes, Y. Kawaguchi, B. Piraino, M. Riella, and S. Vas.** 2000. Adult peritoneal dialysis-related peritonitis treatment recommendations: 2000 update. *Perit. Dial. Int.* **20:**396–411.
6. **Lerner, G. R., B. A. Warady, E. K. Sullivan, and S. R. Alexander.** 1999. Chronic dialysis in children and adolescents. The 1996 annual report of the North American Renal Transplant Cooperative Study. *Pediatr. Nephrol.* **13:**404–417.
7. **Males, B. M., J. J. Walshe, and D. Amsterdam.** 1987. Laboratory indices of clinical peritonitis: total leukocyte count, microscopy, and microbiologic culture of peritoneal dialysis effluent. *J. Clin. Microbiol.* **25:**2367–2371.
8. **Males, B. M., J. J. Walshe, L. Garringer, D. Koscinski, and D. Amsterdam.** 1986. Addi-Chek filtration, BACTEC, and 10-ml culture methods for recovery of microorganisms from dialysis effluent during episodes of peritonitis. *J. Clin. Microbiol.* **23:**350–353.
9. **McCartney, A., J. N. Brunton, and G. L. Warwick.** 1989. *Limulus* ameobocyte lysate (LAL) assay and rapid detection of Gram negative bacterial peritonitis in patients receiving CAPD. *J. Clin. Pathol.* **42:**1115. (Letter.)
10. **Piraino, B., G. R. Bailie, J. Bernardini, E. Boeschoten, A. Gupta, C. J. Holmes, E. J. Kuijper, P. K. Li, W. C. Lye, S. Mujais, D. L. Paterson, M. P. Fontan, A. Ramos, F. Schaefer, and L. Uttley.** 2005. ISPD Ad Hoc Advisory Committee. Peritoneal dialysis-related infections recommendations: 2005 update. *Perit. Dial. Int.* **25:**107–131.
11. **Ryan, S., and S. Fessia.** 1987. Improved methods for recovery of peritonitis-causing organisms from peritoneal dialysate. *J. Clin. Microbiol.* **25:**383–384.
12. **Saubolle, M. A., D. L. Sewell, M. D. Holland, and T. A. Golper.** 1989. Comparison of two commercial broth-culture systems for microbial detection in dialysates of patients on continuous ambulatory peritoneal dialysis. *Diagn. Microbiol. Infect. Dis.* **12:**457–461.
13. **Schaefer, F., G. Klaus, D. E. Mueller-Weifel, and O. Mehls.** 1999. Current practice of peritoneal dialysis: results of a longitudinal survey. Mid European Pediatric Peritoneal Dialysis Study Group (MEPPS). *Perit. Dial. Int.* **19**(Suppl. 2)**:**S445–S449.
14. **Tranæus, A.** 2000. Peritonitis in paediatric continuous peritoneal dialysis, p. 301–347. *In* R. N. Fine, S. R. Alexander, and B. A. Warady (ed.), *CAPD/CCPD in Children*, 2nd ed. Kluwer Academic Publishers, Boston, MA.
15. **U.S. Renal Data Systems.** 2001. *USRDS 2000 Annual Data Report.* National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD.
16. **Van Caseele, P., M. J. Alfaa, and G. K. Harding.** 2000. Assessment of the FAN aerobic bottle for culture of continuous ambulatory peritoneal dialysis fluid using the BacT/Alert system. *Diagn. Microbiol. Infect. Dis.* **36:**85–90.
17. **Vas, S. I.** 1994. Renaissance of tuberculosis in the 1990's: lessons for the nephrologist. *Perit. Dial. Int.* **14:**209–214.
18. **Warady, B. A., E. K. Sullivan, and S. R. Alexander.** 1996. Lessons from the peritoneal dialysis patient database: a report of the North American Pediatric Renal Transplant Cooperative Study. *Kidney Int. Suppl.* **53:**S68–S71.
19. **Warady, B. A., D. Herbert, E. K. Sullivan, S. R. Alexander, and A. Tejani.** 1997. Renal transplantation, chronic dialysis, and chronic renal insufficiency in children and adolescents. The 1995 annual report of the North American Pediatric Transplant Cooperative Study. *Pediatr. Nephrol.* **11:**49–64.
20. **Warady, B. A., F. Schaefer, M. Holloway, S. Alexander, M. Kandert, B. Piraino, I. Salusky, A. Tranæus, J. Divo, M. Honda, S. Mujais, and E. Verrina.** 2000. Consensus guidelines for the treatment of peritonitis in pediatric patients receiving peritoneal dialysis. *Perit. Dial. Int.* **20:**610–624.
21. **Woods, G. L., and J. A. Washington II.** 1987. Comparison of methods for processing dialysate in suspected continuous ambulatory peritoneal dialysis-associated peritonitis. *Diagn. Microbiol. Infect. Dis.* **7:**155–157.

APPENDIX 13.8–1

CAPD and Infections Associated with Automated Peritoneal Dialysis

A. CAPD-associated infections
   1. Types
      a. Infection of the catheter exit site or subcutaneous tunnel
      b. Infection of the peritoneum
      c. Infection of the catheter exit site or subcutaneous tunnel is often a precursor to infection of the peritoneum.
   2. Etiologic agents
      Although bacteria predominate, fungal and mycobacterial infections are reported and are probably more common than the data suggest.
   3. Origin
      a. Common skin microorganisms: access via catheter lumen or periluminal space
      b. Intestinal organisms, transmural access
      c. Hematogenous seeding (streptococci and *M. tuberculosis*)
      d. Vaginal, ascending (yeasts)
      e. Environmental (e.g., tap water, *Mycobacterium chelonae*, *Methylobacterium mesophilicum*, *Stenotrophomonas maltophilia*, and other pseudomonads)

B. Culture negativity
   Even with all the advancements made in culture techniques, 20% of all cases of peritonitis (cloudy dialysate, abdominal pain, etc.) are culture negative. There are several plausible explanations for this.
   1. Dilution
      Large volumes of dialysate may lower the concentration of bacteria to below detection limits of the culture method used.
   2. Inhibitory substances
      Organisms may be present but have their growth inhibited. While not all the antibacterial factors are known, suppression by the sequestration in phagocytic cells and the presence of antimicrobial agents in the fluid are the most documented sources of false-negative cultures.
   3. Exacting growth requirements
      False-negative cultures may also be caused by a lack of suitable growth requirements for the spectrum of etiologic agents.
   4. Avoidance
      To minimize the number of false-negative cultures, the laboratory should provide for the following.
      a. Concentration of microorganisms
      b. Dilution or removal of antimicrobial agents
      c. Lysis of host cells to release intracellular microorganisms
      d. Parallel or tandem cultures for bacteria, fungi, and mycobacteria

C. Optimal culture protocol
   While there is a plethora of different methods for culturing peritoneal dialysis fluid (1–3, 5, 6, 8–11, 13), with no one method proven to be better than the others, a consensus within the renal community has been reached (7, 12). However, a clearly inferior method is simply culturing a small volume of unconcentrated dialysis fluid. Many methods described in the literature have called for a variety of techniques, including lysis-centrifugation, sonication, antimicrobial removal methods, large-volume culture, etc. (1–4, 5, 6, 8–11, 13). The consensus in the community is known as "Consensus Guidelines for the Treatment of Peritonitis in Pediatric Patients Receiving Peritoneal Dialysis" (12) and the "Peritoneal Dialysis-Related Infections Recommendations" (7).
   1. Send the whole bag to the laboratory.
   2. Send a sample to hematology for an unspun leukocyte count and cell differential. The cell differential could be performed by cytofuging 200 μl of effluent dialysis fluid (1:10 to 1:100 dilution in physiologic saline for WBC counts of >1,000) at 8,000 × g for 7 min and evaluating using Pap smear staining.
   3. Concentrate 50 to 100 ml of effluent for Gram stain and culture.
   4. Culture should include broth enrichment (blood culture system, i.e., BACTEC or BacT/Alert).

APPENDIX 13.8–1 (*continued*)

References

1. **Alfa, M. J., P. Degagne, N. Olson, and G. K. Harding.** 1997. Improved detection of bacterial growth in continuous ambulatory peritoneal dialysis effluent by use of BacT/Alert FAN bottles. *J. Clin. Microbiol.* **35:**862–866.
2. **Buggy, B. P.** 1986. Culture methods for continuous ambulatory peritoneal dialysis-associated peritonitis. *Clin. Microbiol. Newsl.* **8:**12–14.
3. **Dawson, M. S., A. M. Harford, B. K. Garner, D. A. Sica, D. M. landwehr, and H. P. Dalton.** 1985. Total volume culture technique for the isolation of microorganisms from continuous ambulatory peritoneal dialysis patients with peritonitis. *J. Clin. Microbiol.* **22:**391–394.
4. **Forbes, B. A., P. A. Frymore, R. T. Kopecky, J. M. Wojtaszek, and D. J. Pettit.** 1988. Evaluation of lysis-centrifugation system for culturing dialysates from continuous ambulatory peritoneal dialysis patients with peritonitis. *Am. J. Kidney Dis.* **11:**176–179.
5. **Ludlam, H. A., T. N. C. Price, A. J. Berry, and I. Phillips.** 1988. Laboratory diagnosis of peritonitis in patients on continuous ambulatory peritoneal dialysis. *J. Clin. Microbiol.* **26:**1757–1762.
6. **Males, B. M., J. J. Walshe, L. Garringer, D. Koscinski, and D. Amsterdam.** 1986. Addi-Chek filtration, BACTEC, and 10-ml culture methods for recovery of microorganisms from dialysis effluent during episodes of peritonitis. *J. Clin. Microbiol.* **23:**350–353.
7. **Piraino, B., G. R. Bailie, J. Bernardini, E. Boeschoten, A. Gupta, C. J. Holmes, E. J. Kuijper, P. K. Li, W. C. Lye, S. Mujais, D. L. Paterson, M. P. Fontan, A. Ramos, F. Schaefer, and L. Uttley.** 2005. ISPD Ad Hoc Advisory Committee. Peritoneal dialysis-related infections recommendations: 2005 update. *Perit. Dial. Int.* **25:**107–131.
8. **Ryan, S., and S. Fessia.** 1987. Improved methods for recovery of peritonitis-causing organisms from peritoneal dialysate. *J. Clin. Microbiol.* **25:**383–384.
9. **Saubolle, M. A., D. L. Sewell, M. D. Holland, and T. A. Golper.** 1989. Comparison of two commercial broth-culture systems for microbial detection in dialysates of patients on continuous ambulatory peritoneal dialysis. *Diagn. Microbiol. Infect. Dis.* **12:**457–461.
10. **Sewell, D. L., T. A. Golper, P. P. B. Hulman, C. M. Thomas, L. M. West, W. Y. Kubey, and L. Holmes.** 1990. Comparison of large volume culture to other methods for isolation of microorganisms from dialysate. *Perit. Dial. Int.* **10:**49–52.
11. **Van Caseele, P., M. J. Alfa, and G. K. Harding.** 2000. Assessment of the FAN aerobic bottle for culture of continuous ambulatory peritoneal dialysis fluid using the BacT/Alert system. *Diagn. Microbiol. Infect. Dis.* **36:**85–90.
12. **Warady, B. A., F. Schaefer, M. Holloway, S. Alexander, M. Kandert, B. Piraino, I. Salusky, A. Tranæus, J. Divo, M. Honda, S. Mujais, and E. Verrina.** 2000. Consensus guidelines for the treatment of peritonitis in pediatric patients receiving peritoneal dialysis. *Perit. Dial. Int.* **20:**610–624.
13. **Woods, G. L., and J. A. Washington II.** 1987. Comparison of methods for processing dialysate in suspected continuous ambulatory peritoneal dialysis-associated peritonitis. *Diagn. Microbiol. Infect. Dis.* **7:**155–157.

13.9 Air Cultures for Fungi

I. PRINCIPLE

Air is the medium that transports fungi from sources such as soil, construction disturbances, or moldy hospital environments (7, 14, 16). Although breathing airborne fungi is common and for most individuals results in no effects on health, hospitalized patients with extreme immune suppression are susceptible to infections with those airborne fungi that can grow at body temperatures (11).

Generally, patients are maintained in controlled environments during periods of severe immune suppression. Environmental controls for protected environments include increased air changes, positive-pressure rooms, and highly filtered air (1, 9, 15, 19, 20). When it is determined that such individuals have experienced a nosocomial infection due to opportunistic airborne filamentous fungi, an investigation is required to determine whether the controlled environment failed or whether there were other breaks in prescribed procedure, e.g., transport of an unmasked patient outside the controlled environment. Failing to detect the latter, the institution may elect to undertake air sampling to determine whether the controlled environment is working effectively.

Air sampling should not be initiated lightly, since the conduct and interpretation of air cultures for fungi are relatively complex. Ubiquitous in all environments, *fungi in controlled environments must be quantitated and their numbers compared with those in the ambient air in contiguous uncontrolled environments.* These results will provide a basis for determining the efficacy of the suspect controlled environment. Although environmental sampling of hospital air for fungi is not recommended on a routine basis, there are a number of instances in which sampling may be judged necessary: (i) when nosocomial infections are due to common environmental airborne fungi (10, 14, 16), (ii) during outdoor or indoor construction in close proximity to susceptible patients, (iii) prior to initial occupancy of special controlled environments, and (iv) when procedures necessary for the cleanest air quality are being established and maintained. In general, microbiological testing should be limited to environments associated with immunocompromised patients in transplant protocols. Laminar-flow rooms for other hospital applications (surgical suites, pharmacy, etc.) are tested with a particle counter and anemometer.

PREANALYTICAL CONSIDERATIONS

II. SPECIMEN

A. Sample site selection

Comparison samples are essential to determine the effectiveness of filtration and provide a rank order concentration of fungi. For example, the level of fungi in a protected environment might be compared with the levels outdoors and in adjacent areas of the hospital that are not controlled environments (5, 9). Where multiple nosocomial infections have occurred, the rooms of those affected might also be compared with the rooms of those unaffected. Sample site selection must be tailored to the goals of the undertaking; understanding the items that follow will help in the design of the sampling strategy.

B. Sample volume

The volume of air sampled is based on the expected number of fungi in the environment in question and determined by the air volume per unit time of the sampler. The sample volume must be of sufficient size to provide quantitative information at the lower limits of detection when the air is relatively clean and at the higher limits of detection when the air is relatively dirty.

Temperature of incubation of cultures is often adjusted to meet the objectives of the study (see below) and will affect the quantitation. During the growing

Table 13.9–1 Air samplers for quantitation of viable fungal spores[a]

Sampler type	Principle	Rate (liters/min)	Comment(s)
Sieve impactor[b]	Impaction on agar plate	28	Low volume precludes short-term collections that might relate to specific activities; effective for high concentrations of spores.
Slit impactor[c]	Impaction on rotating agar plate	30–700	High volume allows short-term collections that might relate spore aerosols to specific activities; effective for low concentrations of spores; quantification may be compromised at high levels of spores.
Centrifugal impactor[d]	Impaction on plastic strips containing agar media	40	Calibration difficult, thus limited to relative determinations
Impingers (glass)	Impingement into liquids	12.5	Low-volume sampling rates and tendency to disrupt clumps limits application to nonclinical sampling.
Filters (cassette)	Filtration of air through 0.2-μm-pore-size filters	1–2 or 140–1,400	Not practical for viable microbes
Settling plates	Gravity		Most significant spores are too small and buoyant to settle. Lack of quantification severely limits utility.

[a] For additional information, see reference 3.

[b] Anderson Samplers Inc., 4215 Wendell Dr., Atlanta, GA 30336.

[c] New Brunswick Scientific Co., Inc., P.O. Box 4005, 44 Talmadge Rd., Edison, NJ 08818; Casella London Ltd., Regent House, Britannia Walk, London NI 7ND, United Kingdom; or Cassella CEL Inc., 17 Old Nashva Rd. #15, Amherst, NH 03031.

[d] Biotest Diagnostics Corp., 6 Daniel Rd. East, Fairfield, NJ 07006.

II. SPECIMEN (*continued*)

season, when crops, lawns, and gardens are not covered with snow or ice, outdoor air is generally found to contain approximately 1,000 CFU/m^3 (upper limits as high as 100,000 CFU/m^3) when cultures are incubated at room temperature and only 1 to 200 CFU/m^3 when cultures are incubated at 37°C.

For indoor areas with minimum filtration, fungal levels are generally about one-third the level detected outside (2). Areas subjected to high-efficiency filtration can be expected to have $<1/10$ the number of fungi found in adjacent indoor areas. If filters are properly installed and maintained (15), filtration systems remove the majority of fungi, and thus it is necessary to collect high-volume samples (>1 m^3; i.e., 35.3 ft^3) (21). However, when there is snow or ice cover outdoors for extended periods, outdoor levels of fungi may be drastically reduced.

C. Air sampling device

Basic information regarding the types of samplers available and their relative advantages and disadvantages is provided in Table 13.9–1. Many air samplers were devised to sample particles rather than fungi. Thus, they are relatively ineffective for sampling air for fungi. Low-volume samplers operating at 1 ft^3/min or less require sampling times of 35 min or more to ensure adequate air sample volume. The extended employee involvement and the attendant noise

II. SPECIMEN (*continued*)

of sampling may make these samplers relatively useless. More-specific information on the various air sampling devices is provided in Appendix 13.9–1.

Settling plates have often been utilized in the past, but they provide data of little relevance. Since airborne particles settle at different rates (6) and since the smallest fungal particles are often the pathogens of greatest concern, these pathogenic agents may be missed. For example, *Aspergillus fumigatus* spores are 2 to 3.5 μm in diameter, and their aerodynamic buoyance is enhanced by the presence of surface echinulations. Thus, only volumetric methods of sampling provide reliable data.

D. Surface sampling

Subsequent sampling of suspect surfaces may be useful if relatively large numbers of similar organisms are found during air sampling. Such sampling may serve to link an obvious source such as a moldy sink, cabinet, or air duct to a problem with airborne fungi (12, 18, 19). Simply swabbing the suspect area and plating the swab onto media are generally sufficient for such follow-up analysis.

III. MEDIA

Use inhibitory mold agar with chloramphenicol (one plate per sample). (Other suitable selective media, particularly those that promote differentiation of the fungi to produce distinguishing identification characteristics, may be substituted.) Consult section 8 of this handbook for specific guidance on identification of fungi. Although more fungi may be recovered on other media such as rose bengal agar (8), extensive subculturing may be necessary to facilitate identification.

ANALYTICAL CONSIDERATIONS

IV. SPECIMEN PROCESSING

Observe standard precautions.

A. Initial processing

With most air sampling devices, the media are inoculated in association with the operation of the sampler. Specific instructions for the use of the device should be followed.

B. Incubation temperature

1. Room temperature

The selection of the temperature of incubation is determined by the goals of the project. Room temperature incubation is selected to establish rank order differences among the areas sampled. The cleanest environment rank order should be established by using room temperature incubation. This will allow for a determination of filtration efficiencies in various controlled environments in question but will not generally allow for identification of the fungi suspected as pathogens.

2. Incubation at 37°C

Incubation at the more elevated temperature will provide a selective environment for the isolation of airborne fungi that tend to be opportunistic pathogens, e.g., *Aspergillus* species (4). In quantitative comparisons, fungi that grow at room temperature are generally 10-fold more numerous than those that grow at 37°C. Inhibiting growth of the nonpathogenic fungi by incubation at the higher temperature will facilitate the detection and identification of the most likely pathogens.

V. QUALITY CONTROL

Periodically calibrate air-sampling equipment for accurate quantitative measure (17). Generally, a flowmeter or critical orifice may be employed to calibrate the volume of air sampled. Calibration of pumps is also a necessity for reliable data interpretation. Individuals seeking calibration advice should contact the manufacturer of the sampling device or consult with an industrial hygienist with experience in the use of the sampling device.

VI. IDENTIFICATION OF FUNGI

It is imperative that these cultures be handled in a biological safety cabinet.

When tests have been conducted with room temperature incubations for the purpose of determining rank orders of fungal colony counts, it is sufficient to quantitate the colonies without identifying the species of fungi present. When the source of a specific fungal infection is sought and incubation is performed at 37°C, colonies exhibiting appropriate gross morphology should be subjected to microscopic examination and subcultured for further identification only if required to fulfill the intent of the investigation. Species identification methods are covered in section 8 of this handbook.

POSTANALYTICAL CONSIDERATIONS

VII. CALCULATIONS

A. Conversion factors

1. 1 ft^3 = 28.4 liters

2. 1 m^3 = 35.3 ft^3

3. 1 ft^3/min = 0.0283 m^3/min

B. Performing calculations

Divide the number of colonies of the total sample by the volume of air sampled to determine the CFU per cubic meter. Where calibration indicates the necessity, adjust air sample volumes by the appropriate factor.

VIII. DATA INTERPRETATION

The ubiquity of fungi in the environment dictates that low levels of fungi not be considered a cause for alarm; thus, methods that detect fungi without quantification will be of no use. Moreover, methods, such as settling plates, that are insufficiently sensitive to detect fungal pathogens provide no useful information.

Rank ordering of fungal levels within the controlled environmental areas, in adjacent uncontrolled areas, and outdoors may allow for a determination to be made relative to a malfunction of filtration devices, interruptions in the positive pressure, leaks to the outdoors, or point sources of fungal spores. Such interpretation must be made in the local environment of concern and must incorporate a knowledge of the effect of snow cover and temperature of incubation. Generally, protected environments with high-efficiency filtration should exhibit levels of fungi approximately a full order of magnitude lower than the levels detected in the adjacent outdoor environment, but this differential level cannot be expected during long periods of snow cover.

When test samples have been incubated at 37°C to specifically detect and identify a fungal pathogen, its presence may help implicate an environmental source, but its absence does not rule out the temporary presence of such a source during the actual period of patient exposure. If only one colony of a pathogen is recovered, repeat sampling is indicated. Multiple-hit pathogens on culture plates are of greater concern than the single isolation of a "random" airborne pathogenic particle. Repeat sampling is indicated if multiple colonies of a pathogen are recovered from air sampling in highly filtered areas. In areas that provide a protective environment (99.97% efficient filtration for 0.3-μm particles, >12 air exchanges/h, and a pressure differential of 0.01 in. water gauge [2.5 Pa.] outflow of air), the total pathogen

VIII. DATA INTERPRETATION (*continued*)

(*A. fumigatus, Aspergillus flavus, Aspergillus terreus, Fusarium* spp., or other potentially opportunistic fungus) level should be maintained at <0.1 CFU/m^3 (22). If total pathogen levels exceed 1.0 CFU/m^3 on several occasions, the air systems or procedural practice in the patient areas requires intensive evaluation.

When gross colony counts rather than fungal pathogens are determined for the most highly filtered areas, fungal levels should not exceed 15 CFU/m^3 for cultures incubated at room temperature or 2 CFU/m^3 for cultures incubated at 37°C. The homogeneous occurrence of an organism suggests a point source from an indoor area (12), and such problems generally arise from situations that include uncontrolled humidity or water leakage. Removal efficiencies of filtration were established for fungi that grow at 37°C (13), and there may one day be sufficient data to conduct rank order studies at this temperature.

Because of the delay in results from microbiological sampling, real-time quantative analysis is useful for assurance that the filters are properly installed, especially during initial preoccupancy testing and epidemiologic outbreak investigation. The particle counter (either optical or condensation based) is useful for filter evaluations and for assurance of proper installation of the filter. The condensation particle counter will assess small leaks which may be due to tears in the filter media. This would not be possible with the air sampling apparatus for viable fungal particles, but it must be remembered that the particle counter should be used only as an indicator of a problem. These devices provide rank order analysis to determine if the area with the highest filtration has the lowest particle counts. This is useful because if such testing is conducted before microbiological testing during the commissioning of new protective areas, problem solving can be accomplished before expensive microbiology testing takes place. The assurance of pressurization for the protected rooms and room air exchange confirmation are essential before testing for viable or nonviable airborne particles (21).

REFERENCES

1. **Barnes, R. A., and T. R. Rogers.** 1989. Control of nosocomial aspergillosus by laminar air flow isolation. *J. Hosp. Infect.* **14:**89–94.
2. **Burge, H. G. (ed.).** 1989. *Guidelines for the Assessment of Bioaerosols in the Indoor Environment.* Conference of Government Industrial Hygienists, Cincinnati, OH.
3. **Chatigny, M. A., J. M. Macher, H. A. Burge, and W. R. Solomon.** 1989. Sampling airborne microorganisms and aeroallergens, p. 199–220. *In* S. V. Hering (ed.), *Air Sampling Instruments for Evaluation of Atmospheric Contaminants,* 7th ed. Conference of Governmental Industrial Hygienists, Cincinnati, OH.
4. **Davies, R. R.** 1971. Air samplers for fungi, pollen and bacteria, p. 455–472. *In* C. Booth (ed.), *Methods in Microbiology-Mycology,* vol. 4. Academic Press, Inc., New York, NY.
5. **Greene, V. W., D. Vesley, R. G. Bond, and G. S. Michaelsen.** 1962. Microbiological contamination of hospital air: quantitative studies. *Appl. Microbiol.* **10:**561–571.
6. **Gregory, P. H.** 1973. *The Microbiology of the Atmosphere,* p. 126–145. John Wiley & Sons, Inc., New York, NY.
7. **Lentino, J. R., M. A. Rosenkranz, J. A. Michaels, V. P. Knrup, H. D. Rose, and M. W. Rytel.** 1986. Nosocomial aspergillosis: a retrospective review. *Am. J. Epidemiol.* **116:**430–437.
8. **Morring, K. L., W. G. Sorenson, and M. D. Attfield.** 1983. Sampling for airborne fungi: a statistical comparison of media. *Am. Ind. Hyg. Assoc. J.* **44:**662–664.
9. **Murray, W. A., A. J. Streifel, T. J. O'Dea, and F. S. Rhame.** 1988. Ventilation for protection of immune compromised patients. *Am. Soc. Heat. Refrig. Air Cond. Eng. Trans.* **94:**1185–1191.
10. **Opal, S. M., A. A. Asp, B. B. Cannady, P. L. Morse, L. J. Burton, and P. G. Hammer.** 1986. Efficacy of infection control measures with hospital construction. *J. Infect. Dis.* **153:**634–637.
11. **Peterson, P. K., P. B. McGlave, and N. K. Ramsey.** 1983. A prospective study of infectious diseases following bone marrow transplantation: emergence of *Aspergillus* and cytomegalovirus as the major causes of mortality. *Infect. Control* **4:**81–89.
12. **Reynolds, S. J., A. J. Streifel, and C. E. McJilton.** 1990. Elevated concentrations of airborne fungi in residential and office environments. *Am. Ind. Hyg. Assoc. J.* **51:**601–604.
13. **Rhame, F. S., M. Mazzarella, A. J. Streifel, and D. Vesley.** 1990. Evaluation of commercial air filters for fungal spore removal efficiency. *Abstract 3rd Int. Conf. Nosocomial Infect.*

REFERENCES (*continued*)

14. **Rhame, F. S., A. J. Streifel, J. H. Kersey, and P. B. McGlave.** 1984. Extrinsic risk factors for pneumonia in the patient at high risk of infection. *Am. J. Med.* **76:**42–52.
15. **Rose, D. H.** 1972. Mechanical control of hospital ventilation and *Aspergillus* infections. *Am. Rev. Respir. Dis.* **105:**306–307.
16. **Sarubbi, F. A., H. B. Kopf, B. Wilson, M. R. McGinnis, and W. A. Rutala.** 1982. Increased recovery of *Aspergillus flavus* from respiratory specimens during hospital construction. *Am. Rev. Respir. Dis.* **125:**33–38.
17. **Stewart, W. H.** 1959. *Sampling Microbial Aerosols.* Monograph no. 60, Public Health Service publication no. 686. U.S. Department of Health, Education, and Welfare. U.S. Government Printing Office, Washington, DC.
18. **Streifel, A. J., P. P. Stevens, and F. S. Rhame.** 1987. In-hospital source of airborne *Penicillium* species spores. *J. Clin. Microbiol.* **25:**1–4.
19. **Streifel, A. J., D. Vesley, and F. S. Rhame.** 1990. Occurrence of transient high levels of airborne fungal spores, July 29–August 3. *Proc. 5th Int. Conf. Indoor Air Qual. Climate* **1:**207–212.
20. **Streifel, A. J., D. Vesley, F. S. Rhame, and B. Murray.** 1989. Control of airborne fungal spores in a university hospital. *Environ. Int.* **15:**221–227.
21. **Streifel, A. J.** 2000. Healthcare indoor air quality: guidance for infection control. *HPAC Eng.* **72:**28–35.
22. **Thio, C. L., D. Smith, W. G. Merz, A. J. Streifel, G. Bova, L. Gay, C. B. Miller, and T. M. Perl.** 2000. Refinements of environmental assessment during an outbreak investigation of invasive aspergillosis in a leukemia and bone marrow transplant unit. *Infect. Control Hosp. Epidemiol.* **21:**18–23.

SUPPLEMENTAL READING

Streifel, A. J. 1988. *Aspergillus* and construction, p. 198–217. *In* R. B. Kundsin (ed.), *Architectural Design and Indoor Microbial Pollution.* Oxford University Press, New York, NY.

Streifel, A. J. 1999. Design and maintenance of hospital ventilation systems and the prevention of airborne nosocomial infections, p. 1211. *In* C. G. Mayhall (ed.), *Hospital Epidemiology and Infection Control.* Lippincott Williams and Wilkins, Philadelphia, PA.

APPENDIX 13.9–1

Features of Mechanical Volumetric Sampling Devices

A. Sieve impactors

1. General description

   Sieve impactors are efficient air particle collectors (2, 5, 6) and are commercially available (*see* Table 13.9–1). The Andersen device is the most commonly used volumetric sampler. This sampling device uses inertial impaction on the agar surface to collect viable particles. Each stage of the sampler has 200 or 400 holes through which the particles travel, and the holes get smaller as the stage number descends. This provides the basis for the differentiation of various particle sizes. The sieve impactors differentiate respirable (<5-μm-diameter) viable particles from nonrespirable (>5-μm-diameter) particles when at least two stages of the sampler are used. If the sampler sieve impactor has six stages, the particles above stage 3 are considered nonrespirable, while the particles above stage 6 are respirable. Because of the design and aerodynamic nature of airborne particles, stage 3 removes particles that are >5 μm in diameter, allowing the smaller remaining particles to become impacted on stage 6. Because of a statistical correction formula, this sampler allows reasonable accuracy in sampling of areas with high concentrations of airborne fungi. The correction is applied whenever >19 colonies are counted on the sample plate. Because of the reimpaction potential of incoming viable particles, a table for conversion is available (1).

2. Disadvantages

   The sampler generally operates at 1.0 ft^3/min. In a relatively clean environment, the sample time for determining 1.0 CFU of pathogens per m^3 would have to be extended to >35 min in order to collect 35.3 ft^3 (or 1 m^3) of air. Sieve impactors generally employ a vacuum pump and are a noise nuisance in an occupied patient room. For long sampling times, patient room vacuum may be replaced by employing an airflow that restricts the critical orifice interfaced to the air sampler.

B. Slit impactor

1. General properties

   The slit impactor is an efficient particle collector that utilizes inertial force to impact viable particles onto the surface of the agar. These devices do not differentiate particle

APPENDIX 13.9–1 (*continued*)

sizes (7). They have a rotating disk on which a standard-sized plate (150 by 15 mm) of agar medium is placed. The rotation of the plate under the stationary slit allows for production of appropriate time-concentration relationships. Slit impactors have preset sample volume rates ranging from 30 to 700 liters/min; thus, variation of sample time will allow for quantitation. Since the sample volume ranges as high as 25 ft^3/min, these collectors take only minutes. Thus, they may be used to distinguish changes in concentration over brief periods, and such changes might be referable to local activities that induce them.

2. Disadvantage
   Accuracy is limited where there are high concentrations of fungal spores.

C. Centrifugal sampler
1. General properties
   The centrifugal impactor utilizes the same principles as other impactors. A strip containing wells of agar medium is placed around the sampling head. The compact portable nature of this device and its quiet operation make it ideal for inconspicuous operation (3).
2. Disadvantages
   Calibration is done at the factory, and quantitation of air volume is thus not possible except at the factory. Thus, only relative comparisons of fungal contamination between different areas can be made.
   Working with a welled strip of media makes identification difficult and requires extra subculturing.

D. Filtration
This method is primarily used for evaluation of airborne minerals and respirable dust (4). Because of their smallness and low-volume sample rate (~1 liter/min), filter cassettes are not practical for sampling viable microbes.

E. Impingement
Because of their low-volume sampling rates and tendency to disrupt viable particle clumps, the application of these devices is limited to areas where high concentrations are suspected, e.g., turkey barns.

F. Rotorods
This device employs impaction on a sticky glass surface that is rotated in ambient outdoor air. Rotorods are not practical for low-level indoor sampling. Generally employed for air analysis of spores and pollen in allergy studies (7), the device measures numbers of nonviable particles.

References

1. **Andersen, A. A.** 1958. New sampler for collection, sizing, and enumeration of viable airborne particles. *J. Bacteriol.* **76:**471–484.
2. **Burge, H. G. (ed.).** 1989. *Guidelines for the Assessment of Bioaerosols in the Indoor Environment.* Conference of Government Industrial Hygienists, Cincinnati, OH.
3. **Casewell, M. W., N. Desai, and E. J. Lease.** 1985. The use of the Reuter centrifugal air sampler for the estimation of bacterial air counts in different hospital locations. *J. Hosp. Infect.* **7:**250–260.
4. **Fields, N. G., G. S. Oxborrow, J. R. Puleo, and C. M. Herring.** 1974. Evaluation of membrane filter field monitors for microbiological air sampling. *Appl. Microbiol.* **27:**517–520.
5. **Gregory, P. H.** 1973. *The Microbiology of the Atmosphere,* p. 126–145. John Wiley & Sons, Inc., New York, NY.
6. **Rhame, F. S., A. J. Streifel, J. H. Kersey, and P. B. McGlave.** 1984. Extrinsic risk factors for pneumonia in the patient at high risk of infection. *Am. J. Med.* **76:**42–52.
7. **Stewart, W. H.** 1959. *Sampling Microbial Aerosols.* Monograph no. 60, Public Health Service publication no. 686. U.S. Department of Health, Education, and Welfare. U.S. Government Printing Office, Washington, DC.

13.10 Microbiological Assay of Environmental and Medical-Device Surfaces

I. PRINCIPLES

A. Recommendations against routine assays ("monitoring")

Although a number of textbooks in the 1970s recommended routine environmental monitoring in a variety of settings, the vast majority of these practices have proven expensive and of little value (2, 5, 8, 9, 16, 17, 20). Routine monitoring is defined in this context as those procedures that are random and undirected and that differ from those targeted for defined purposes (5). This procedure is not intended in any way to discourage sampling and assay of environmental or medical-device surfaces for which specimen collection, culture, and data interpretation are conducted in accordance with defined protocols. The only routine environmental microbiological assay regimen generally recommended today is for QA purposes: (i) monitoring of sterilization processes by using bacterial spores (biological indicators), (ii) monthly cultures of water and dialysate fluids in the hemodialysis unit (*see* procedure 13.7), and (iii) periodic monitoring of dental-unit water supplies or filtered rinsing water in automated endoscope reprocessors (note that there are no current established standards for the situations listed in item iii, but such monitoring is often done in settings where water quality is a major concern). All other environmental microbiological assays should be performed only in response to epidemiologic investigations suggesting that environmental or medical-device surfaces may be microbial reservoirs or sources of health care-associated disease transmission. Also, the monitoring must be in conformance with relevant policy of the institution (*see* procedures 13.2 and 13.3). Microbiological assays may also be helpful in testing the effectiveness of new or modified cleaning and/or disinfecting procedures. Selection of appropriate sampling and culturing techniques is critical if results are to be meaningful. A number of methods for sampling various types of surfaces and devices have been described. Because of the lack of standard guidelines or protocols as well as the complexity and variety of surfaces and medical devices, the microbiologist will necessarily select the appropriate methodology by reviewing the current literature and using scientific judgment and common sense. Moreover, no assays of environmental or medical-device surfaces should be done unless criteria regarding possible results of the cultures and a corresponding corrective action plan have been established (14, 17, 20).

B. Monitoring plan

No environmental monitoring should be conducted without prior approval of the Infection Control Committee (or, at a minimum, a designated person who has consulted with a qualified microbiologist) and the existence of a carefully formulated and detailed plan of action (14). Items to consider when developing such a plan are summarized in Table 13.10–1. In addition to an objective, a summary of the current literature, and a bibliography, the written plan should include clear descriptions

Table 13.10–1 Considerations before undertaking environmental surface sampling

Background information from the literature and present activities (i.e., preliminary results from an epidemiologic investigation)
Location and accessibility of surfaces to be sampled
Method of sample collection and the appropriate equipment for this task
Number of replicate samples needed
Number and type of control or comparison samples needed
Parameters of the sample assay method and whether the sampling will be qualitative, quantitative, or both
An estimate of the maximum allowable microbial numbers and types on surface(s) sampled (refer to the Spaulding classification for devices and surfaces [23] summarized in item V)
Some anticipation of a preliminary corrective action plan

of (i) location(s) and methods of sample collection, including possible problems with physical access for adequate sampling; (ii) frequency of sample collection (related to time and/or clinical indications); (iii) sample assay method(s), including whether the assays will be quantitative or qualitative; (iv) sample volumes to be assayed; (v) media (selective or nonselective), diluents, rinse fluids, and neutralizers as appropriate; (vi) incubation times and conditions and allowable time lapse between sample collection and assay; (vii) a listing of other physical or chemical factors pertinent to a particular setting that may affect culture results; and (viii) an estimate of the maximum allowable microbial numbers and types on the surface(s) sampled.

PREANALYTICAL CONSIDERATIONS

II. MEDIA, REAGENTS, AND EQUIPMENT

A. Media

Virtually all media (preprepared, dehydrated, or individual components), reagents, and equipment appropriate for environmental microbiological culturing are available from a variety of commercial suppliers of media and equipment serving the clinical microbiology laboratory community.

1. Media appropriate for quantitative determinations of microorganisms
   - **a.** Nutrient-rich, nonselective agar media (e.g., TSA or BHI agar plates with or without 5% sheep or rabbit blood supplement for enhanced recovery of fastidious pathogens)
   - **b.** Nutrient-rich, nonselective broth media (as above) with or without supplements (as above) for saturation of absorbent pads placed beneath membrane filters
   - **c.** Selective agar or broth media (as above) for culture of specific groups of microorganisms (e.g., MAC or Cetrimide or Sabouraud agar or broth)
   - **d.** Addition of disinfectant neutralizers to culture media may be appropriate (*see* item III.B.1.b below).

2. Media appropriate for qualitative determinations for specific groups of microorganisms
   - **a.** Nonselective broth media, as necessary, for initial enrichment of low levels of microorganisms recovered from surfaces
   - **b.** Selective broth media (e.g., Cetrimide or MAC) to detect the presence of specific groups of microorganisms

3. Media appropriate for culturing environmental microorganisms that are expected to be physically damaged or "stressed" (e.g., such as those microorganisms recovered from finished treated or raw water ["naturally occurring" water microorganisms])
   - **a.** Avoid using high-nutrient-content media (e.g., blood agar, TSA) in these situations, as these media may actually inhibit the growth of damaged organisms (1).
   - **b.** Media with reduced nutrient content (e.g., R2A agar, diluted peptone) to recover microorganisms from environments with minimal nutrient concentrations (1)

B. Reagents

Special reagents necessary for environmental and medical-device sampling are minimal and consist primarily of rinse or dilution fluids (e.g., nonbacteriostatic saline [0.85%, wt/vol, sodium chloride]) or various buffer solutions (with or without specific neutralizers of disinfectants), preprepared or custom-made (11).

C. Equipment

Equipment necessary for the vast majority of environmental or medical-device sampling is available in most well-equipped clinical microbiology laboratories and includes a variety of glass- or plasticware (e.g., petri plates, beakers, flasks, screw-cap tubes and bottles, pipettes), incubators, an ultrasonic bath, vortex mixers, membrane filter holder assemblies, a vacuum source with a vacuum flask and "traps" to protect the vacuum source, a biological safety cabinet, and so forth. For membrane filter assays, a variety of prepackaged sterile filter holders, filters, and sample tubes as well as culture plates and media are available from a number of commercial sources. Sterile gloves are appropriate for use during aseptic sampling techniques. Tweezers, forceps, scissors, or other devices for manipulating sampling materials should be single-use disposables or sterilized, preferably by steam autoclaving, prior to use. Some means for spot decontamination of tweezers, scissors, etc., should also be available

II. MEDIA, REAGENTS, AND EQUIPMENT (*continued*)

(e.g., ceramic oven devices used for inoculating loop "flaming"). In the past, natural gas Bunsen burners or propane torches were commonly available in laboratories. Open-flame burners, however, may not be allowed in modern laboratory facilities for safety reasons. Additionally, use of such burners in biological safety cabinets may interfere with the proper airflow in the cabinet.

ANALYTICAL CONSIDERATIONS

III. COLLECTION AND ASSAY OF SPECIMENS

◩ **NOTE:** Observe proper aseptic procedures.

A. General

Collect microbiological samples by using a sterile swab, sponge, or "wipe" cloth subsequently eluted in a rinse fluid (commonly termed swab-rinse, sponge-rinse, or wipe-rinse method, respectively), by directly eluting an item with rinse fluid (rinse method), or by pressing an agar medium surface directly on a surface to be sampled (i.e., replicate organism direct agar contact [RODAC] method). A summary of surface sampling methods is shown in Table 13.10–2. Whichever

Table 13.10–2 Comparison of surface sampling methods[a]

Method	Location for use	Assay technique	Procedure notes	Points of interpretation	Availability of standards	References
Sample-rinse						
Moistened swab-rinse	Nonabsorbent surfaces, corners, crevices, devices, instruments	Dilutions; qualitative or quantitative assays	Assay multiple measured areas or devices with separate swabs	Report results per measured area or, if assaying an object, per the entire sample site	Yes for food industry; no for health care	10–12, 18
Moistened sponge-rinse	Large areas, housekeeping surfaces (e.g., floors, walls)	Dilutions; qualitative or quantitative assays	Vigorously rub a sterile sponge over the surface	Report results per measured area	Yes for food industry; no for health care	10–12, 18
Moistened wipe-rinse	Large areas, housekeeping surfaces (e.g., countertops)	Dilutions; qualitative or quantitative assays	Use a sterile wipe	Report results per measured area	Yes for food industry; no for health care	10–12, 18
Direct immersion	Small items capable of being immersed	Dilutions; qualitative or quantitative assays	Use membrane filtration if rinse volume is large and anticipated microbiological concentration is low	Report results per item	No	
Containment	Interior surfaces of containers, tubes, bottles, etc.	Dilutions; qualitative or quantitative assays	Use membrane filtration if rinse volume is large	Evaluate both the type(s) and numbers of microorganisms	Yes for food/industrial applications for containers prior to fill	
RODAC	Previously cleaned and sanitized flat, nonabsorbent surfaces; not suitable for irregular surfaces	Direct assay	Overgrowth occurs if used on heavily contaminated surfaces; use neutralizers in the agar if surface disinfectant residuals are present	Provides direct, quantitative results; minimum of 15 plates per average hospital room	No	11, 13

[a] See also Appendix 13.10–1.

III. COLLECTION AND ASSAY OF SPECIMENS (*continued*)

technique is chosen, observe proper aseptic procedure throughout the sampling and assay processes.

B. Rinse fluid

1. Choice of fluid

a. Surfaces without residual disinfectant or other toxic substances

(1) Sterile nonbacteriostatic saline (0.85% [wt/vol] sodium chloride) or a buffer solution

(2) General multipurpose broth medium (e.g., TSB or BHI broth) supplemented with 0.5% beef extract (3, 16)

b. Surfaces with residual disinfectant or other toxic substances

Many environmental or instrument surfaces have residues of disinfectants (e.g., phenolics, hypochlorites, iodophors, quaternary ammonium compounds, hydrogen peroxide, formaldehyde, glutaraldehyde) that may require incorporation of specific neutralizers into the rinse medium, particularly when the rinse medium itself serves as a culture medium for enrichment in qualitative assays. Use of specific neutralizers in rinse or recovery media is often necessary to eliminate toxic carryover in the assay that may reduce the numbers of microorganisms and give an erroneous result (Table 13.10–3). More comprehensive discussions of the use of disinfectant neutralizers have been published elsewhere (12, 21). Commercially available recovery media having a spectrum of neutralizing agents (e.g., Dey-Engley medium) (11), nonspecific neutralization of disinfectants by incorporation of protein supplements in rinse media (e.g., 0.5% beef extract), or double-strength broth media (e.g., 2× TSB or BHI broth) may be advocated when the exact nature of residual chemicals is not known. A combination of 0.07% lecithin and 0.5% polysorbate (Tween) 80 is often recommended as a routine supplement to rinse or recovery media (Table 13.10–3) (3, 17). Unfortunately, specific neutralizers themselves may be toxic to certain groups of microorganisms. For example, sodium thiosulfate will inhibit the growth of some streptococci (21), and Dey-Engley medium may prevent the growth of certain mycobacteria (6). An effective approach to the problem of residual disinfectant or other toxic substances on surfaces being sampled is to employ the membrane filter assay method with extensive postfiltration rinsing along with adequate controls to ensure that rinsing is effective.

2. Culture methods for rinse fluids (1, 3, 11)

a. Quantitative culture, agar spread plate method

(1) Make serial 10-fold dilutions of the rinse fluid in tubes of sterile medium.

(2) Pipette 0.1 ml of each dilution (e.g., undiluted, 1:10, 1:100) onto a standard agar plate surface.

Table 13.10–3 Neutralizers of common disinfectant chemicals[a,b]

Disinfectant(s)	Neutralizer
Glutaraldehyde	Glycine
Formaldehyde	Glycine
Phenolics	Polysorbate (Tween) 80
Quaternary ammonium compounds	Lecithin + polysorbate (Tween) 80[c]
Chlorine	Sodium thiosulfate
Iodine	Sodium thiosulfate
Hydrogen peroxide	Catalase

[a] Reference 21.

[b] Used in lieu of or in combination with membrane filtration assay methods.

[c] Often recommended as a routine supplement for rinse or recovery media (3, 17). Letheen broths and agars are commercial alternatives.

III. COLLECTION AND ASSAY OF SPECIMENS (*continued*)

(3) Use a separate, sterile, bent spreader ("hockey stick") to distribute the inoculum on each plate surface.
(4) Dry the plates in a biological safety cabinet to prevent extraneous contamination, and invert them for incubation.
(5) Incubate plates under conditions and for periods of time sufficient to recover implicated microbial populations.
(6) Examine plates with a well-lighted binocular dissecting microscope or a back-lighted magnified colony-counting device to discern small colonies.
(7) Count colonies on plates having between 30 and 300 colonies.
(8) Report the numbers of microorganisms per unit or unit area sampled (taking into account the dilution and volume of diluent assayed).

b. Quantitative culture, membrane filter method
(1) Make dilutions of rinse fluid as explained in item III.B.2.a(1) above.
(2) Filter portions (1 ml, or more if desired) of each dilution through a membrane filter (0.45- or 0.22-μm pore size, 47-mm diameter [filters with gridded surfaces facilitate colony counting]).
(3) Rinse filters thoroughly by suctioning additional sterile rinse medium (with or without specific or nonspecific chemical neutralizers, as dictated by the sampling regimen) through them.
(4) Using sterile forceps, place filters sample side up on petri plates containing the agar medium of choice (avoid trapping air between the filter and the agar surface) or on sterile absorbent pads saturated with the appropriate broth medium.
(5) Incubate filters appropriately, and use a well-lighted dissecting microscope to count colonies on filters having between 20 and 200 colonies.
(6) Report the numbers of microorganisms as explained in item III.B.2.a.(8) above.

c. Quantitative culture, pour plate method
Quantitative culture using the pour plate method is precise and relatively simple but may be cumbersome and time-consuming in a clinical microbiological laboratory. Up to 2 to 3 ml of sample or diluted sample fluid may be placed in an empty petri plate and then mixed with molten agar medium (brought to 44 to 46°C; a thermostatically controlled water bath is required). After solidification, plates are inverted and incubated as usual. Subsurface colonies are discrete and obviate problems of spreading and colony coalescence. Subsurface growth, however, may be slower than on the agar surface, colony morphologies are atypical (e.g., "flying saucer" shaped), and retrieval of colony material for subculture may be difficult. Details of this culture method are given elsewhere (1).

d. Qualitative culture (to detect low numbers of selected groups of microorganisms in heterogeneous microbial populations [3])
(1) Use an appropriate broth nutrient medium rather than 0.85% sodium chloride or buffer as a rinse fluid for sample collection.
(2) Transfer 0.5 ml of the rinse fluid onto the surface of an appropriate agar medium (e.g., MAC for selective culture of coliforms or Cetrimide agar for culture of *Pseudomonas* spp.).
(3) Tilt the plate to distribute the inoculum over the agar surface, dry agar surface in a biological safety cabinet, and invert plate for incubation.
(4) Incubate the broth rinse fluid at an appropriate recovery temperature.
(5) Subculture rinse fluid as in item III.B.2.d.(2) above at intervals of 4 and 24 h.

III. COLLECTION AND ASSAY OF SPECIMENS (*continued*)

(6) If rinse fluid appears turbid after 4 or 24 h of incubation, streak a loopful of fluid on separate plates of the selected subculture medium.

(7) After appropriate incubation, examine subculture plates with a well-lighted dissecting microscope to detect small colonies.

C. Swab-rinse sampling method (3, 4, 7, 10–12, 16, 17)

◩ **NOTE:** It must be acknowledged that the recommended agitation methods and durations for elution of microorganisms from sample collection devices (e.g., swabs, sponges, cloths) will result in much less than 100% recovery (15). Once an agitation method and its duration are chosen, however, their application should be consistent among specimens.

1. Sampling large, flat, nonabsorbent surfaces

a. Cut a square hole of specified size (e.g., 2 by 2 in.) in a piece of heavy paper (e.g., craft or butcher paper) as a sampling template, and make one for each sample to be taken.

b. Wrap templates, and sterilize them in an autoclave.

c. Use sterile swabs of nonabsorbent cotton or polyester fiber (approximately 0.5 by 2 cm on a 12- to 15-cm applicator stick).

d. Moisten the swab in a test tube containing 4 to 5 ml of sterile rinse medium by first immersing the swab head in rinse medium and then wringing out excess fluid by pressing and rotating the swab on the inside surface of the tube above the fluid.

e. Place template on selected area, using a separate template for each sampling site.

f. Slowly rotate the moistened swab on the surface while pulling the swab toward you in close parallel streaks within the template opening to effect a shearing action between the surface and the swab.

g. Repeat the swab motion with the same swab in streaks at right angles to the first streaks.

h. Place the swab head in a tube of rinse fluid of known volume (e.g., 10 ml) by cutting the plastic applicator stick with sterile scissors or by using the inside lip of the tube to break a wooden applicator stick below the portion that was handled.

i. Cap the tube, and then shake end to end 50 times, or process for 30 s on a vortex mixer at high speed.

j. Assay the rinse fluid, and report results as explained in item III.B.2 above.

2. Sampling irregular or recessed surfaces (e.g., tubes, containers, or external or internal surfaces of medical devices)

a. Develop a plan for sampling each surface or device to ensure consistency from sample to sample for comparison purposes (e.g., 10 cm upward from the distal tip of the flexible fiber-optic endoscope insertion tube's outer surface, or swab rotated 10 times in the suction valve seat).

b. Collect swab specimens, and elute as explained in steps III.C.1.h and i above;

c. Assay rinse fluid, and report results as explained in item III.B.2 above.

D. Sponge-rinse (11) and wipe-rinse (18) sampling methods

1. The sponge-rinse and wipe-rinse methods are useful for sampling relatively large environmental surfaces (e.g., in food-handling areas during an outbreak of *Salmonella* infections).

2. Sterilize individually packaged squares (e.g., 5 by 5 cm) of cellulose sponge or disposable nonwoven fabric (rayon) wiping cloths (approximately 15 by 25 cm) by autoclaving (sponges and wipes must *not* be the types preloaded with cleaning or disinfecting agents).

3. Use sterile crucible tongs or sterile latex gloves to handle sponges or wipes.

III. COLLECTION AND ASSAY OF SPECIMENS (*continued*)

4. Moisten the sterile sampling materials with approximately 10 ml of rinse fluid (nonbacteriostatic saline, buffer, or nutrient medium with or without disinfectant neutralizers as discussed above).
5. Vigorously rub each designated area (up to several square meters) in a consistent manner.
6. Place the sponge or wipe into a sterile, sealable plastic bag or mason-type jar with a liquid-tight lid.
7. Add 50 to 100 ml of sterile rinse fluid to the bag or jar.
8. Massage the plastic bag or shake the jar vigorously for a least 1 min to elute the microorganisms.
9. Assay the rinse fluid, and report results as explained in item III.B.2 above.

E. Rinse sampling method (direct immersion)

1. The rinse sampling method using direct immersion is one of the most efficient methods for recovering surface microorganisms from items small enough to be placed into a culture tube or flask and thoroughly exposed to a rinse solution.
2. Using sterile forceps or gloves, place the item to be sampled into a container of sterile rinse fluid of known volume.
3. Shake the container vigorously for 1 min (use a plastic or unbreakable container if breakage of glass is possible), or use an ultrasonic cleaning bath to facilitate removal of microorganisms from occluded surfaces (19).
4. Assay rinse fluid, and report results as explained in item III.B.2 above.

F. Rinse sampling method (containment)

1. The rinse sampling method using containment is used for sampling bottles or containers (e.g., nebulizer reservoirs) or respiratory therapy tubing.
2. Place a suitable volume of rinse fluid (e.g., 10 to 50 ml) in the bottle or tubing, and seal appropriately with sterile lids or rubber stoppers.
3. Vigorously shake sealed item end to end approximately 50 times.
4. If necessary, decant the sample fluid into a sterile collection vessel.
5. Assay rinse fluid, and report results as explained in item III.B.2 above.

G. RODAC sampling-culture method (11)

1. The RODAC technique is very convenient and less cumbersome than other methods (e.g., item III.C.1 above) for estimating the sanitary quality of flat, smooth surfaces (e.g., floors, tabletops). Reserve this sampling method for surfaces that have been recently cleaned and sanitized, since heavily contaminated surfaces may result in rapid overgrowth of the plates.
2. Purchase special sterile plastic plates that when filled with 16.5 ml of agar medium will form a convex agar meniscus. (RODAC plates are commercially available empty or prefilled with a selected number of media; other similar plates may hold less medium. Follow manufacturers' directions accordingly.)
3. Fill empty plates with a general culture medium (e.g., TSA with or without added disinfectant neutralizers) for total counts or with a selective medium (e.g., MAC) for detecting specific groups of microorganisms.
4. Use a minimum of 15 plates for an average hospital room, with sampling sites chosen randomly to prevent biasing of results.
5. Press a plate firmly, without circular or linear movement, against a dry surface.
6. Count colonies after 48 h of incubation at the selected temperature.

IV. CULTURE WORKUP

A. Selection of colonies

At least two colonies representative of each morphological type present on culture plates should be picked for subsequent identification and further characterization.

B. Characterization of isolates

If the sampling has been done in response to an epidemiologic investigation of a disease outbreak, identification of isolates to the species level is mandatory, and characterization beyond the species level should be seriously considered (17). Methods for identification of microorganisms are given in other sections of this handbook.

POSTANALYTICAL CONSIDERATIONS

V. INTERPRETATION (9)

When interpreting sampling results for environmental and medical-device surfaces, it is important to keep the general principles of the Spaulding classification in mind (23).

A. Critical medical instruments

Critical medical instruments (e.g., surgical instruments) break the blood barrier or penetrate to normally sterile areas of the body and carry a high degree of infection risk if contaminated at the time of use. The generally acceptable microbiological standard for these items is the absence of all viable microbial forms, including bacterial spores (i.e., no microbial growth should be observed when an instrument previously subjected to a validated sterilization cycle is sampled).

B. Semicritical medical instruments

Semicritical instruments (e.g., flexible fiber-optic endoscopes, laryngoscope blades) generally come into contact only with mucous membranes during use and have a moderate degree of infection risk if they are contaminated at the time of use. The minimum acceptable microbiological standard for this class of instruments is the absence of all microorganisms except an occasional bacterial sporeformer not recognized as an overt pathogen (e.g., certain species of the genus *Bacillus* or *Clostridium*).

C. Noncritical medical instruments

Noncritical medical instruments (e.g., blood pressure cuffs, stethoscopes) come into contact only with intact skin during use and carry a low risk of disease transmission if contaminated. The basic criterion for safety is visible cleanliness, and a generally acceptable microbiological standard for this category is the absence of recognized pathogens.

D. (Noncritical) environmental surfaces (9)

Assuming at least minimal levels of hygiene and aseptic technique, environmental surfaces (e.g., the outer surfaces and controls of medical devices such as X-ray or hemodialysis machines, housekeeping surfaces [floors, walls, tabletops, etc.]) do not usually come into direct contact with the patient, and if they do, it is usually with intact skin. These surfaces pose the least risk of disease transmission, but this risk may be categorized into two levels. The first level would comprise surfaces that are touched frequently during patient care ("high-touch" surfaces, such as adjustment knobs or handles on hemodialysis machines, instrument carts, X-ray machines, dental units, or even bed rails). If highly contaminated, these surfaces could play a role in secondary cross-contamination via hands of health care workers or contact with medical instruments. The lowest level of risk would be represented by housekeeping surfaces (e.g., floors, walls, tabletops, window sills). The basic criterion for safety at both levels would be visible cleanliness and the absence of recognized pathogens (5, 9, 22, 23). In

V. INTERPRETATION (9) (*continued*)

all cases, the growth of microorganisms in a particular assay may represent accidental contamination during collection or assay, and proper controls and caveats need to be provided for these considerations.

VI. ACKNOWLEDGMENTS

We thank Donald Vesley, School of Public Health, University of Minnesota, Minneapolis, and George Mills (retired), Center for Devices and Radiological Health, U.S. Food and Drug Administration, Rockville, MD, for their review and thoughtful comments.

REFERENCES

1. **American Public Health Association, American Water Works Association, Water Environment Foundation.** 1999. Part 9000. Microbiological examination, p. 9-1–9-41. *In* A. D. Eaton, L. S. Clesceri, and A. E. Greenberg (ed.), *Standard Methods for the Examination of Water and Wastewater,* 20th ed. American Public Health Association, Washington, DC.
2. **American Public Health Association Committee on Microbial Contamination of Surfaces.** 1975. Environmental microbiologic sampling in the hospital. *Health Lab. Sci.* **12:**234–235.
3. **Bartlett, R. C., D. H. M. Groeschel, D. C. Mackel, G. F. Mallison, and E. H. Spaulding.** 1974. Microbiological surveillance, p. 845–851. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), *Manual of Clinical Microbiology,* 2nd ed. American Society for Microbiology, Washington, DC.
4. **Bond, W. W., N. J. Petersen, and M. S. Favero.** 1977. Viral hepatitis B: aspects of environmental control. *Health Lab. Sci.* **14:**235–252.
5. **Centers for Disease Control and Prevention and Healthcare Infection Control Practices Advisory Committee (HICPAC).** 2003. Guidelines for environmental infection control in health-care facilities. *Morb. Mortal. Wkly. Rep.* **52**(RR-10)**:**1–48.
6. **Cole, E. C., W. A. Rutala, L. Nessen, N. S. Wannamaker, and D. J. Weber.** 1990. Effect of methodology, dilution, and exposure time on the tuberculocidal activity of glutaraldehyde-based disinfectants. *Appl. Environ. Microbiol.* **56:**1813–1817.
7. **Favero, M. S.** 1971. Microbiologic assay of space hardware. *Environ. Biol. Med.* **1:**27–56.
8. **Favero, M. S., and W. W. Bond.** 1991. Sterilization, disinfection, and antisepsis in the hospital, p. 183–200. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), *Manual of Clinical Microbiology,* 5th ed. American Society for Microbiology, Washington, DC.
9. **Favero, M. S., and W. W. Bond.** 2001. Disinfection of medical and surgical materials, p. 881–917. *In* S. S. Block (ed.), *Disinfection, Sterilization, and Preservation,* 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
10. **Favero, M. S., W. W. Bond, N. J. Petersen, K. R. Berquist, and J. E. Maynard.** 1974. Detection methods for study of the stability of hepatitis B antigen on surfaces. *J. Infect. Dis.* **129:**210–212.
11. **Favero, M. S., D. A. Gabis, and D. Vesley.** 1984. Environmental monitoring procedures, p. 47–61. *In* M. L. Speck (ed.), *Compendium of Methods for the Microbiological Examination of Foods,* 2nd ed. American Public Health Association, Washington, DC.
12. **Favero, M. S., J. J. McDade, J. A. Robertsen, R. K. Hoffman, and R. W. Edwards.** 1968. Microbiological sampling of surfaces. *J. Appl. Bacteriol.* **31:**336–343.
13. **Hall, L. B., and R. G. Lyle.** 1971. Foundations of planetary quarantine. *Environ. Biol. Med.* **1:**5–8.
14. **Isenberg, H. D.** 1977. Significance of environmental microbiology in nosocomial infections and the care of hospitalized patients, p. 220–234. *In* V. Lorian (ed.), *Significance of Medical Microbiology in the Care of Patients.* Williams & Wilkins, Baltimore, MD.
15. **McCullough, N. V., L. M. Brosseau, D. Vesley, and J. H. Vincent.** 1998. Improved methods for generation, sampling, and recovery of biological aerosols in filter challenge tests. *Am. Ind. Hyg. Assoc. J.* **59:**234–241.
16. **McGowan, J. E., Jr., and B. G. Metchock.** 1999. Infection control epidemiology and clinical microbiology, p. 107–115. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), *Manual of Clinical Microbiology,* 7th ed. ASM Press, Washington, DC.
17. **McGowan, J. E., Jr., and R. A. Weinstein.** 1998. Role of the laboratory in control of nosocomial infection, p. 143–164. *In* J. V. Bennett and P. S. Brachman (ed.), *Hospital Infections,* 4th ed. Lippincott-Raven Publishers, Philadelphia, PA.
18. **Petersen, N. J., D. E. Collins, and J. H. Marshall.** 1974. Evaluation of skin cleansing procedures using the wipe-rinse technique. *Health Lab. Sci.* **11:**182–197.
19. **Puleo, J. R., M. S. Favero, and N. J. Petersen.** 1967. Use of ultrasonic energy in assessing microbial contamination of surfaces. *Appl. Microbiol.* **15:**1345–1351.
20. **Rhame, F. S.** 1998. The inanimate environment, p. 299–324. *In* J. V. Bennett and P. S. Brachman (ed.), *Hospital Infections,* 4th ed. Lippincott-Raven Publishers, Philadelphia, PA.
21. **Russell, A. D.** 2001. Principles of antimicrobial activity and resistance, p. 31–56. *In* S. S. Block (ed.), *Disinfection, Sterilization, and Preservation,* 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.

REFERENCES (*continued*)

22. **Rutala, W. A.** 1996. APIC guideline for selection and use of disinfectants. 1994, 1995, and 1996 APIC Guidelines Committee. Association for Professionals in Infection Control and Epidemiology, Inc. *Am. J. Infect. Control* **24:**313–342.

23. **Spaulding, E. H.** 1972. Chemical disinfection and antisepsis in the hospital. *J. Hosp. Res.* **9:**5–31.

APPENDIX 13.10–1

Sampling of Complex Medical Instruments

Although sampling of smooth, easily accessible environmental surfaces is rather straightforward, efficient sampling of certain medical instruments (or individual components of the instruments) may be hindered by the functional design, materials, construction, expense, and fragility of the devices. For example, the suction, air, water, elevator, or CO_2 channels and other complex or occluded components of flexible fiber-optic and video gastrointestinal endoscopes (2, 3), their accessories (e.g., biopsy forceps, snares) (1), and support equipment (e.g., water supplies, automated endoscope reprocessors) (7) generally present several major and sometimes unique dilemmas in obtaining adequate, pertinent microbiological samples. Description of exact methods for microbiological assay of various physically complex medical devices such as these is far beyond the scope of this procedure, but the following considerations and suggestions are among the minimal criteria for an adequate, meaningful assay of these and similar medical devices.

Prior to sampling protocol formulation, determine the exact configuration of the device as well as access routes to pertinent areas by thorough examination of the instruction manual and, possibly, consultation with the manufacturer. Knowledge of the exact methods and materials routinely used in the between-patient reprocessing of the instrument as well as the length of time between processing and microbiological assay will be essential to choosing appropriate sampling or assay methods and also to accurate interpretation of results.

The use of a sterile swab or brush or even ultrasonic energy where possible to dislodge residual material in or on the device should be considered in the sampling protocol. For instance, a swab-rinse technique is appropriate for sampling the exterior surfaces of the flexible endoscope, the distal opening of the suction-biopsy channel port, or the valve seats in any of the ports. Swab samples from these last two locations, however, may not be appropriate for quantitative assay and may not be representative of the microbial flora of the interior of the channels. A more adequate sample could be obtained in the following manner (i.e., a "flush/brush/flush" technique) from the interior surface of the suction-biopsy channel segment from the biopsy valve to the distal tip of the instrument (2, 4). (*Note:* There are three accessible segments in the suction-biopsy channel system: from the biopsy valve to the distal tip, from the suction valve [in the viewing head of the instrument] distally to the juncture with the biopsy port, and from the suction valve proximally through the umbilical cord to the power light suction source.) Introduce a portion (e.g., 5 ml) of sterile rinse fluid (e.g., buffer solution, buffer solution with neutralizers, or TSB supplemented with lecithin and polysorbate [Tween] 80) into the biopsy valve port, and let it flow downward through the channel. Collect rinse fluid exiting the distal tip in a sterile container. Using aseptic technique and sterile gloves, insert a sterile (disposable [preferable] or autoclaved reusable) cleaning brush into the biopsy port, and brush through the entire length of the channel several (e.g., three or four) times. Rinse the brush tip in sterile rinse fluid each time it exits the distal tip with measured portions of sterile rinse fluid, and finally, rinse the channel with another portion of sterile rinse fluid. If the brush is disposable, sterile scissors may be used to cut the brush off into the collection vessel. The collected fluids and brush tip should then be thoroughly vortexed or exposed to ultrasonic energy to disrupt particulate material and break up clumped microorganisms, thereby ensuring the most accurate determination of CFU per milliliter. Since most of these devices are routinely very difficult to clean, a second brushing and rinsing with a fresh sterile brush in the sampling regimen to ensure a representative sample may be considered if particulate material is seen exiting the lumen during the final flush. Unfortunately, it is not possible to pass a brush through other channels (air, water, CO_2) of the vast majority of flexible gastrointestinal endoscopes, so a simple rinse procedure is all that is possible in these instances. In the latter instances where brushing of the channel lumens is impossible, it must be considered during data interpretation that simple flushing is the least efficient method of sampling. With a simple flush-through technique, a number of fluid dynamic factors inherent to sampling intact lumens of any diameter (e.g., velocity,

APPENDIX 13.10–1 (*continued*)

viscosity, density, surface tension of the eluent) severely limit turbulent flow and shear forces necessary to overcome the adhesion forces of tightly bound patient material and microbial biofilms. Stated differently, a positive quantitative result in this instance most likely does not reflect the entire microbial content of the lumen. Likewise, a negative culture certainly does not indicate that the intact lumen is either "clean" or "sterile" (2).

Small items in the endoscopic set (e.g., valves, biopsy forceps) can be sampled by using an immersion rinse technique (*see* item III.E above), preferably augmented by ultrasonic energy to ensure adequate release and suspension of microorganisms (8). The long, springlike shaft of unsheathed biopsy forceps or other similar accessories can be coiled under the surface of rinse fluid in a flask before the flask is exposed to ultrasonic energy. This will also aid in separating the spring coils and release of material trapped inside the spring. Accessories such as this having plastic sheaths and no access ports (most are single-use disposables) cannot be accurately assayed quantitatively or qualitatively unless they are physically destroyed by aseptic cutting into small segments to ensure penetration of elution fluid and ultrasonic energy (or culture broth) into the lumen spaces.

Similar considerations and strategies for sampling and culturing are appropriate for other physically complex medical devices (e.g., hemodialysis machines, dental instruments, respiratory therapy equipment). Whatever the source, the rinse fluid sample should be assayed immediately if possible or as soon as practical to ensure minimal effects of possible residual disinfectant or other toxic chemicals. For quantitative determinations, a membrane filter assay (see above) followed by flushing of each filter with additional portions of sterile rinse fluid is recommended.

Note on using certain clinical assay methods in environmental sampling protocols

Most methods for bacteriological assays of specimens in clinical laboratories are those designed primarily for human source material. For example, it is common to use a rapid and inexpensive semiquantitative method to detect low-level bacteriuria (urine with $<10^5$ CFU/ml). Platinum loops calibrated to deliver fluid volumes of approximately 1/100 or 1/1,000 ml are used to streak plates of blood, MAC, EMB, or other agars (5). When done in a consistent and specific manner, the technique appears to provide sufficient speed, economy, sensitivity, and precision for diagnostic purposes when human source specimens having a microbial load between 10^3 and 10^5 CFU/ml are assayed (6). However, it is clearly inappropriate when a clinical technique such as this is applied in an environmental microbiology research setting where patients are being exposed to potentially contaminated rinse fluids during diagnostic or surgical procedures (9). The "standard" clinical method in this instance does not employ the appropriate media or incubation time and temperature for optimal recovery of waterborne microorganisms, nor does it have the necessary sensitivity, precision, or replicate size to accurately detect microbial populations of less than 10^3 to 10^4 CFU/ml. Accordingly, the "loop-streak" method is clearly an inappropriate technique for the vast majority of all environmental microbiological assays and especially inappropriate for invocation of the term "sterile." Readers are therefore cautioned to adopt a critical, multidisciplinary approach whenever adapting any standard clinical laboratory specimen collection and assay method to environmental microbiology settings.

References

1. **Alfa, M. J.** 2000. Methodology of reprocessing reusable accessories, p. 361–378. *In* R. A. Kozarek (ed.), *Gastrointestinal Endoscopy Clinics of North America: Endoscopic Disinfection and Reprocessing of Endoscopic Accessories.* W. B. Saunders, Philadelphia, PA.
2. **Bond, W. W.** 2000. Overview of infection control problems: principles in gastrointestinal endoscopy, p. 199–213. *In* R. A. Kozarek (ed.), *Gastrointestinal Endoscopy Clinics of North America: Endoscopic Disinfection and Reprocessing of Endoscopic Accessories.* W. B. Saunders, Philadelphia, PA.
3. **Bond, W. W., B. J. Ott, K. A. Franke, and J. E. McCracken.** 1991. Effective use of liquid chemical germicides on medical devices: instrument design problems, p. 1099–1106. *In* S. S. Block (ed.), *Disinfection, Sterilization, and Preservation,* 4th ed. Lea & Febiger, Philadelphia, PA.
4. **Deva, A. K., J. Vickery, J. Zou, R. H. West, W. Selby, R.A. Benn, J. P. Harris, and Y. E. Cossart.** 1998. Detection of persistent vegetative bacteria and amplified viral nucleic acid from in-use testing of gastrointestinal endoscopes. *J. Hosp. Infect.* 39:149–157.

APPENDIX 13.10–1 (*continued*)

5. **Forbes, B. A., and P. A. Granato.** 1995. Processing specimens for bacteria, p. 265–281. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 6th ed. ASM Press, Washington, DC.
6. **Middleton, R. M., III, W. Huff, D. A. Brickley, and M. B. Kirkpatrick.** 1996. Comparison of quantitative cultures to semi-quantitative loop cultures of bronchoscopic protected specimen brush samples. *Chest* **109:**1204–1209.
7. **Muscarella, L. F.** 2000. Automatic flexible endoscope reprocessors, p. 245–257. *In* R. A. Kozarek (ed.), *Gastrointestinal Endoscopy Clinics of North America: Endoscopic Disinfection and Reprocessing of Endoscopic Accessories.* W. B. Saunders, Philadelphia, PA.
8. **Puleo, J. R., M. S. Favero, and N. J. Petersen.** 1967. Use of ultrasonic energy in assessing microbial contamination of surfaces. *Appl. Microbiol.* **15:**1345–1351.
9. **Wilcox, C. M., K. Waites, and E. S. Brookings.** 1996. Use of sterile compared with tap water in gastrointestinal endoscopic procedures. *Am. J. Infect. Control* **24:**407–410.

13.11 Surveillance Cultures from Immunocompromised Hosts

I. PRINCIPLE

Surveillance cultures from immunocompromised patients provide a service that can directly impact patient care and outcome in this patient population, but a rational, planned approach must be considered in order to optimize resources and interpretive impact. While some studies suggest that surveillance cultures may be of value, other studies suggest that routine surveillance may not be helpful. Differing conclusions from these studies may be due to insufficient data or inconclusive definitions of test conditions rather than from opposing data for similar populations. Selective surveillance, depending on occurrences at a particular time, can be useful in predicting anticipated difficulties in immunocompromised individuals. Surveillance could be part of studies to define the natural history of colonization by microorganisms and infections during pretreatment and after treatment of the immunosuppressed patient.

Several studies in the 1980s suggested that surveillance cultures of urine, stool, or respiratory specimens from this population could be accurate predictors of subsequent systemic infection due to gram-negative organisms (5) and yeasts (13, 15) in neutropenic patients. However, other studies have questioned the value of surveillance cultures in predicting bacterial and fungal infections either in transplant recipients (4, 14) or in patients likely to develop chemotherapy-induced mucositis (14). Selective surveillance appears to be most helpful in identifying antimicrobial-resistant organisms, particularly with the increase in infections with gram-positive bacteria (1, 7). Routine stool surveillance cultures for vancomycin-resistant enterococci (VRE) have now been suggested for patients at risk for infection with those organisms (10, 11). When stool colonization is identified, those patients could be isolated to prevent nosocomial spread of the organism. Methicillin-resistant *Staphylococcus aureus* (MRSA) colonization of the anterior nares was found to be a significant predictor of MRSA infection in liver transplant recipients (3).

Surveillance cultures can also be an important part of impact studies. For example, rectal cultures routinely collected from liver and kidney transplant recipients helped document that VRE colonization can persist for months to years (12); cultures of stool provided evidence that antimicrobial chemotherapy targeted to intestinal anaerobic bacteria in marrow transplant recipients reduced the severity of acute graft-versus-host disease (2); and surveillance cultures were useful in confirming the efficiency of suppression of body microbiota by antimicrobial agents (4) and the frequent occurrence of yeast colonization after bone marrow transplantation in spite of double prophylaxis with oral amphotericin B and fluconazole (9), the usefulness of cultures in predicting who will be at risk for development of mucositis (6), and the possibility that mucous membranes might be sources of strains of coagulase-negative staphylococci causing bacteremia (8).

PREANALYTICAL CONSIDERATIONS

II. SPECIMENS

Observe standard precautions.

A. General surveillance culture for bacteria and yeasts

A combination of sites should be selected and decided upon in coordination with the medical staff utilizing the culture results. Samples may include specimens of urine and stool and specimens from oropharyngeal, vaginal, and central catheter line sites. All swab specimens are collected using the techniques for obtaining these specimens for routine culture. Swabs should be submitted in transport media that is routinely used for other routine swab cultures.

B. Selective bowel culture

A rectal swab or stool specimen is collected and submitted as for routine culture.

II. SPECIMENS (*continued*)

C. Selective surveillance cultures
Samples for selective surveillance cultures are collected from specific sites for VRE and MRSA; usually rectal and oropharyngeal swabs are obtained. Other sites may be selected depending on the current protocol(s). Samples are collected and transported the same as for general surveillance cultures.

III. SCHEDULE OF SPECIMEN SUBMISSION

The schedule of submission is dependent on the coordination of the medical staff and infection control and laboratory personnel. Some examples are at admission; before and after, e.g., bowel decontamination, total body radiation, and resolution of neutropenia; and during or after isolation.

IV. MEDIA

A. General surveillance culture for bacteria and yeasts
BAP, CHOC, MAC, colistin-nalidixic acid agar, BHI, BHI supplemented agar, anaerobic blood agar, or equivalent alternative media are used.

B. Selective bowel culture
Media can be tailored to select for organisms appropriate for the specific bowel decontamination regimen or to select organisms resistant to specific antimicrobial regimens, for example, Mueller-Hinton agar (MHA) with blood and vancomycin and cefepime. Always inoculate a nonselective medium, such as BAP, along with the selective media.

C. Selective surveillance cultures
1. MRSA:
See section 13.17.
2. VRE:
See section 13.18.

ANALYTICAL CONSIDERATIONS

V. INCUBATION

Observe standard precautions.

A. General surveillance culture for bacteria and yeasts
Incubate culture at 35°C in 5% CO_2 and examine after 24 and 48 h of incubation.

B. Selective bowel culture
Incubate culture at 35°C in 5% CO_2. Examine after 24 and 48 h of incubation. Incubate anaerobic cultures at 35°C in an anaerobic environment. Examine after 48 h of incubation. Additional incubation may be required depending on expected anaerobic growth.

C. Selective surveillance cultures
1. Incubate at 35°C in 5% CO_2. Examine after 24 h of incubation.
2. Oxacillin screen agar
Incubate at 35°C (no higher) in ambient air for a full 24 h. Examine for any evidence of growth, which indicates resistance.

VI. CULTURE WORKUP

A. Quantitation
Semiquantitate the colonies according to the laboratory protocol for quantitating microorganisms, e.g., few (1 to 10 CFU), moderate (11 to 100 CFU), and many (>100 CFU).

B. Identification
1. Yeasts
Isolate and identify all yeasts to the species level.
2. Gram-negative bacilli
Identify all gram-negative bacilli to the species level. If the intent of the various cultures is to determine the presence or absence of microorganisms, a presumptive identification only may be necessary. Alternatively, the gram-negative bacilli may be initially identified to the species level, with presumptive identification on subsequent cultures.

VI. CULTURE WORKUP (*continued*)

3. Gram-positive bacteria

a. Skin microbiota

Gram-positive organisms are not identified, except for specific bacteria associated with infections in this patient population.

b. MRSA

See procedure 13.17.

c. VRE

See procedure 13.18.

VII. SUSCEPTIBILITY TESTING

Perform routine susceptibility testing according to the laboratory policies on gram-negative bacilli and gram-positive cocci. Reporting of the antimicrobial agents should follow laboratory policy for selection reporting.

POSTANALYTICAL CONSIDERATIONS

VIII. REPORTING RESULTS

Report presumptive and definitive identification along with semiquantitation of each species.

IX. INTERPRETATION

Colonization does not imply infection but may predict systemic infection. Organisms that are identified as resistant to the antimicrobial agent(s) in the patient bowel decontamination regimen may warrant altering the antimicrobial agent regimen. Isolation of selective organisms may also alert the infection control practitioners of a possible nosocomial spread. The infection control policies may be implemented for isolation of the patient.

X. QUALITY IMPROVEMENT

A. QC

Routine medium QC testing is sufficient if selective media are appropriately tested for their selectivity.

B. QA

Surveillance culturing should be continuously monitored as to the value and use of the results. Monitoring should be performed as a team approach with the medical staff and infection control and laboratory personnel for the specific protocol regimen.

REFERENCES

1. **Aksu, G., M. Z. Ruhi, H. Akan, S. Bengisun, C. Ustun, O. Arslan, and H. Ozenci.** 2001. Aerobic bacterial and fungal infections in peripheral blood stem cell transplants. *Bone Marrow Transplant.* **27:**201–205.
2. **Beelen, D. W., A. Elmaagacli, K. D. Muller, H. Hirche, and U. W. Schaefer.** 1999. Influence of intestinal bacterial decontamination using metronidazole and ciprofloxacin or ciprofloxacin alone on the development of acute graft-versus-host disease after marrow transplantation in patients with hematologic malignancies: final results and long-term follow-up of an open-label prospective randomized trial. *Blood* **93:**3267–3275.
3. **Chang, F. Y., N. Singh, T. Gayowski, S. D. Drenning, M. M. Wagener, and I. R. Marino.** 1998. Staphylococcus aureus nasal colonization and association with infections in liver transplant recipients. *Transplantation* **65:**1169–1172.
4. **Czirok, E., G. Y. Prinz, R. Denes, P. Remenyi, and A. Herendi.** 1997. Value of surveillance cultures in a bone marrow transplantation unit. *J. Med. Microbiol.* **46:**785–791.
5. **Daw, M. A., P. Munnelly, S. R. McCann, P. A. Daly, F. R. Falkiner, and C. T. Keane.** 1988. Value of surveillance cultures in the management of neutropenic patients. *Eur. J. Clin. Microbiol. Infect. Dis.* **7:**742–747.
6. **Feld, R.** 1997. The role of surveillance cultures in patients likely to develop chemotherapy-induced mucositis. *Support Care Cancer* **5:**371–375.
7. **Haahr, V., N. A. Peterslund, and J. K. Moller.** 1997. The influence of antimicrobial prophylaxis on the microbial and clinical findings in patients after autologous bone marrow transplantation. *Scand. J. Infect. Dis.* **29:**623–626.

REFERENCES (*continued*)

8. **Herwaldt, L. A., R. J. Hollis, L. D. Boyken, and M. A. Pfaller.** 1992. Molecular epidemiology of coagulase-negative staphylococci isolated from immunocompromised patients. *Infect. Control Hosp. Epidemiol.* **13:**86–92.
9. **Hoppe, J. E., M. Klausner, T. Klingebiel, and D. Niethammer.** 1997. Retrospective analysis of yeast colonization and infections in paediatric bone marrow transplant recipients. *Mycoses* **40:**47–54.
10. **Kapsur, D., D. Dorsky, J. M. Feingold, R. D. Bona, R. L. Edwards, J. Aslanzadeh, P. J. Tutschka, and S. Bilgrami.** 2000. Incidence and outcome of vancomycin-resistant enterococcal bacteremia following autologous peripheral blood stem cell transplantation. *Bone Marrow Transplant.* **25:**147–152.
11. **Koc, Y., D. R. Snydman, D. S. Schenkein, and K. B. Miller.** 1998. Vancomycin-resistant enterococcal infections in bone marrow transplant recipients. *Bone Marrow Transplant.* **22:**207–209.
12. **Patel, R., S. L. Allen, J. M. Manahan, A. J. Wright, R. A. Krom, R. H. Wiesner, D. H. Persing, F. R. Cockerill, and R. L. Thompson.** 2001. Natural history of vancomycin-resistant enterococcal colonization in liver and kidney transplant recipients. *Liver Transplant.* **7:**27–31.
13. **Pfaller, M., I. Cabezudo, F. Koontz, M. Bale, and R. Gingrich.** 1987. Predictive value of surveillance cultures for systemic infection due to *Candida* species. *Eur. J. Clin. Microbiol.* **6:**628–633.
14. **Rotstein, C., D. Higby, K. Killion, and E. Powell.** 1988. Relationship of surveillance cultures to bacteremia and fungemia in bone marrow transplant recipients with Hickman or Broviac catheters. *J. Surg. Oncol.* **39:**154–158.
15. **Sandford, G. R., W. G. Merz, J. R. Wingard, P. Charache, and R. Saral.** 1980. The value of fungal surveillance cultures as predictors of systemic fungal infections. *J. Infect. Dis.* **142:**503–509.

13.12 Culture of Intravascular Devices

I. PRINCIPLE

Intravascular catheters are used to provide continuous vascular access to permit blood sampling; to administer blood products, medications, total parenteral nutrition, and other fluids; and, in the case of pulmonary artery catheters, to permit hemodynamic monitoring of cardiac function. Because these devices penetrate the integument, they put the patient at significant risk for development of device-related infection. The insertion site becomes colonized by bacteria from the patient's own skin or by microorganisms carried on the hands of medical personnel (5). Organisms can also gain access through the lumen of the catheter following contamination of the hub (4) or infusion of contaminated fluids. Invading organisms can then colonize the intravascular catheter surfaces in the form of a biofilm and produce local infection and, in a significant number of cases, bacteremia, fungemia, suppurative phlebitis, or septic thrombosis (5). (*See* Appendix 13.12–1 for further discussion.)

PREANALYTICAL CONSIDERATIONS

II. SPECIMENS

Observe standard precautions.

A. Types

1. Long catheters

These catheters are surgically inserted into the central vein to provide access for administration of therapeutic agents, total parenteral nutrition, and blood products (6).

a. Long term

(1) Percutaneous catheters: Broviac, Hickman, Raaf, Groshong, Quinton (Fig. 13.12–1)

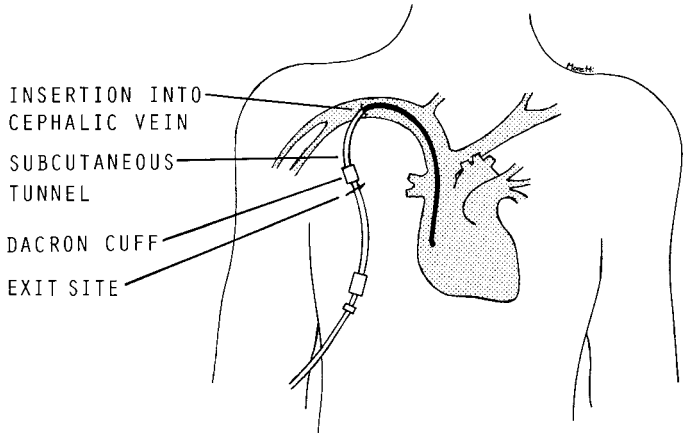

Figure 13.12–1 Placement of surgically implanted long percutaneous catheter (illustration by Carol Moretti).

II. SPECIMENS (*continued*)

These catheters are surgically inserted and subcutaneously tunnelled to a chest wall exit site. A Dacron cuff facilitates growth of fibrous tissue to the catheter and inhibits migration of microorganisms along the surface of the catheter (6).

(2) Subcutaneous ports: Infus-A-Port, Port-A-Cath, Med-i-Port (Fig. 13.12–2)

These systems can be entered by inserting a needle through the septum of the reservoir (6).

b. Short-term percutaneous catheters: Swan-Ganz (plus introducer), Intracath, Cordis, multilumen, Udall (for hemodialysis) (Fig. 13.12–1)

2. Short catheters

These catheters are inserted into various sites and are used for short-term vascular access.

a. Peripheral
b. Steel needles
c. Umbilical
d. Arterial

B. Collection and transport

1. Collection

To prevent contamination by skin microorganisms and antimicrobial ointment, clean the skin insertion site with iodophor and alcohol prior to removal of the cannula. Remove the cannula in an aseptic manner once the alcohol has dried. If purulence of the catheter exit site is evident, send pus for culture and Gram stain (5).

a. Long catheters

Two portions of long catheters should be sent for culture: the distal intravascular tip and the proximal transcutaneous segment. Each segment should be approximately 2 to 3 cm long (Fig. 13.12–3).

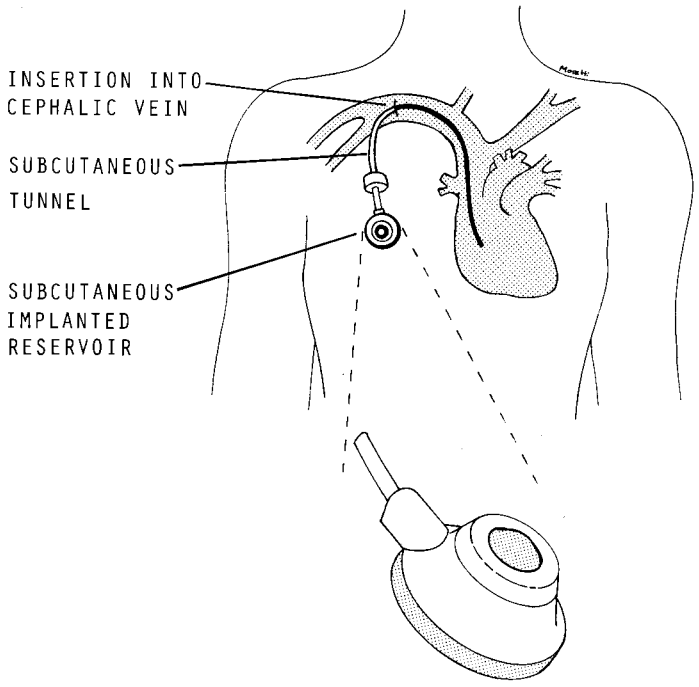

Figure 13.12–2 Placement of surgically implanted subcutaneous port (illustration by Carol Moretti).

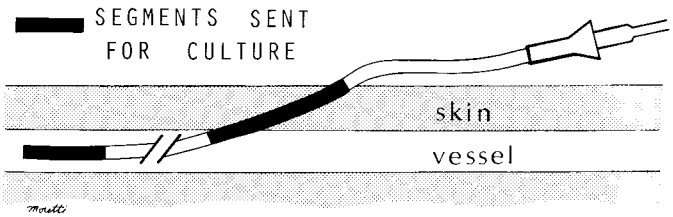

Figure 13.12–3 Segments of long catheter for culture (illustration by Carol Moretti).

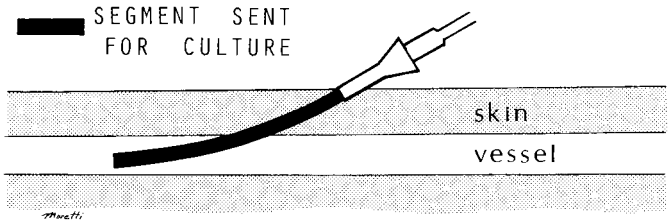

Figure 13.12–4 Segment of short catheter for culture (illustration by Carol Moretti).

II. SPECIMENS (*continued*)

b. Short catheters
The cannula is cultured in its entirety following removal of the hub. To remove the hub, use sterile scissors, or snap off steel needles with a sterile hemostat (5) (Fig. 13.12–4).

2. Transport
Transport catheter tips in a sterile container. If tips are cut to proper length, there is no need to bend them for insertion into transport container. Culture tips within 2 h of collection to prevent desiccation of microorganisms.

III. EQUIPMENT, SUPPLIES, AND REAGENTS

A. 100-mm 5% BAP
B. Forceps
C. 95% alcohol
D. Sterile TSB
E. 100-µl pipette and sterile tips
F. Sterile 1-ml syringe and needle
G. Sterile test tubes (12 by 75 mm) with caps

ANALYTICAL CONSIDERATIONS

IV. PROCEDURE

Observe standard precautions.

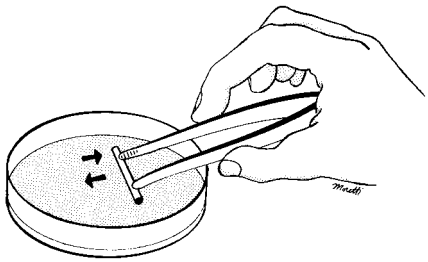

Figure 13.12–5 Maki roll technique (illustration by Carol Moretti).

A. Semiquantitative culture, Maki method

1. Dip forceps in 95% alcohol, and flame sterilize. Allow to cool.
2. If the catheter segment is too long to be rolled on the plate, cut it in half with sterile scissors and culture each segment separately.
3. Use sterile forceps to transfer catheter tip from transport container to a BAP.
4. Using slight pressure, roll catheter tip back and forth across agar surface at least four times. It is essential that the catheter tip have good contact with the surface of the plate. If tip is bent and hard to roll, use forceps to pick up tip, and rub all surfaces onto plate (Fig. 13.12–5).
5. Dip forceps in 95% alcohol, and flame sterilize.
6. Incubate plate for at least 72 h at 35°C in a CO_2 incubator. It is not necessary to routinely culture for anaerobic bacteria, fungi, or mycobacteria.

B. Culture of implanted subcutaneous ports

At this time, there is no standardized method available for culturing implanted subcutaneous ports. If sepsis is suspected, quantitative blood cultures may be done without necessitating removal of the device. If the port is subsequently

IV. PROCEDURE (*continued*)

removed, the distal catheter tip is also cultured (7). When a subcutaneous infection is suspected, culture an aspirate or tissue sample of the wound site. There is no evidence that suggests that culturing the port itself is of any value.

C. Culture of the lumen

1. Aseptically transfer catheter segment from transport container to a sterile test tube (12 by 75 mm).
2. Use a sterile syringe to draw up 1 ml of sterile TSB.
3. Place needle into lumen of catheter, and dispense broth through catheter segment.
4. Cap tube, and vortex to dislodge adherent bacteria.
5. Serially dilute sample 100-fold.
6. Inoculate 100 μl of each dilution onto a 100-mm BAP. Cross-streak for well-isolated colonies.
7. Incubate plate for at least 72 h at 35°C in a CO_2 incubator. It is not necessary to routinely culture for anaerobic bacteria, fungi, or mycobacteria.

D. Quantitative cultures of catheter segments (8–10)

1. Aseptically transfer catheter segment to 10 ml of broth.
2. Sonicate for 1 min.
3. Vortex sample for 15 s.
4. Plant 100 μl to BAP cross streaking for isolated colonies.
5. Incubate at 35°C in CO_2 for 72 h.

V. INTERPRETATION

A. Semiquantitative culture, Maki method

Identify and perform susceptibility testing on each organism that produces ≥15 colonies. *Note:* Some authors use five colonies as the cutoff (2, 3).

B. Culture of implanted subcutaneous ports

Identify and perform susceptibility testing on all organisms isolated.

C. Culture of the lumen

Calculate the number of CFU per milliliter by multiplying the number of colonies by 10 times the dilution factor and then dividing by the volume of broth used (1 ml). Identify and perform susceptibility testing on all organisms present at >1,000 CFU/ml (1).

D. Quantitative cultures of catheter segments

Calculate the number of CFU/ml (= colonies/plate × 100). Identify and do susceptibility tests on all those with >1,000 CFU/ml. Selectively identify those with <1,000 CFU/ml.

VI. LIMITATION OF THE PROCEDURE

Fastidious organisms will not be detected.
CHOC may help detect these organisms.

VII. QUALITY CONTROL

Perform QC on media according to your laboratory procedure. Report catheter-associated infections to the appropriate committee (e.g., Infection Control, Catheter Care) for QA review.

POSTANALYTICAL CONSIDERATIONS

VIII. REPORTING RESULTS

A. Semiquantitative culture, Maki method

For each organism that produces >15 CFU, include actual colony count with the organism identification and susceptibility report. If there is confluent growth, report as such.

B. Culture of implanted subcutaneous ports

Report qualitatively (e.g., few, moderate, many) when organism identification and susceptibility are reported.

C. Culture of lumen

For each organism that produces >1,000 CFU/ml, include dilution amount with the identification and susceptibility report.

D. Quantitative cultures of catheter segments

Report with full identification and susceptibilities all those with >1,000 CFU/ml. Report all *S. aureus* and *Candida* species with colony count. For other organisms, list with minimal investigations as <1,000 CFU/ml.

REFERENCES

1. **Cleri, D. J., M. L. Corrado, and S. J. Seligman.** 1980. Quantitative culture of intravenous catheters and other intravascular inserts. *J. Infect. Dis.* **141:**781–786.
2. **Collignon, P. J., R. Chan, and R. Munro.** 1987. Rapid diagnosis of intravascular catheter-related sepsis. *Arch. Intern. Med.* **147:**1609–1612.
3. **Collignon, P. J., N. Soni, I. Y. Pearson, W. P. Woods, R. Munro, and T. C. Sorrell.** 1986. Is semiquantitative culture of central vein catheter tips useful in the diagnosis of catheter-associated bacteremia? *J. Clin. Microbiol.* **24:**532–535.
4. **Liñares, J., A. Sitges-Serra, J. Garau, J. L. Perez, and R. Martin.** 1985. Pathogenesis of catheter sepsis: a prospective study with quantitative and semiquantitative cultures of catheter hub and segments. *J. Clin. Microbiol.* **21:**357–360.
5. **Maki, D. G.** 1980. Sepsis associated with infusion therapy, p. 207–253. *In* S. Karran (ed.), *Controversies in Surgical Sepsis.* Praeger, New York, NY.
6. **Miller, K. B.** 1988. Cardiovascular system update, p. 1168–1181. *In* R. Berg (ed.), *The APIC Curriculum for Infection Control Practices*, vol. 3. Kendall/Hunt, Dubuque, IA.
7. **Moore, C. L., K. A. Erickson, L. B. Yanes, M. Franklin, and L. Gonsalves.** 1986. Nursing care and management of venous access ports. *Oncol. Nurs. Forum* **13:**35–39.
8. **Raad, I., M. F. Sabbagh, K. H. Rand, and R. J. Sherertz.** 1992. Quantitative tip culture methods and the diagnosis of central venous catheter-related infections. *Diagn. Microbiol. Infect. Dis.* **15:**13–20.
9. **Raad, I., H. Hanna, and D. Maki.** 2007. Intravascular catheter-related infections: advances in diagnosis, prevention, and management. *Lancet Infect. Dis.* **7:**645–657.
10. **Siegman-Igra, Y., A. M. Anglim, D. E. Shapiro, K. A. Adal, B. A. Strain, and B. M. Farr.** 1997. Diagnosis of catheter-related bloodstream infections: a meta-analysis. *J. Clin. Microbiol.* **35:**928–936.

SUPPLEMENTAL READING

Collignon, P. J., and R. Munro. 1989. Laboratory diagnosis of intravascular catheter associated sepsis. *Eur. J. Clin. Microbiol. Infect. Dis.* **8:**807–814.

Coutleé, F., C. Lemieux, and J. F. Paradis. 1988. Value of direct catheter staining in the diagnosis of intravascular-catheter-related infection. *J. Clin. Microbiol.* **26:**1088–1090.

Hamory, B. H. 1987. Nosocomial bloodstream and intravascular device-related infections, p. 283–319. *In* R. P. Wenzel (ed.), *Prevention and Control of Nosocomial Infections*, vol. 1. The Williams & Wilkins Co., Baltimore, MD.

Kristinsson, K. G., I. A. Burnett, and R. C. Spencer. 1989. Evaluation of three methods for culturing long intravascular catheters. *J. Hosp. Infect.* **14:**183–191.

APPENDIX 13.12–1

Device-Related Infections

Intravascular-device-related infections are most often caused by endogenous skin microbiota (Table 13.12–A1). Some organisms, such as coagulase-negative staphylococci and *Corynebacterium jeikeium,* that were previously considered contaminants are now recognized as major pathogens in device-related infections. Accurate diagnosis of device-related infection can be difficult, since true infection must be distinguished from contamination of the catheter during its removal from the patient. When device-related infection is suspected, the entire catheter (short catheters) or 2- to 3-cm segments from both the distal tip and the transcutaneous portions (long catheter) should be submitted for culture (5). The clinical microbiology laboratory must be able to identify and quantitate the microorganisms present.

Qualitative culture of the catheter, in which the segment is incubated in liquid medium, is a sensitive technique for diagnosis of infection but provides no information about the number of microorganisms present (7); i.e., the presence of a single contaminant can lead to a positive culture. In an effort to improve the specificity of catheter cultures, quantitative techniques that demonstrate a sensitivity comparable to that of broth culture but with superior specificity and predictive value have been developed. Detection of catheter-related infections is also possible by microscopic examination of the catheter surfaces following Gram (2) or acridine orange (10) staining. This allows rapid detection, preliminary characterization, and quantitation of the organisms present. Although these staining techniques show promise, they are not feasible for all types of catheters and are technically quite demanding and labor-intensive.

Maki et al. (7) developed a semiquantitative technique in which the catheter segment is rolled back and forth across the surface of a BAP. Colonies are counted after incubation, and the presence of $\geq$15 indicates local infection. Because the technique samples only the exterior portion of the catheter, it may not detect infections originating in the lumen of the catheter. It has been suggested that colonization of the lumen may be an important reservoir of infection in some catheter infections (1, 3, 4, 8). Cleri et al. (1) developed a quantitative technique in which the catheter lumen is flushed with liquid medium and agitated vigorously to dislodge adherent microorganisms. The suspension is then serially diluted and plated. The presence of $>$1,000 CFU/ml suggests bacteremia. The theoretical advantage of this approach is that both the interior and exterior surfaces of the catheter are sampled. Thus, a modified procedure for such culture is also included. Rello et al. suggest that using both the semiquantitative culture of the exterior catheter surface and the quantitative culture of the lumen increases the detection of catheter colonization (9).

Table 13.12–A1 Bloodstream pathogens suggestive of intravenous-infusion-related septicemia[a]

Probable source	Pathogens
Peripheral intravenous catheters	Coagulase-negative staphylococci, *Staphylococcus aureus,* enterococci, *Candida* spp.
Central venous catheters	*Candida* spp., coagulase-negative staphylococci, *S. aureus, Corynebacterium* spp. (especially *C. jeikeium*), *Klebsiella* spp., *Acinetobacter* spp., *Fusarium* spp., *Malassezia furfur*

[a] Modified from reference 6.

APPENDIX 13.12–1 (*continued*)

References

1. **Cleri, D. J., M. L. Corrado, and S. J. Seligman.** 1980. Quantitative culture of intravenous catheters and other intravascular inserts. *J. Infect. Dis.* **141:**781–786.
2. **Cooper, G. L., and C. C. Hopkins.** 1985. Rapid diagnosis of intravascular catheter-associated infection by direct Gram staining of catheter segments. *N. Engl. J. Med.* **312:**1142–1147.
3. **Haslett, T. M., H. D. Isenberg, E. Hilton, V. Tucci, B. G. Kay, and E. M. Vellozzi.** 1988. Microbiology of indwelling central intravascular catheters. *J. Clin. Microbiol.* **26:**696–701.
4. **Liñares, J., A. Sitges-Serra, J. Garan, J. L. Perez, and R. Martin.** 1985. Pathogenesis of catheter sepsis: a prospective study with quantitative and semiquantitative cultures of catheter hub and segments. *J. Clin. Microbiol.* **21:**357–360.
5. **Maki, D. G.** 1980. Sepsis associated with infusion therapy, p. 207–253. *In* S. Karran (ed.), *Controversies in Surgical Sepsis.* Praeger, New York, NY.
6. **Maki, D. G.** 1989. Pathogenesis, prevention, and management of infections due to intravascular devices used for infusion therapy, p. 161–177. *In* A. L. Bisno and F. A. Waldvogel (ed.), *Infections Associated with Indwelling Medical Devices.* American Society for Microbiology, Washington, DC.
7. **Maki, D. G., C. E. Weise, and H. W. Sarafin.** 1977. A semiquantitative culture method for identifying intravenous-catheter-related infection. *N. Engl. J. Med.* **296:**1305–1309.
8. **Needham, P. M.** 1990. Intraluminal colonization of a Groshong catheter. *Clin. Microbiol. Newsl.* **12:**189–190.
9. **Rello, J., J. M. Gatell, J. Almirall, J. M. Campistol, J. Gonzalez, and J. Puig de la Bellacasa.** 1989. Evaluation of culture techniques for identification of catheter-related infection in hemodialysis patients. *Eur. J. Clin. Microbiol. Infect. Dis.* **8:**620–622.
10. **Zufferey, J., B. Rime, P. Francioli, and J. Bille.** 1988. Simple method for rapid diagnosis of catheter-associated infection by direct acridine orange staining of catheter tips. *J. Clin. Microbiol.* **16:**175–176.

13.13 Culture of Blood Bank Products

I. PRINCIPLE

Transfusion of blood and blood components is usually a safe and effective form of therapy. However, untoward effects can occur. These untoward effects, called "transfusion reactions," may present immediately or be delayed, and they may or may not be immunologically mediated. A serious complication of transfusion therapy is the transmission of infection by many different kinds of microorganisms, such as viruses (hepatitis) (7), spirochetes (*Borrelia burgdorferi*) (4), and other bacteria, for example, *Yersinia* spp. This procedure focuses only on culture of suspected bacterial agents in blood and blood products. A list of some bacterial agents implicated in transfusion reactions is shown in Table 13.13–1.

The use of a closed system for blood collection in sterile disposable bags, the implementation of rigid standards for blood bank operations, and more stringent attention to skin disinfection have contributed to the reduction of bacterial contamination. Although bacterial contamination of blood and blood products is rare, it remains a significant concern (15). (*See* Appendix 13.13–1 for further discussion.)

Table 13.13–1 Bacterial organisms implicated in transfusion reactions

Organism(s)	Reference(s)
Coagulase-negative staphylococci	1, 12
Enterobacter agglomerans	2
Enterobacter cloacae	5, 6, 19
Escherichia coli	3, 12
Flavobacterium meningosepticum	6
Klebsiella pneumoniae	8, 12
Propionibacterium acnes	19
Pseudomonas aeruginosa	16
Pseudomonas cepacia	16
Pseudomonas fluorescens	11, 18
Pseudomonas putida	18
Salmonella enterica sv. choleraesuis	6, 14
Salmonella enterica sv. heidelberg	9
Staphylococcus aureus	1, 12
Staphylococcus epidermidis	6
Yersinia enterocolitica	2, 10, 17

PREANALYTICAL CONSIDERATIONS

II. MATERIALS

A. Aerobic and anaerobic blood culture bottles
One of each type of bottle constitutes one set. The broth medium used in the culture bottles is dependent on the blood culture system used in your laboratory. If the lysis centrifugation system is in use, additional plated media for room temperature (25°C) and anaerobic incubation should be employed.

B. BAP
C. CHOC plates
D. Anaerobic blood agar plates
E. Gram stain reagents
F. Alcohol
G. Iodophor
H. Needle and 20-ml syringe

ANALYTICAL CONSIDERATIONS

III. PROCEDURE

Observe standard precautions.

A. *Wear gloves and a face shield at all times when handling blood or blood products.* For each unit of blood and/or blood component to be cultured, have two sets of blood culture bottles. A set is one aerobic and one anaerobic bottle. Alternatively, one adult Isolator 10 tube may be used.

B. Cleanse the rubber septum of each blood culture bottle and the port(s) of the blood bag with alcohol and/or iodophor, scrubbing with the antiseptic for at least 1 min.

C. Using a needle and syringe, aseptically remove approximately 20 ml of blood or blood product from the suspect unit.

D. Inoculate approximately a 3- to 5-ml sample into each of the four blood culture bottles. If less than 20 ml of blood is available for culture, dispense equal amounts into the blood culture bottles. The final dilution of blood to culture broth should be 1:10. Reserve approximately 0.5 to 1 ml of blood for Gram stain. If lysis centrifugation is the blood culture method in use, prepare plates for 35°C, room temperature, and anaerobic incubation. Observe daily for up to 4 days for aerobic organisms and for up to 6 days for anaerobic organisms.

E. Prepare a slide for Gram stain (*see* procedure 3.2.1). If organisms are observed, directly inoculate plated media (CHOC and blood agar), and incubate at 35°C in CO_2.

F. Depending on the blood culture system, use one of the following methods (13).

1. Manual method

a. Incubate one set of blood culture bottles at 35°C in CO_2 and the other set at 25°C.

b. Daily, for up to 7 days, examine bottles macroscopically (visually) for growth.

c. Perform blind subculture at 18 and 48 h. Cleanse the bottle septum as described above. Remove approximately 0.2 to 0.5 ml of blood and inoculate a CHOC plate. Incubate the CHOC plate for 48 h under the incubation conditions for the bottle sampled before discarding it as negative. At 48 h, perform a blind subculture onto an anaerobic blood agar plate. Incubate this plate in an anaerobic environment for 48 h before discarding it as negative.

d. If macroscopic examination of the bottles indicates growth (turbidity, hemolysis, gas, etc.), subculture the bottle to a blood agar plate, CHOC plate, and anaerobic blood agar plate. Incubate the blood agar and CHOC plates at 35°C in CO_2. Incubate the anaerobic blood agar plate in an anaerobic environment at 35°C. Prepare a Gram stain from the bottle at the same time. Subculture the bottles incubated at room temperature onto plates incubated at both room temperature and 35°C.

III. PROCEDURE (*continued*)

e. If the Gram stain indicates the presence of bacteria, appropriate presumptive tests should be performed from the blood culture broth, but final confirmatory identification must be based on the evaluation of a pure culture isolate.

2. Automated blood culture system

If using a continuous monitoring system or a daily-read system, follow the manufacturer's recommendations for reading the blood culture bottles. If bottles are flagged as positive, use subculturing methods as described above. Examine the bottles macroscopically before discarding as negative.

POSTANALYTICAL CONSIDERATIONS

IV. INTERPRETATION

A negative culture makes it very unlikely that the blood or blood product was significantly contaminated with bacteria at the time of transfusion. However, a positive culture does not positively identify infection as the etiology of the transfusion reaction, since the organism cultured may be a minor contaminant. A positive culture of the blood or blood product does not establish the source of the contamination (blood collection, blood bank, ward, etc.) unless both blood bank and patient's blood samples yield identical organisms.

Whenever clinical circumstances lead to culture of a blood unit, the recipient patient's blood should be cultured simultaneously. Since intravascular therapy has been implicated as the source of sepsis, the catheter and fluid should be cultured as well (8). The supplier of the blood, blood product, intravenous fluid, etc., should always be notified if the culture of the specific product is positive.

REFERENCES

1. **Anderson, K. C., M. Lew, B. Gorgone, J. Martel, C. Leamy, and B. Sullivan.** 1986. Transfusion-related sepsis after prolonged platelet storage. *Am. J. Med.* **81:**405–410.
2. **Arduino, M., L. Bland, M. Tripple, S. Aguero, and M. Favero.** 1989. Growth and endotoxin production of *Yersinia enterocolitica* and *Enterobacter agglomerans* in packed erythrocytes. *J. Clin. Microbiol.* **27:**1483–1485.
3. **Arnow, P., L. Weiss, D. Weil, and N. Rosen.** 1986. *Escherichia coli* sepsis from contaminated platelet transfusion. *Arch. Intern. Med.* **146:**321–324.
4. **Badon, S. J., R. O. Fister, and R. G. Cable.** 1989. Survival of *Borrelia burgdorferi* in blood products. *Transfusion* **29:**581–582.
5. **Blajchman, M. A.** 2000. Reducing the risk of bacterial contamination of cellular blood components. *Dev. Biol. Stand.* **102:**183–193.
6. **Buchholz, D. H., V. M. Young, N. R. Friedman, J. A. Reilly, and M. R. Mardiney.** 1973. Detection and quantitation of bacteria in platelet products stored at ambient temperature. *Transfusion* **13:**268–275.
7. **Conrad, M.** 1981. Diseases transmissible by blood transfusions: viral hepatitis and other infectious disorders. *Semin. Hematol.* **18:**122–145.
8. **Elin, R. J., W. B. Lundberg, and P. Schmidt.** 1975. Evaluation of bacterial contamination in blood processing. *Transfusion* **15:**260–265.
9. **Heal, J. M., S. Singal, E. Sardisco, and T. Mayer.** 1986. Bacterial proliferation in platelet concentrates. *Transfusion* **26:**388–390.
10. **Jacobs, J., D. Jamaer, J. Vandevan, M. Wouters, C. Vermylen, and J. Vandepitte.** 1989. *Yersinia enterocolitica* in donor blood: a case report and review. *J. Clin. Microbiol.* **27:**1119–1121.
11. **Murray, A. E., C. A. Bartzokas, A. J. Shepherd, and F. M. Roberts.** 1987. Blood transfusion-associated *Pseudomonas fluorescens* septicaemia: is this an increasing problem? *J. Hosp. Infect.* **9:**243–248.
12. **Punsalang, A., J. Heal, and P. Murphy.** 1989. Growth of gram positive and gram negative bacteria in platelet concentrates. *Transfusion* **29:**596–599.
13. **Reisner, B. S., G. L. Woods, R. B. Thomson, Jr., D. H. Larone, L. S. Garcia, and R. Y. Shimizu.** 1999. Specimen processing, p. 64–67. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 7th ed. ASM Press, Washington, DC.
14. **Rhame, F., R. Root, J. MacLowry, T. Dadisman, and J. Bennett.** 1973. Salmonella septicemia from platelet transfusion. *Ann. Intern. Med.* **78:**633–641.

REFERENCES (*continued*)

15. **Saxena, S., V. Odono, J. Uba, J. Nelson, W. Lewis, and I. Shulman.** 1990. The risk of bacterial growth in units of blood that have warmed to more than 10°C. *Am. J. Clin. Pathol.* **94:**80–84.
16. **Steere, A., J. Tenney, D. Mackel, M. Snyder, S. Polakavetz, M. Dunne, and R. Dixon.** 1977. Pseudomonas species bacteremia caused by contaminated normal human serum albumin. *J. Infect. Dis.* **135:**729–731.
17. **Stenhouse, M. A. E., and L. V. Milner.** 1982. *Yersinia enterocolitica:* a hazard in blood transfusion. *Transfusion* **22:**396–398.
18. **Tabor, E., and R. Gerety.** 1984. Five cases of pseudomonas sepsis transmitted by blood transfusion. *Lancet* **i:**1403.
19. **Younes, H. J., and P. N. Samuelson.** 1978. Bacteriologic examination of autologous blood. *South. Med. J.* **71:**1232–1234.

SUPPLEMENTAL READING

Hamill, T. R., S. G. Hamill, and M. P. Busch. 1990. Effects of room temperature exposure on bacterial growth in stored red cells. *Transfusion* **30:**302–306.

APPENDIX 13.13–1

Sources of Bacterial Contamination of Blood and Blood Products

Both warm- (mesophilic) and cold (psychrophilic)-growing organisms have been incriminated in bacterial transfusion reactions. Predominance of gram-negative bacilli in blood for transfusion has been attributed to the ability of these organisms to proliferate at 0 to 6°C, the temperatures at which blood is stored. *Yersinia enterocolitica*, *Pseudomonas fluorescens*, *Pseudomonas putida,* and *Enterobacter* species are examples of such organisms. Some gram-negative bacteria produce endotoxins whose presence in the bloodstream may cause rapid and sometimes irreversible shock (3). Even low levels of bacterial contamination can result in a blood product that contains high concentrations of endotoxin and/or bacteria after 2 to 3 weeks of storage. Gram-positive organisms grow poorly in the cold and rarely contaminate blood stored under these conditions. On the other hand, platelets are routinely stored for long periods at room temperature (22°C) and thus provide an excellent medium for this type of bacterial growth. As a result, platelets are now the most common cause of transfusion-related sepsis caused by organisms such as diphtheroids, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Pseudomonas* spp., and *Enterobacter cloacae* (2). Documented cases of bacterial contamination are listed in Table 13.13–1.

Contamination of blood products can occur via several routes: skin microbiota (1, 4), environmental organisms introduced at the time of collection or during processing (1, 5), or a bacteremia (asymptomatic) in the blood of a healthy donor. Water baths in which frozen blood products are thawed can also be a source of contamination (5). The American Association of Blood Banks recommends keeping blood bag ports dry and water baths clean to avoid contamination by this route (6).

The clinical manifestations associated with bacterial contamination mimic those seen in a hemolytic transfusion reaction unrelated to infection. After immunological and other noninfectious sources are ruled out, bacterial contamination should be considered. The blood product must be examined for evidence of bacterial contamination (change in color or presence of clots). In addition, Gram stain and cultures should be performed.

References

1. **Anderson, K. C., M. Lew, B. Gorgone, J. Martel, C. Leamy, and B. Sullivan.** 1986. Transfusion-related sepsis after prolonged platelet storage. *Am. J. Med.* **81:**405–410.
2. **Buchholz, D. H., V. M. Young, N. R. Friedman, J. A. Reilly, and M. R. Mardiney.** 1973. Detection and quantitation of bacteria in platelet products stored at ambient temperature. *Transfusion* **13:**268–275.
3. **Elin, R. J., W. B. Lundberg, and P. Schmidt.** 1975. Evaluation of bacterial contamination in blood processing. *Transfusion* **15:**260–265.
4. **Gibson, T., and W. Norris.** 1958. Skin fragments removed by injection needles. *Lancet* **ii:**983–985.
5. **Murray, A. E., C. A. Bartzokas, A. J. Shepherd, and F. Roberts.** 1987. Blood transfusion-associated *Pseudomonas fluorescens* septicaemia: is this an increasing problem? *J. Hosp. Infect.* **9:**243–248.
6. **Walker, R. (ed.).** 1990. *Technical Manual*, 10th ed., p. 472–473. American Association of Blood Banks, Arlington, VA.

13.14 Microbiological Assessment of Orthopedic Surgery Sites

I. PRINCIPLE

The severe morbidity associated with infection following implantation of orthopedic prosthetic devices or repair of bone fractures at septic trauma sites requires early and accurate microbiological assessment of these sites for successful management. Although no procedural or interpretive standards for culture of these sites have been established, the following is a set of procedures often used by surgical teams to assess microbial load or, along with other laboratory findings, to confirm the presence of an infection. These procedures include (i) enumeration of bacteria in tissue from a contaminated trauma site prior to repair of fracture, (ii) intraoperative culture of a site selected for implantation of a prosthesis, (iii) culture of fluid removed from the joint, and (iv) intraoperative culture of a failed prosthesis site.

PREANALYTICAL CONSIDERATIONS

II. SPECIMEN

Observe standard precautions.

Gram stain any purulent material that arrives.

A. Collection

1. Septic trauma site
   Collect 1 cm^3 of tissue at debridement from the trauma site that is not grossly contaminated. Biopsy material, including that obtained by punch biopsy (for quantitative analysis), from this site can also be used. Make sure the biopsy tissue is not submitted in formalin.
2. Implantation site
   Samples are collected during surgery after exposure of the implantation site. Use sterile swabs to sample surfaces of the site that will receive the prosthesis. Although no standard has been set, four to six samples could be taken depending upon the practice of the surgical team.
3. Septic prosthesis site
   a. Fluid withdrawn by aspiration from joint. Cleanse the surface of the skin through which the specimen will be aspirated as you would for collecting blood for culture. A needle and syringe should be used to withdraw fluid from the joint space. Because of the high viscosity of purulent fluid found in an infected site, a large-bore needle, 18 gauge, should be used.
   b. Samples collected after exposure of the prosthesis and site at or prior to surgery—multiple swab samples from the site, including the prosthesis and surrounding tissue. Four to six samples may be collected. This procedure is usually performed because acute infection of the prosthesis is not apparent. Other materials might be collected during surgery, including tissue surrounding the device, granulomatous tissue, capsular material, or pus. These materials should be sent in sterile containers as described below.

II. SPECIMEN (*continued*)

4. The whole prosthesis may be removed and sent to the laboratory for sonication and culture. The prosthesis must be collected into an appropriate sterile container such as a sterilized 1-liter polypropylene jar.

B. Specimen transport

1. For tissue specimens, use a sterile, airtight container, such as a plastic jar, plastic tube, or *sterile* Vacutainer tube. Do not push the tissue into the sheath of a swab transport system—small pieces of tissue can be lost, and large pieces are difficult to remove. Large pieces of tissue, 1 cm^2 or larger, need only be protected from desiccation. Smaller pieces of tissue, such as those obtained by punch biopsy, should be placed onto a piece of sterile gauze moistened with sterile, nonbacteriostatic saline and then placed into a container as described above.
2. Swabs used to collect samples should be part of a commercial swab transport system. The system should be sterile inside and out to minimize the possibility of contaminating the surgical field during use. Preferred systems use a simple liquid transport medium, not a semisolid. Smears for Gram stain should be prepared at the site and accompany the culture swabs to the laboratory.
3. Depending upon infection control practices of the institution, fluid withdrawn by aspiration can be sent to the laboratory in the syringe used to collect the sample. The needle must be removed or should be a type that is shielded during transport. Preferably, transfer the fluid to a secondary device, such as a sterile tube, commercial fluid transport system, or, if standard practice in your laboratory, a blood culture bottle. The Isolator tube may also be used, but host cells will be destroyed, thus limiting the ability to evaluate the specimen for infection.

III. MATERIALS

A. Septic trauma site

1. Sterile disposable plastic tissue grinder or mechanical homogenizer with sterile sample bag
2. Inoculating loop or glass rod "hockey stick"
3. Sterile pipettes, 1 and 2 ml
4. Dilution blanks, 9.9 ml of sterile 0.85% NaCl
5. TSA with 5% defibrinated sheep blood agar plate (SBAP)
6. CDC anaerobic sheep blood agar plate or another nonselective anaerobic agar medium supplemented with hemin and vitamin K

B. Specimens collected on swabs

1. BAP
2. Tube of fluid THIO medium

C. Fluid withdrawn by aspiration

Follow standard practice in your laboratory for processing specimens collected by aspiration of normally sterile body sites.

D. Tissue from septic implantation site

Follow standard practice in your laboratory for processing tissue specimens.

E. Explanted prosthesis

1. 400 ml of Ringer's solution
2. Aerobic and anaerobic BAP
3. Ultrasound bath with sonicator (frequency, 40 ± 2 kHz; power density, 0.22 ± 0.04 W/cm^2)
4. Vortex

ANALYTICAL CONSIDERATIONS

IV. SPECIMEN PROCESSING

Observe standard precautions.

A. Initial processing

Assess the integrity of the sample and transport device. Note the type of device received, along with a brief description of the appearance and approximate volume of the specimen.

B. Septic trauma site—quantification of organisms in tissue from a contaminated trauma site

1. Weight the barrel of a sterile tissue grinder to at least 0.0l g.

IV. SPECIMEN PROCESSING (*continued*)

2. With sterile forceps, aseptically place the piece of tissue being cultured into the barrel and reweigh to determine the mass of the sample. This value will be used in calculating CFU per gram of original sample.
3. If a mechanical homogenizer is used, take the weight of a sterile sample bag supplied with the device.
4. For a sample that weighs ≤1.0 g add 0.5 ml of sterile, nonbacteriostatic saline; for larger samples, add 1.0 ml.
5. Thoroughly grind or homogenize the sample.
6. Withdraw the entire volume of the homogenate into a sterile pipette large enough to determine the total volume.
7. Dispense 0.1 ml of the homogenate into a 9.9-ml dilution blank.
8. Shake vigorously or vortex the mixture to disperse the homogenate. This yields a 1:100 dilution of the original homogenate.
9. Transfer 0.2 ml of the final dilution to each of two BAPs and two anaerobic agar plates.
10. Using a sterile inoculating loop or glass rod hockey stick, spread the inoculum over the surface of each plate, and allow it to dry completely before moving plates into the incubator.

C. Implantation site

1. Multiple swab samples are usually received for culture. Each sample should be processed individually; do not combine the samples.
2. Rinse each swab into a tube of THIO by inserting the swab about 2 cm below the surface and gently squeezing it against the wall of the tube while twisting it. Discard the swab.
3. No Gram stain is performed.

D. Fluid withdrawn by aspiration

Process fluid withdrawn by aspiration as a routine body fluid culture, making certain to include culture media and incubation conditions to recover both fastidious and nonfastidious aerobic and anaerobic bacteria, and a Gram stain. Refer to your standard laboratory protocol and appropriate sections in this handbook for details of these procedures.

E. Tissue collected from septic implantation site

Process tissue collected from a septic implantation site as a routine tissue specimen, making certain to include culture media and incubation conditions to recover both fastidious and nonfastidious aerobic and anaerobic bacteria, and Gram stain. Processing should include dispersion of the material by homogenization or grinding. Refer to your standard laboratory protocol or procedure 3.13.2 for details of these procedures.

F. Explanted prosthesis

1. Add 400 ml of Ringer's solution to container with explanted material.
2. Vortex for 30 s.
3. Sonicate for 5 min.
4. Vortex for 30 s.
5. Plant 0.5 ml of Ringer's solution to aerobic and anaerobic BAP, and incubate at 35 to 37°C in 5 to 7% carbon dioxide aerobically and anaerobically for 5 and 7 days, respectively.
6. Centrifuge 200 ml of Ringer's solution at 2,600 rpm for 15 min. Gram stain the deposit.

V. INCUBATION

Incubate aerobic agar plates for 18 to 24 h at 35°C under an atmosphere enriched with carbon dioxide (5 to 8%) and elevated humidity (60 to 80% relative humidity). Incubate anaerobic plates for 48 h at 35°C in an anaerobic atmosphere. Incubate THIO at 35°C for 24 h.

VI. LABORATORY EVALUATION

A. Septic trauma site

1. 24 h

a. Examine aerobic plates for growth.

b. If discrete, mature colonies are present, count all and calculate the total colony count in the original sample as described below in item VI.A.4.

c. Gram stain each distinct colony type. A complete identification of each type of bacterium present is not necessary at this time, nor is susceptibility testing. This procedure is a measure of the bacteriological burden at the site known to be contaminated. Culture plates should be saved in the event that additional workup is requested.

d. If organism identifications are requested, proceed according to your standard laboratory procedures or appropriate sections of this handbook.

e. If the growth on the plates is too small to evaluate, or if there was no growth, incubate the plates for an additional 24 h.

f. At the end of the first day's work, prepare a preliminary report of the count of bacteria, if possible, along with Gram stain results. Make sure to indicate that anaerobic culture is pending.

2. 48 h

a. Examine primary plates incubated from the previous day for growth. If, at this time, the primary plates have no growth, discard them.

b. Examine primary anaerobic plates for growth. To confirm the anaerobic nature of colonies observed on these plates, subculture a representative colony onto aerobic and anaerobic agar and incubate accordingly.

c. Gram stain each distinct colony type.

d. At the end of the second day's work, update the preliminary report with any additional findings.

3. 72 h

a. Examine the subcultures set up the previous day. Compare growth and colony morphology seen on the subculture plates with those seen on the primary aerobic and anaerobic plates to determine the actual anaerobic populations in the original sample.

b. Do a final reconciliation of the total count to include the true anaerobic component.

c. Discard all plates except those containing isolates that will be saved in case additional workup is requested.

4. Calculations

Determine the number of organisms using the following equation (modified from reference 2): CFU/gram of tissue $= (N \times D \times V \times 5)/W$, where N is the number of colonies on the agar plate, D is the reciprocal of the dilution, V is the total volume of the original homogenate divided by 0.1, 5 is the factor to compensate for a 0.2-ml inoculum size, and W is the weight of the original sample.

5. Report

The final report should include the calculated total microbial count as CFU per gram of tissue at the original site, indicating growth of aerobic and anaerobic microbiota as appropriate, and listing of the types seen on Gram stain.

B. Implantation site

1. 24 h

a. Examine the inoculated broths for evidence of growth.

b. Prepare a smear from each broth showing growth, and Gram stain.

c. Subculture each broth onto BAP and incubate for 18 to 24 h.

d. Enter a preliminary report describing the Gram stain morphology of organism detected in how many tubes out of how many are growth positive.

VI. LABORATORY EVALUATION (*continued*)

e. Reincubate any broths with no growth.

f. At the end of the first day's work, prepare a preliminary report of the number of positive broth cultures, along with Gram stain results.

2. 48 h

a. Examine broths. Where growth is now evident, process as described above.

b. Examine subcultures from positive broths from the previous day.

c. Identify these organisms. Refer to your laboratory protocols or appropriate sections of this handbook for identification methods.

d. Depending upon laboratory practice, antimicrobial susceptibility testing may be done at this time or may be delayed until additional instructions are received from the requesting physician.

e. Save any isolates in accord with laboratory policy for any requests for additional workup.

f. Reincubate any subcultures from positive broths from the previous day.

g. Discard broths with no growth.

h. At the end of the second day's work, update the preliminary report with any additional findings.

3. 72 h

a. Examine all reincubated plates for evidence of growth.

b. Proceed with identification of any new growth.

c. Discard all plates with no growth.

d. At the end of the third day's work, update the preliminary report with any additional findings.

4. Fourth and fifth days

a. Examine the broth cultures each day and proceed as described above when growth appears.

b. If no growth appears after day 5, discard the broth and report as no growth.

5. Report

Prepare a final report including any the identities of organisms isolated from the broth cultures, the number of broths in which each isolate was found, and the results of any antimicrobial susceptibility testing done.

C. Septic implantation site

Swab cultures from this site should be evaluated as described in item VI.B. Culture of fluid and tissue are evaluated as defined for routine sterile body fluid or tissue specimen. Refer to your standard laboratory protocols or appropriate sections of this handbook.

D. Explanted prosthesis

Examine plates daily for growth. Quantify numbers of organisms per plate. Perform identification and susceptibility testing by standard procedures.

POSTANALYTICAL CONSIDERATIONS

VII. LIMITATIONS OF THE PROCEDURE

As with any culture procedure, the quality of the results obtained is directly related to the care taken in collecting and transporting the specimen, which is beyond the control of the laboratory. Specimens obtained from patients on antimicrobial therapy may yield false-negative results. Fastidious organisms, or organisms that undergo slow growth, such as *Mycoplasma* spp., certain mycobacteria, or fungi, may not be recovered using these methods.

VIII. INTERPRETATION

As always, clinical correlation of results rests with the physician. The following are general guidelines for interpretations of culture results derived from relevant studies in the literature.

A. If the patient has received no antimicrobial therapy for several weeks prior to collection of the specimen, culture of multiple specimens of periprosthetic tissue is 100% diagnostic, while culture of arthrocentesis fluid is 85 to 98% sensitive for detection of infection at the site of the prosthesis (1).

B. A colony count of >100,000 CFU/g of tissue excised from a contaminated trauma site is predictive of septic complications at the healing site unless closure is delayed and accompanied by appropriate antimicrobial therapy (2).

C. Any organism isolated from a supposedly sterile site should be considered potentially significant, even organisms such as coagulase-negative staphylococci and *Corynebacterium* spp., especially if the organism is (i) isolated in large numbers or (ii) isolated repeatedly from the same site. For swab samples, three or more positive swabs growing identical organisms from a normally sterile site are indicative of infection (1, 3).

D. Explanted prosthesis

1. Gram stain has a reported sensitivity of approximately 45% but a specificity of 100% for infected prostheses.

2. Quantitative cultures of >5 colonies per plate are indicative of an infected prosthesis.

IX. QUALITY CONTROL AND QUALITY ASSURANCE

A. QC

1. Culture media

Standard procedures for verifying the quality and performance of culture media used in these procedures should be performed and documented. Of particular importance is sterility testing of media prepared in-house and for those to which sterile enrichments are added after sterilization.

2. Reagents and equipment

Procedures for ensuring the quality and performance of reagents and equipment used for these analyses must be performed and documented.

B. QA

Results of the culture procedures described above can be used to monitor one aspect of the quality of care. Reduction of surgical procedure failure rates attributed to postsurgical infection are best evaluated by a multidiscipline team including hospital infection control, the microbiology laboratory, and the medical department delivering care. Action taken to improve care, if deemed necessary, and follow-up culturing to assess the impact of such actions should be decided upon by the same team.

REFERENCES

1. **Atkins, B. L., N. Athanasou, J. J. Deeks, D. W. M. Crook, H. Simpson, T. E. A. Peto, P. McLardy-Smith, A. R. Berendt, and The OSIRIS Collaborative Study Group.** 1998. Prospective evaluation of criteria for microbiological diagnosis of prosthetic-joint infection at revision arthroplasty. *J. Clin. Microbiol.* **36:**2932–2939.
2. **Cooney, W. P., III, R. H. Fitzgerald, Jr., J. H. Dobyns, and J. A. Washington II.** 1982. Quantitative wound cultures in upper extremity trauma. *J. Trauma* **33:**112–117.
3. **Cuckler, J. M., A. M. Star, A. Alavi, and R. B. Noto.** 1991. Diagnosis and management of the infected total joint arthroplasty. *Orthop. Clin. N. Am.* **22:**523–530.
4. **Trampuz, A., K. E. Piper, M. J. Jacobson, A. D. Hanssen, K. K. Unni, D. R. Osmon, J. N. Mandrekar, F. R. Cockerill, J. M. Steckelberg, J. F. Greenleaf, and R. Patel.** 2007. Sonication of removed hip and knee prostheses for diagnosis of infection. *N. Engl. J. Med.* **357:**654–663.

SUPPLEMENTAL READING

Atkins, B. L., N. Athanasou, H. Simpson, P. McLardy-Smith, D. W. M. Crook, A. R. Brenedt, and The OSIRIS Collaborative Study Group Oxford. 1997. Is the Gram stain useful in diagnosing infection at elective revision arthroplasty? *J. Infect.* **34:**75.

Atkins, B. L., and I. C. Bowler. 1998. The diagnosis of large joint sepsis. *J. Hosp. Infect.* **40:**263–274.

Duff, G. P., P. F. Lachiewicz, and S. S. Kelley. 1996. Aspiration of the knee joint before revision arthroplasty. *Clin. Orthop.* **331:**132–139.

Gruninger, R. P. 1989. Diagnostic microbiology in bone and joint infections, p. 42–51. *In* R. B. Gustilo, R. P. Gruninger, and D. T. Tsukayama (ed.), *Orthopaedic Infection: Diagnosis and Treatment.* W. B. Saunders Co., Philadelphia, PA.

Kortekangas, P., H. T. Aro, and O. P. Lehtonen. 1995. Synovial fluid culture and blood culture in acute arthritis. A multi-case report of 90 patients. *Scand. J. Rheumatol.* **24:**44–47.

Steckelberg, J. M., and D. R. Osmon. 1994. Prosthetic joint infections, p. 259–290. *In* A. L. Bisno and F. A. Waldvogel (ed.), *Infections Associated with Indwelling Medical Devices*, 2nd ed. American Society for Microbiology, Washington, DC.

13.15 Quantitative Culture of Small-Bowel Contents

I. PRINCIPLE

The microbial content of the normal gastrointestinal tract varies with the precise site (2, 5). The concentration of bacteria in gastric aspirates from fasting patients is fewer than 10^3/ml. Duodenal and jejunal organisms are normally present at fewer than 10^5/ml (3) and consist of primarily gram-positive bacteria (staphylococci, streptococci, and lactobacilli) and yeasts. Members of the family *Enterobacteriaceae* are occasionally present in small numbers. Strict anaerobes are notably absent. Postprandially, the microbial content of the proximal small bowel is increased by the introduction of food and oropharyngeal microorganisms. The ileal microbiota more closely resembles that of the colon, with the ileocecal valve incompletely regulating backwash from the colon. In the distal ileum, the bioload consists of 10^7 to 10^8 aerobes and anaerobes per ml, whereas in the colon, a significant rise in anaerobes to 10^{10} to 10^{11} organisms per ml and of aerobes to 10^8 organisms per ml is found (3). (*See* Appendix 13.15–1 for further discussion.)

PREANALYTICAL CONSIDERATIONS

II. SPECIMEN

A. Specimen collection

The small-bowel aspirate for bacteriological culture is obtained endoscopically with a sterile catheter or simple sterile tube (3, 4). Be careful not to inject air into the bowel during collection so that anaerobic growth will not be compromised.

B. Specimen volume

Collect at least 1 ml of fluid.

C. Specimen transport

No special transport device is necessary, but anaerobic transport devices may be used. Specimens should be delivered immediately to the laboratory and processed as soon as possible (1). If immediate processing or transport is not possible, refrigerate and process within 2 h.

D. Specimen rejection

Reject all specimens not properly transported. Do not accept any solid fecal matter; accept only fluid bowel contents.

III. MEDIA

A. Plated media

1. TSA with sheep blood and/or CHOC
2. Nonselective anaerobic agar such as CDC anaerobic blood agar or brucella blood agar

B. Broth diluents

1. Schaedler broth
2. Other broths, such as Mueller-Hinton or even THIO, may be substituted.

ANALYTICAL CONSIDERATIONS

IV. SPECIMEN PROCESSING

Observe standard precautions.

A. Dilution

1. Invert specimen at least three times, or vortex for 10 s to mix well.
2. Prepare 10^{-2} dilution by placing 0.01 ml of specimen into 0.99 ml of Schaedler broth. (Use a 0.01-ml calibrated loop or 10-μl pipette.) Mix well.
3. Prepare 10^{-4} dilution by placing 0.01 ml of the 10^{-2} dilution into 0.99 ml of Schaedler broth. Mix well.
4. Prepare 10^{-6} dilution by placing 0.01 ml of the 10^{-4} dilution into 0.99 ml of Schaedler broth. Mix well.

B. Plating

1. Remove 0.01 ml from the 10^{-2} dilution to one each of a blood agar or CHOC plate and an anaerobic blood agar plate. Inoculate the broth across the center of the plate, and then streak it back and forth perpendicularly to the original streak. Label the plates with the proper dilution factor.
2. Repeat with the 10^{-4} and 10^{-6} dilutions.

C. Incubation

1. Place blood agar and/or CHOC plates in a CO_2 incubator at 35°C for 16 to 24 h.
2. Place anaerobic blood agar plates into any available anaerobic incubation system at 35°C for 72 h.

V. RESULTS

A. Evaluation of cultures: quantitation of aerobes

After incubation, examine aerobic plates and count the colonies on the plate containing 30 to 300 colonies. Discard other plates. To calculate the number of organisms per milliliter, use the formula given below.

B. Calculations

Organisms/milliliter of specimen = number of colonies/plate ×

$$\frac{1}{\text{dilution of plate counted}} \times \frac{1}{\text{volume plated}}$$

Example: If there are 10 colonies on the 10^{-4} dilution agar plate, then

$$10 \times \frac{1}{10^{-4}} \times \frac{1}{10^{-2}} = 10^{7} \text{ organisms per ml.}$$

Report this figure for total aerobes.

C. Quantitation of anaerobes

Select the anaerobic plate containing 30 to 300 colonies. Discard other plates. Choose one colony representing each type present, note the colony count, and subculture aerobically and anaerobically. Incubate for 48 h under appropriate atmospheres. If there is growth anaerobically but not aerobically, the growth represents the strict anaerobes. Count the anaerobic colonies on the plate, and compute the organisms per milliliter by using the above formula. Report this figure for total anaerobes.

POSTANALYTICAL CONSIDERATIONS

V. RESULTS (*continued*)

D. Reporting results

Add aerobic and anaerobic counts to produce total bacterial count. Report results as follows.

total aerobic count = ___ organisms/ml
total anaerobic count = ___ organisms/ml
total bacterial count = ___ organisms/ml

If there are fewer than 10 colonies on the 10^{-2} dilution plate, report "fewer than 10^5 organisms/ml." If there are more than 100 colonies present on the 10^{-6} dilution and they cannot be estimated, report "more than 10^{10} organisms/ml." If plates are overgrown with swarming *Proteus* spp., report "no quantitation possible."

E. Identification and susceptibility testing

Since the goal of this procedure is to detect overgrowth, no identification or susceptibility testing need be done routinely. All plates with representative organisms should be saved for 7 days so that additional testing can be done if indicated. To save time, some microbiologists proceed with identification tests immediately.

VI. LIMITATIONS OF THE PROCEDURE

In this procedure, the dilution protocol does not reveal the presence of organisms present at normal levels (fewer than 10^5/ml) in the duodenal fluid. Since the goal of the procedure is to detect overgrowth rather than to evaluate normal conditions, this approach minimizes the effort while maximizing information applicable to detection of the overgrowth syndrome.

This test does not detect parasites, which require a separate examination. Very strict anaerobes and fastidious organisms also may not be detected by this technique. Since the sole purpose of this test is to detect bacterial overgrowth of the small intestine, other applications of the information provided may not be appropriate.

VII. INTERPRETATION

A total colony count of fewer than 10^5 organisms per ml in a duodenal-fluid specimen is considered a normal result. A total colony count of more than 10^5 organisms per ml may indicate that overgrowth of bacteria has occurred in the small bowel.

VIII. QUALITY CONTROL AND QUALITY ASSURANCE

A. QC

QC is conducted for the volumetric devices and media as described elsewhere in this handbook.

B. QA

This test would not normally be subject to a QA monitor, as it should be a rare test. Excessive test ordering should, however, precipitate a QA intervention.

REFERENCES

1. **DeSilva, D. G. H., P. Wills, R. H. George, D. C. A. Candy, and A. S. McNeish.** 1985. Effect of transport and storage on jejunal fluid microflora. *J. Diarrhoeal Dis. Res.* **3:**205–208.
2. **Gorbach, S. L., and R. Levitan.** 1970. Intestinal flora in health and gastrointestinal diseases, p. 252–275. *In* G. B. J. Glass (ed.), *Progress in Gastroenterology*. Grune and Stratton, Inc., New York, NY.
3. **King, C. E., and P. P. Toskes.** 1979. Small intestine bacterial overgrowth. *Gastroenterology* **76:**1035–1055.
4. **McEvoy, A., J. Duttan, and O. F. W. James.** 1983. Bacterial contamination of the small intestine is an important cause of occult malabsorption in the elderly. *Br. Med. J.* **287:**789–793.
5. **Thadepalli, H., S. M. A. Law, V. T. Bach, T. K. Matsui, and A. K. Mandal.** 1979. Microflora of the human small intestine. *Am. J. Surg.* **138:**845–850.

SUPPLEMENTAL READING

Gracey, M. 1979. The contaminated small bowel syndrome: pathogenesis, diagnosis and treatment. *Am. J. Clin. Nutr.* **32:**234–243.

Isaacs, P., and B. Jardine. 1986. Gram staining in the rapid diagnosis of small bowel bacterial contamination. *Med. Lab. Sci.* **43:**386–387.

Schneider, A., B. Novis, V. Chen, and G. Leichtman. 1985. Value of the ^{14}C-D-xylose breath test in patients with intestinal bacterial overgrowth. *Digestion* **32:**86–91.

APPENDIX 13.15–1

Bacterial Overgrowth in the Small Bowel

There is a common misconception that the stomach and small intestine are sterile; close examination of the literature (2, 6) shows that modifiers such as "functionally" or "effectively" are generally employed in association with the term "sterile." For someone working with colon or oral microbiota, where dilutions of 10^{-8} are necessary to achieve isolated colonies, specimens from the stomach (10^3/ml), duodenum-jejunum (10^5/ml), and even ileum (10^7 to 10^8/ml) would appear sterile.

Overgrowth of bacteria in the healthy small bowel is prevented by various natural regulatory mechanisms. The most important appears to be normal small-intestine peristalsis. Other mechanisms include gastric acid, gastrointestinal tract mucus, bile, and inhibitory properties of the gastric and jejunal mucosa (5).

Surgical procedures (3, 7) or physiological conditions that interfere with intestinal motility are a primary cause of microbial overgrowth (2, 4, 6). Numerous other conditions, both anatomic (e.g., diverticular strictures, fistulas) and nonanatomic (e.g., scleroderma, diabetes, cirrhosis) (5), also contribute. Age may also be a risk factor for bacterial overgrowth and its consequences (11). Once overgrowth has occurred, metabolic consequences follow, and diarrhea, steatorrhea, anemia, and malabsorption, which is manifested clinically as weight loss and malnutrition (5, 8), may result.

Bacterial overgrowth may be detected directly by quantitative culture of fluid from the duodenum or indirectly by detection of microbial metabolites with breath tests (9, 12). Cultures of duodenal aspirates or string test samples (10) may indicate overgrowth (1, 8). Such cultures are considered important as infection control cultures because they represent an adverse microbiological consequence of a medical procedure.

References

1. **Anonymous.** 1981. Questions and answers. *Clin. Microbiol. Newsl.* **3:**61–62.
2. **Bjorneklett, A.** 1983. Small bowel bacterial overgrowth syndrome. *Scand. J. Gastroenterol. Suppl.* **85:**83–93.
3. **Bjorneklett, A., O. Fausa, and T. Midtuedt.** 1983. Small bowel bacterial overgrowth in the postgastrectomy syndrome. *Scand. J. Gastroenterol.* **18:**277–287.
4. **Bjorneklett, A., T. Hoverstad, and T. Hovig.** 1985. Bacterial overgrowth. *Scand. J. Gastroenterol. Suppl.* **109:**123–132.
5. **Drude, R. B., Jr., and C. Hines, Jr.** 1980. The pathophysiology of intestinal overgrowth syndromes. *Arch. Intern. Med.* **140:**1349–1352.
6. **Hill, M.** 1985. Normal and pathological microbial flora of the upper gastrointestinal tract. *Scand. J. Gastroenterol.* **111**(Suppl.)**:**1–6.
7. **Kelly, D. G., S. F. Phillips, K. A. Kelly, W. M. Weinstein, and M. J. R. Gilchrist.** 1983. Dysfunction of the continent ileostomy: clinical features and bacteriology. *Gut* **24:**193–201.
8. **King, C. E., and P. P. Toskes.** 1979. Small intestine bacterial overgrowth. *Gastroenterology* **76:**1035–1055.
9. **King, C. E., and P. P. Toskes.** 1984. Breath tests in the diagnosis of small intestine bacterial overgrowth. *Crit. Rev. Clin. Lab. Sci.* **21:**269–281.
10. **Liebman, W. M., and P. Rosenthal.** 1983. Evaluation of the string test in intestinal bacterial overgrowth. *Am. J. Dis. Child.* **137:**1177–1178.
11. **McEvoy, A., J. Duttan, and O. F. W. James.** 1983. Bacterial contamination of the small intestine is an important cause of occult malabsorption in the elderly. *Br. Med. J.* **287:**789–793.
12. **Rumessen, J. J., E. Gudmand-Hayer, E. Bachman, and T. Justesen.** 1985. Diagnosis of bacterial overgrowth of the small intestine. *Scand. J. Gastroenterol.* **20:**1267–1275.

13.16 Phenotypic Characterization of Organisms for Epidemiologic Purposes

13.16.1 Introduction

Numerous methods exist for characterizing microorganisms beyond the species level. Methods include but are not limited to determining antimicrobial resistance patterns, biotyping, serotyping, and using molecular techniques such as plasmid analysis and restriction enzyme analysis of plasmid or chromosomal DNA. These methods are used when it is necessary to separate organisms more finely for clinical and epidemiologic purposes. In order to have wide application, laboratory techniques for typing organisms in the clinical microbiology laboratory must be easy to perform with readily available reagents and materials. A valid typing technique must be reproducible, standardized, and stable over time. It must be sensitive enough to distinguish epidemiologically related and unrelated strains. No single method of strain subtype delineation has proved to be ideal. Most often, a combination of several systems of characterization is used to evaluate a group of organisms. The fundamental principle in the application of any technique to the typing of organisms is that the entire group must be tested as a batch. All organisms must be run in parallel on the same day by the same laboratory and the same personnel with identical reagents. (*See* Appendix 13.16–1 on p. 13.16.5.1 for further discussion.)

13.16.2 Dienes Typing for *Proteus* spp.

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Two different strains of *Proteus* form a line of inhibition where the swarms meet. Two identical *Proteus* organisms swarm together smoothly.

II. MATERIALS

A. Test isolates of swarming *Proteus* spp.
B. Blood agar plates
C. Motility media

III. QUALITY CONTROL

A. Negative control: the same *Proteus mirabilis* strain (isolate) spotted twice on the plate; no inhibition
B. Positive control: two different *Proteus* strains spotted opposite each other; inhibition line (not identical)

ANALYTICAL CONSIDERATIONS

IV. PROCEDURE

Observe standard precautions.

A. The organisms to be tested must be swarming. If this characteristic has been lost, passage the organisms once through motility medium to help them recover the ability to swarm.
B. Inoculate by placing a loopful of each of two to four test isolates near the edges of a blood agar plate. The spots should be about 6 cm apart (Fig. 13.16.2–1). All pairs of organisms to be compared must be spotted on the same plate.
C. Incubate the plates for 18 to 24 h at 35°C.
D. Observe plates for a line of inhibition at the conjunction of the swarming zones. The line is more easily seen at the edges of the plate. Organisms that swarm together smoothly are similar. Those that inhibit each other are different.
E. Strains that fail to swarm or that swarm too slowly cannot be typed by this technique.

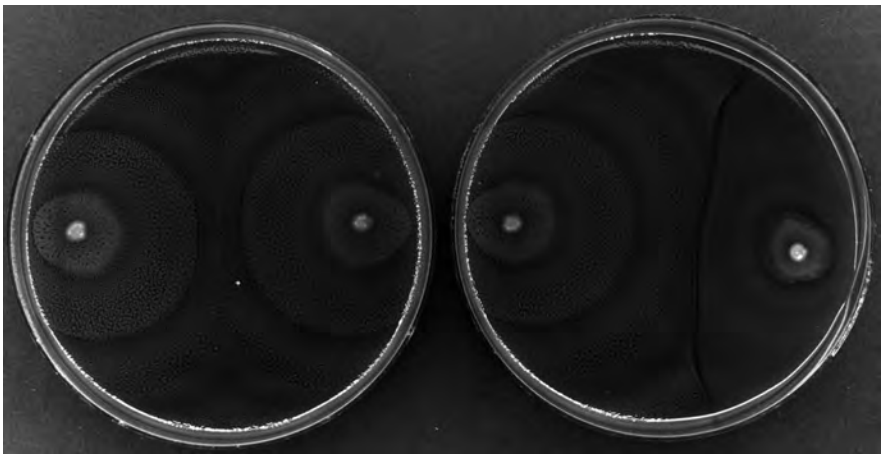

Figure 13.16.2–1 Dienes typing: negative (left) and positive (right) tests.

SUPPLEMENTAL READING

Hickman, F. W., and J. J. Farmer III. 1976. Differentiation of *Proteus mirabilis* by bacteriophage typing and the Dienes reaction. *J. Clin. Microbiol.* **3:**350–358.

Tracy, O., and E. J. Thomson. 1972. An evaluation of three methods of typing organisms of the genus *Proteus. J. Clin. Pathol.* **25:**69–72.

13.16.3 Slime Test for Staphylococci

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Slime, a heteropolysaccharide loosely associated with cells of some staphylococci, promotes adherence to smooth surfaces. The presence of slime can be detected on glass tube surfaces by staining with safranin. Slime production can be used as a phenotypic strain market but may not predict the potential pathogenic nature of an isolate.

II. MATERIALS

A. TSB (BBL)
B. Glass screw-cap round-bottom tubes (16 by 125 mm)
C. Safranin O stock (2.5 g of safranin O in 100 ml of 95% ethanol)
D. Safranin working solution (1:10 dilution of stock in water)
E. Pipette

III. QUALITY CONTROL

Test at least weekly.

A. Positive: slime-producing *Staphylococcus epidermidis*
B. Negative: *Staphylococcus haemolyticus*

ANALYTICAL CONSIDERATIONS

IV. PROCEDURE

Observe standard precautions.

A. Inoculate a 5-ml tube of TSB with a few representative colonies. Incubate at 35°C for 48 h.
B. Aspirate all broth, being careful not to disturb the sides of the tube.
C. Add 5 to 10 drops of safranin, recap, and gently rotate tube to stain all inner surfaces. Invert tube to drain.
D. Slime production is present if a visible stained film lines the walls of the tube. A ring at the liquid-air interface is not considered positive.
E. Grade as follows.
Negative: no staining of walls
1+: very slight color on side of tube
2+: light staining along walls of tube
3+: heavy staining along walls of tube
F. Grades negative and 1+ are reported as slime test negative; grades 2+ and 3+ are reported as slime test positive.

SUPPLEMENTAL READING

Christensen, G. D., J. T. Parisi, A. L. Bisno, W. A. Simpson, and E. H. Beachey. 1983. Characterization of clinically significant strains of coagulase-negative staphylococci. *J. Clin. Microbiol.* **18:**258–269.

Christensen, G. D., W. A. Simpson, A. L. Bisno, and E. H. Beachey. 1982. Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. *Infect. Immun.* **37:**318–326.

13.16.4 Synergistic Hemolysis

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Some isolates of staphylococci produce a hemolysin that will give synergistic or complete hemolysis with beta-toxin-producing *Staphylococcus intermedius*. This test can be used as a strain marker.

II. MATERIALS

A. BAP
B. *S. intermedius* ATCC 49052
C. *Staphylococcus epidermidis* negative control

III. QUALITY CONTROL

A. Test with every batch.
B. Negative control: all test isolates tested in duplicate with *S. epidermidis* negative control
C. Positive control: *S. epidermidis*, which produces synergistic hemolysis

ANALYTICAL CONSIDERATIONS

IV. PROCEDURE

Observe standard precautions.

A. Inoculate a BAP with *S. intermedius* ATCC 49052 as a line down the center of the plate.
B. Inoculate a second BAP with the negative control, *S. epidermidis*, in the same way.
C. Streak each test strain in a line perpendicular to but not touching the center lines, about 0.5 cm from the inoculum (Fig. 13.16.4–1).
D. Incubate at 35°C. Read plates at 16 to 18, 24, and 48 h.
E. A zone of complete hemolysis within the partial hemolysis zone of *S. intermedius* is a positive test. It will appear as an arrowhead similar to that produced in the CAMP test for identification of group B streptococci.
F. All tests must be negative with the *S. epidermidis* negative control strain.
G. Staphylococcal isolates can be designated as displaying the following.
  1. Complete hemolysis: beta-hemolysis without *S. intermedius*
  2. Synergistic hemolysis: clear zone with *S. intermedius*
  3. No hemolysis

Figure 13.16.4–1 Synergistic hemolysis test: positive tests, left and upper right; weak positive test, lower right; negative test, center right.

SUPPLEMENTAL READING

Bergan, T., and J. R. Norris. 1978. *Methods in Microbiology*, vol. 10. Academic Press, Inc., New York, NY.

Brokopp, C. D., and J. J. Farmer. 1979. Typing methods for *Pseudomonas aeruginosa*, p. 89–133. *In* R. G. Doggett (ed.), *Pseudomonas aeruginosa.* Academic Press, Inc., New York, NY.

Hebert, G. A., R. C. Cooksey, N. C. Clark, B. C. Hill, W. R. Jarvis, and C. Thornsberry. 1988. Biotyping coagulase-negative staphylococci. *J. Clin. Microbiol.* **26:**1950–1956.

Hebert, G. A., and G. A. Hancock. 1985. Synergistic hemolysis exhibited by species of staphylococci. *J. Clin. Microbiol.* **22:**409–415.

Pitt, T. L. 1988. Epidemiological typing of *Pseudomonas aeruginosa. Eur. J. Clin. Microbiol. Infect. Dis.* **2:**238–247.

13.16.5 Appendix to Procedure 13.16

APPENDIX 13.16–1

Biotyping and Antibiograms

Biotyping and antibiograms are readily available in most clinical microbiology laboratories. Caution must be used in the application of these for organism characterization. Most identification systems and antibiotic panels are designed to identify species and to define clinically useful antibiotics and are not sensitive or reproducible enough for strain delineation by themselves. There are several simple and practical additional tests that can be used to subtype selected nosocomial microorganisms in most clinical microbiology laboratories. Four of these tests, Dienes test for swarming *Proteus* spp., pyocin typing for *Pseudomonas aeruginosa*, and synergistic hemolysis and slime tests for staphylococci, provide excellent results and are relatively easy to perform. Procedures and references are provided in this procedure. Additional methods of characterization and variations on biotyping and antibiograms designed for specific species are in use in many research laboratories. In the future, some of these will be in the realm of the clinical laboratory as the problems of stability, reproducibility, and labor intensity are solved.

13.17 Screening for Methicillin-Resistant *Staphylococcus aureus*

I. PRINCIPLE

Screening patients for colonization with methicillin-resistant *Staphylococcus aureus* (MRSA) is often used during outbreaks to identify and isolate colonized/infected patients in an attempt to prevent further spread on health care units and within health care facilities. The utility of ongoing surveillance programs and universal admission screening is controversial at present (1, 3–5, 8, 9) but they are used in some jurisdictions. The microbiology laboratory must provide input into screening programs that are implemented and be reimbursed for procedures that are part of these programs. The sensitivity of any screening program depends on the specific body sites sampled, the number of sites sampled, and the methods used for detection. The laboratory costs for screening initiatives are often high, so these initiatives should not be undertaken at the expense of less costly infection control practices.

II. MEDIA, REAGENTS, AND EQUIPMENT

A. Agar media

Virtually any medium that supports the growth of *S. aureus* can be used to detect MRSA. However, selective media with or without an enrichment broth step are generally used to decrease workload and (in some cases) increase sensitivity (2, 6, 7). A variety of chromogenic media to distinguish between *S. aureus* and coagulase-negative staphylococci with incorporated antibiotics (oxacillin or cephamycins) to prevent the growth of MRSA are available (*see* Appendix 13.17.1–1). The laboratory should evaluate the relative sensitivity of several media to ensure optimal sensitivity and specificity.

B. Broth enrichment

Inoculation of screening samples into broth enrichment before planting to agar media can increase the recovery of low counts of MRSA. The relative yield is lower with newer chromogenic selective agar for screening for MRSA. In addition, the use of broth enrichment delays culture results by 18 to 24 h. Laboratories need to make decisions about the utility of broth enrichment based on the sensitivity of their direct screening methods and the prevalence of MRSA in their screening samples. If the negative predictive value of direct plating is greater than 95%, the addition of an enrichment step is probably not cost effective in most settings.

Multiple broth types have been evaluated including Todd-Hewitt, TSB with 6.5% NaCl, and Mueller-Hinton with 6.5% NaCl with or without antibiotics. There are few comparative clinical trials comparing broth types.

C. Direct nucleic acid amplification

Screening swabs for MRSA can be processed using nucleic acid amplification methods for the detection of *mecA*$^+$ *S. aureus*. Proprietary methods use the detection of segments of the SCC*mec* incorporated into the *S. aureus* genome to ensure that *mecA* in coagulase-negative staphylococci are not detected. While highly sensitive, commercial MRSA nucleic acid amplification is associated

II. MEDIA, REAGENTS, AND EQUIPMENT (*continued*)

with false-positive results, requiring culture confirmation (5). Costs of commercial nucleic acid amplification assays are higher than the cost of culture. The major advantage of this method is the shorter turnaround time for reporting of results.

PREANALYTICAL CONSIDERATIONS

III. SPECIMEN COLLECTION

A. Selection of culture sites

A variety of sites can be used for screening for MRSA colonization. Most studies show nasal, rectal/groin, throat swabs, and open skin lesions to be the most sensitive sites. More than one surface site may have to be sampled to achieve optimal sensitivity. The collection of throat and rectal swabs is associated with patient discomfort/intolerance, and the decision to use these samples must be made in conjunction with the staff who will be required to do the collection.

B. Collection of specimens

Swabs of dry skin surfaces should be premoistened with sterile nonbacteriostatic saline or transport medium prior to collection. Both nares can be sampled using a single swab rotated on the surface. If a rectal swab is collected, insertion into the area of the anal sphincter is all that is required (unlike screening for vancomycin-resistant enterococci, where fecal material should be obtained). Specimens should be labeled clearly as MRSA screening samples to ensure that appropriate media are inoculated.

C. Laboratory transport and storage

S. aureus survives well in transport media at both room temperature and 4°C for up to 96 h. However, for clinical screening, samples should be transported to the testing location as quickly as possible to avoid delays in reporting.

IV. SPECIMEN REJECTION

Specimens should be rejected if unlabeled or mislabeled, in accordance with standard laboratory rejection criteria. In addition, MRSA colonization is a prolonged state in most hospitalized patients, and repeat swabs are not warranted unless an attempt at decolonization has been made or a significant length of time has passed.

ANALYTICAL CONSIDERATIONS

V. SPECIMEN PROCESSING

A. Direct plating

Swabs can be plated directly with streaking for isolated colonies. Plates should be incubated aerobically for a minimum of 24 h before being read. Colonies typical of *S. aureus* (if nonselective medium is being used) should be confirmed by the usual methods and tested for methicillin resistance using CLSI-compliant methods.

For samples plated to medium selective for MRSA, isolates typical for MRSA (depending on the medium used) should be confirmed as *S. aureus*, and methicillin resistance should be confirmed using the standard latex agglutination method.

B. Enrichment broth

Swabs can be inoculated into broth medium and incubated for 18 to 24 h at 35°C. Some studies have looked at pooling all screening specimens from a single patient into one container of broth in an attempt to reduce costs. After initial incubation, broth must be inoculated onto culture medium, streaked for individual colonies, and identified as above.

V. SPECIMEN PROCESSING (*continued*)

C. Nucleic acid amplification

Swabs should be processed as indicated by the manufacturer. Samples that are positive or invalid should be plated to agar medium to determine if MRSA is present.

VI. INTERPRETATION

A. Agar plates

Results are reported as positive or negative for methicillin-resistant *S. aureus*.

B. Nucleic acid amplification

Because of problems with specificity, positive reports should be reported as presumptively positive for methicillin-resistant *S. aureus*, with a final report to follow.

POSTANALYTICAL CONSIDERATIONS

VII. RESULT REPORTING

Screening results need to be conveyed to the Infection Control Program as well as the admitting physician. The Infection Control Program takes the primary responsibility for ensuring that patients are placed in appropriate isolation to prevent nosocomial transmission.

VIII. ISOLATE BANKING

Infection Control Programs may need to have MRSA isolates typed for epidemiologic or medico-legal reasons. The laboratory should retain at least one isolate for each patient for a length of time determined in collaboration with the Infection Control Program.

IX. DATA STORAGE AND RETRIEVAL

Most laboratory information systems can store and easily retrieve information on patients identified to be colonized or infected with MRSA. Data from point prevalence studies during outbreaks and cumulative data from admission screening programs should be reported regularly to the Infection Control Committee.

REFERENCES

1. **Buhlmann, M., K. Bogli-Stuber, S. Droz, and K. Muhlemann.** 2008. Rapid screening for carriage of methicillin-resistant *Staphylococcus aureus* by PCR and associated costs. *J. Clin. Microbiol.* **467:**2151–2154.
2. **Cherkaoui, A., G. Renzi, P. Francois, and J. Schrenzel.** 2007. Comparison of four chromogenic media for culture-based screening of methicillin-resistant *Staphylococcus aureus*. *J. Med. Microbiol.* **56:**500–503.
3. **Dancer, S. J.** 2008. Considering the introduction of universal MRSA screening. *J. Hosp. Infect.* **69:**315–320.
4. **Harbarth, S., C. Fankhauser, J. Schrenzel, J. Christenson, P. Gervaz, C. Bandiera-Clerc, et al.** 2008. Universal screening for methicillin-resistant *Staphylococcus aureus* at hospital admission and nosocomial infection in surgical patients. *JAMA* **299:**1149–1157.
5. **Jeyaratnam, D., C. J. Whitty, K. Phillips, D. Liu, C. Orezzi, U. Ajoku, et al.** 2008. Impact of rapid screening tests on acquisition of methicillin-resistant *Staphylococcus aureus*: cluster randomised crossover trial. *BMJ* **336:**927–930.
6. **Krishna, B. V., M. Smith, A. McIndeor, A. P. Gibb, and J. Dave.** 2008. Evaluation of chromogenic MRSA medium, MRSA select and oxacillin resistance screening agar for the detection of methicillin-resistant *Staphylococcus aureus*. *J. Clin. Pathol.* **61:**841–843.
7. **Lee, S., Y. J. Park, J. H. Yoo, J. Kahng, I. H. Jeong, and Y. M. Kwon.** 2008. Comparison of culture screening protocols for methicillin-resistant *Staphylococcus aureus* (MRSA) using a chromogenic agar (MRSA-Select). *Ann. Clin. Lab. Sci.* **38:**254–257.
8. **Pegues, D. A.** 2008. Rapid screening for methicillin-resistant *Staphylococcus aureus* (MRSA) did not reduce MRSA acquisition rate. *ACP J. Club.* **149:**14.
9. **Wenzel, R. P., G. Bearman, and M. B. Edmond.** 2008. Screening for MRSA: a flawed hospital infection control intervention. *Infect. Control Hosp. Epidemiol.* **29:**1012–1018.

APPENDIX 13.17–1

Media and methods for Surveillance Cultures for MRSA

Mannitol-Salt Agar

dissolved in 1 liter of distilled water:

digest of casein	5.0 g
digest of animal tissue	5.0 g
beef extract	1.0 g
sodium chloride	75.0 g
mannitol	10.0 g
phenol red	0.025 g
agar	15.0 g

This medium can be supplemented with 2 to 6 µg of oxacillin per ml or 2 to 8 µg of cefoxitin per ml to make it selective for MRSA.

Other proprietary methods and media

MRSA-Select ……Bio-Rad Laboratories, Marnes-la-Coquette, France
Brilliance MRSA agar ……Oxoid, Basingstoke, United Kingdom
BBL CHROMagar MRSA ……BD Diagnostics, Franklin Lakes, NJ
MRSA ID ……bioMérieux, La Balme Les Grottes, France
BD GeneOhm MRSA Assay ……Becton Dickenson, San Diego, CA
Xpert MRSA ……Cepheid, Sunnyvale, CA

13.18 Screening for Vancomycin-Resistant Enterococci

I. PRINCIPLE

Vancomycin-resistant enterococci (VRE) (also referred to as glycopeptide-resistant enterococci) have emerged as significant nosocomial pathogens. Immunosuppression and indwelling devices are risk factors for invasive infections (3, 6). Transmission on hospital wards is the major source of spread of these antibiotic-resistant organisms. Infection rates can be reduced by screening high-risk groups (4). Patients colonized with VRE are isolated to prevent transmission. The laboratory, in conjunction with the Infection Control Program, should decide which groups of patients to screen and the frequency of screening depending on the local epidemiology, unless regulations state otherwise (8).

II. MEDIA, REAGENTS, AND EQUIPMENT

A. Agar media

A variety of selective media are available for the detection of VRE. Laboratories should verify which medium works best in their setting. In general, a medium with vancomycin as well as other inhibitory substances is the best choice (5). New chromogenic agar media have been developed to improve sensitivity and reduce the confirmatory work that was required for previously used agar media (2).

B. Broth enrichment

A broth enrichment step can be used prior to inoculation onto selective agar. Using a broth enrichment step prolongs the turnaround time for results and may not increase the recovery rate from patient samples (1, 5); however, it may improve recovery from environmental samples (5).

C. Direct nucleic acid amplification

Screening swabs for the presence of *vanA* and *vanB*, responsible for vancomycin resistance in *Enterococcus faecalis* and *E. faecium*, are currently under development. One commercial assay has been found to be sensitive enough for clinical use (7). However, false-positive results are a problem. The cost effectiveness of PCR methods has not been established to date.

PREANALYTICAL CONSIDERATIONS

III. SPECIMEN COLLECTION

A. Selection of culture sites

The primary specimen used for screening is the rectal or stool swab. Other open skin lesions and mucosal surfaces can be sampled as well, but the sensitivity of the test is low when these samples are used. Nasal swabs are not suitable for VRE screening.

B. Collection of specimens

Swabs of dry skin surfaces should be premoistened with sterile nonbacteriostatic saline or transport medium prior to collection. If a rectal swab is used, it should be inserted into the rectum such that it becomes contaminated with fecal material. Specimens should be labeled clearly as VRE screening samples to ensure that appropriate media are inoculated.

III. SPECIMEN COLLECTION (*continued*)

C. Laboratory transport and storage

Enterococci survive well in transport media at both room temperature and 4°C for up to 96 h. However, for clinical screening, samples should be transported to the testing location as quickly as possible to avoid delays in reporting.

IV. SPECIMEN REJECTION

Specimens should be rejected if unlabeled or mislabeled, in accordance with standard laboratory rejection criteria. In addition, VRE colonization is a prolonged state in most hospitalized patients, and repeat swabs are not warranted unless an attempt at decolonization has been made or a significant length of time has passed.

ANALYTICAL CONSIDERATIONS

V. SPECIMEN PROCESSING

A. Direct plating

Swabs can be plated directly onto selective agar with streaking for isolated colonies. Plates should be incubated aerobically for a minimum of 24 h before being read.

Isolates typical of enterococci growing on the selective agar should be confirmed as *E. faecalis* or *E. faecium* by standard methods. In addition, these isolates must be confirmed as vancomycin resistant using CLSI approved methodology. Confirmed positive samples are reported as positive for VRE. The admitting service and Infection Control should be notified immediately to institute isolation precautions.

Specimens with no growth or growth of other species of enterococci with low-level intrinsic resistance to vancomycin are reported as negative for VRE.

B. Enrichment broth

Swabs can be inoculated into broth medium containing 6 μg of vancomycin per ml and incubated for 18 to 24 h at 35°C. The broth then must be plated to VRE selective agar to identify the presence of VRE.

C. Nucleic acid amplification

Swabs should be processed as indicated by the manufacturer. Samples that are positive or invalid should be plated to agar media to determine if VRE are present and to isolate organisms for epidemiologic typing if necessary.

ANALYTICAL CONSIDERATIONS

VI. INTERPRETATION

A. Agar plates

Results are reported as positive or negative for vancomycin-resistant enterococci.

B. Nucleic acid amplification

Laboratories using PCR for screening will have to determine the positive predictive value of the assay and, in conjunction with Infection Control, determine whether to report results presumptively or await confirmatory testing.

POSTANALYTICAL CONSIDERATIONS

VII. RESULT REPORTING

Screening results need to be conveyed to the Infection Control Program as well as the admitting physician. The Infection Control Program takes the primary responsibilities for ensuring that patients are placed in appropriate isolation to prevent nosocomial transmission.

VIII. ISOLATE BANKING

Infection Control Programs may need to have VRE isolates typed for epidemiologic or medico-legal reasons. The laboratory should retain at least one isolate for each patient for a length of time determined in collaboration with the Infection Control Program.

IX. DATA STORAGE AND RETRIEVAL

Most laboratory information systems will have the ability to store and easily retrieve information on patients identified to be colonized or infected with VRE. Data from point prevalence studies during outbreaks and cumulative data from admission screening programs should be reported regularly to the Infection Control Committee.

REFERENCES

1. **Brown, D. F., and E. Walpole.** 2003. Evaluation of selective and enrichment media for isolation of glycopeptide-resistant enterococci from faecal specimens. *J. Antimicrob. Chemother.* **51:**289–296.
2. **Grabsch, E. A., S. Ghaly-Derias, W. Gao, and B. P. Howden.** 2008. Comparative study of selective chromogenic (chromID VRE) and bile esculin agars for isolation and identification of *vanB*-containing vancomycin-resistant enterococci from feces and rectal swabs. *J. Clin. Microbiol.* **46:**4034–4036.
3. **Olivier, C. N., R. K. Blake, L. L. Steed, and C. D. Salgado.** 2008. Risk of vancomycin-resistant Enterococcus (VRE) bloodstream infection among patients colonized with VRE. *Infect. Control Hosp. Epidemiol.* **29:**404–409.
4. **Price, C. S., S. Paule, G. A. Noskin, and L. R. Peterson.** 2003. Active surveillance reduces the incidence of vancomycin-resistant enterococcal bacteremia. *Clin. Infect. Dis.* **37:**921–928.
5. **Reisner, B. S., S. Shaw, M. E. Huber, C. E. Woodmansee, S. Costa, P. S. Falk, et al.** 2000. Comparison of three methods to recover vancomycin-resistant enterococci (VRE) from perianal and environmental samples collected during a hospital outbreak of VRE. *Infect. Control Hosp. Epidemiol.* **21:**775–779.
6. **Sakka, V., S. Tsiodras, L. Galani, A. Antoniadou, M. Souli, I. Galani, et al.** 2008. Risk-factors and predictors of mortality in patients colonised with vancomycin-resistant enterococci. *Clin. Microbiol. Infect.* **14:**14–21.
7. **Stamper, P. D., M. Cai, C. Lema, K. Eskey, and K. C. Carroll.** 2007. Comparison of the BD GeneOhm VanR assay to culture for identification of vancomycin-resistant enterococci in rectal and stool specimens. *J. Clin. Microbiol.* **45:**3360–3365.
8. **Weber, S. G., S. S. Huang, S. Oriola, W. C. Huskins, G. A. Noskin, K. Harriman, et al.** 2007. Legislative mandates for use of active surveillance cultures to screen for methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci: position statement from the Joint SHEA and APIC Task Force. *Infect. Control Hosp. Epidemiol.* **28:**249–260.

SECTION 14

Quality Assurance, Quality Control, Laboratory Records, and Water Quality

SECTION EDITOR: *Judy A. Daly*

14.1. Quality Assessment and Improvement (Quality Assurance)
Ron B. Schifman and Stephen G. Jenkins 14.1.1
14.1.1 Process Improvement • *Judy A. Daly* 14.1.1.1

14.2. Quality Control
Stephen G. Jenkins and David L. Sewell 14.2.1

14.3. Laboratory Records
Stephen G. Jenkins and David L. Sewell 14.3.1

14.4. Preparation and Quality Control of Laboratory Water
John L. Kempf, David L. Sewell, and Stephen G. Jenkins 14.4.1

On 24 January 2003, the *Federal Register* published the Centers for Medicare and Medicaid Services and the CDC's final rule on the requirements for laboratory director personnel qualifications and certain QC provisions under the Clinical Laboratory Improvement Amendments of 1988. The final rule, effective 24 February 2003, mandated that all new directors of high-complexity laboratories who hold a doctoral degree also be board certified. The rule grandfathered certain non-board-certified individuals with a doctoral degree who had served or were serving as the director of a laboratory performing high-complexity testing on the effective date of the rule.

This final rule also consolidated and reorganized the requirements for patient test management, QC, and QA and modified the consensus required for grading proficiency testing challenges. The resulting changes to the QC provisions became effective on 24 April 2003. To the extent possible, this section reflects the final rule changes.

14.1 Quality Assessment and Improvement (Quality Assurance)

I. DEFINITIONS, PRINCIPLES, AND OBJECTIVES

QA is a process of monitoring the functional components of a system and correcting defects when unacceptable performance is identified. Quality is characteristically assessed by specifying performance indicators and setting targets (thresholds) for acceptable proficiency. Limits may be set so that action is taken only when the number of deficiencies exceeds a specified threshold, or a limit may be defined as a sentinel event that requires review and action whenever it is encountered. The functional attributes of a QA plan are listed in Table 14.1–1.

Quality management also entails quality improvement objectives, which address methods of continuously improving reliability, efficiency, and utilization of laboratory services (Fig. 14.1–1). The importance of quality improvement should not be underestimated, since experience in other service industries has proven that the most effective and long-lasting improvements are those achieved by anticipating and preventing problems rather than by identifying and correcting defects after they have occurred (6, 7, 21, 22, 38, 40). This procedure will describe basic quality assessment and quality improvement procedures, but not QC procedures, for reagents, methods, and equipment.

II. MATERIALS AND RESOURCES

A. Personnel

Active participation by all individuals working in the system is essential to meet quality standards and continuously improve performance. Table 14.1–2 describes the roles of various participants in an institution's quality management structure.

B. Information

Quality assessment is information intensive, requiring multiple sources of data and a substantial amount of record keeping in order for compliance with goals to be demonstrated. Worksheets are helpful for documenting results and observations. A computerized system for managing and analyzing the large volume of data associated with quality assessment is a valuable and labor-saving resource.

Table 14.1–1 Attributes of a QA plan

Planned, systematic, and ongoing
Comprehensive
Based on indicators and criteria with consensus approval
Routine surveillance and analysis of data
Documentation of problem identification and resolution
Continuous activity to ensure that quality performance is sustained
Integrated and interdepartmental

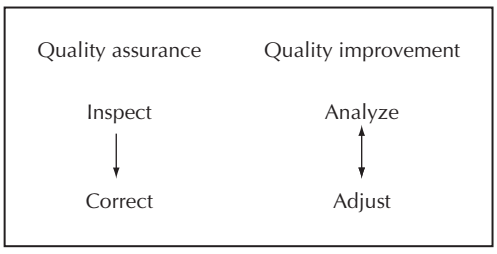

Figure 14.1–1 Differences between QC and quality improvement QA objectives are met by continuously monitoring a process, identifying defects, and correcting them. Quality improvement objectives are met by analyzing and understanding how a process works so that adjustments can be made to prevent defects from occurring.

Table 14.1–2 Roles of personnel in quality management organization

Personnel	Function
Hospital administrator	Provides leadership and support for institutional quality improvement
Chief of staff and clinical executive committee	Reviews and approves policies and procedures required to achieve quality improvement goals
QA committee	Fosters interdisciplinary communication, facilitates problem solving, and documents results of QA activities
Laboratory director; pathologist	Allocates resources and assigns personnel for departmental quality improvement activities, delineates departmental goals, reviews procedures, interprets QA data, and acts as liaison with other institutional units
Section supervisor	Implements procedures and manages data in accordance with QA goals; provides recommendations to director for improving products and services
Microbiologist-technologist	Participates in monitoring and evaluating laboratory services; provides recommendations for improving products and services

II. MATERIALS AND RESOURCES *(continued)*

C. Problem-solving skills

Quality improvement is often implemented by analyzing various components of a process with the aim of looking for ways to permanently remove obstacles to success (4, 30). Data collection, data analysis, and creative problem solving are the key components of this process. Information derived from QA procedures may be useful for detecting flaws in the system, but rarely does it point to a specific solution. The most valuable resources in problem solving are the observations by and recommendations from those who regularly work with the processes under consideration. Problem solving often requires the participation of members of other departments in the institution. Tools for successful problem solving include a thorough understanding of how the process works, good communication skills, consensus building, and, perhaps most importantly, a mind open to new ideas.

D. Procedure development

Because QA is an information-driven process, substantial attention has focused on choosing indicators. A clinical indicator (or monitor) is a measurable variable related to some aspect of care (5). Initiation of the process establishing a quality assessment and improvement procedure by specifying indicators prior to defining how the information will actually be used decreases the possibility of achieving a successful QA program (30).

Objectives for quality improvement should be clearly established *before* sources of data to be used for monitoring and evaluation are defined. This avoids the natural tendency to develop a procedure based on ease of data collection rather than on the potential utility of data derived from the process for dealing with a specific quality assessment and improvement objective. It is important to understand that useful indicators may require access to sources of information (such as charts and clinical staff) that are not readily available from the laboratory database (26). Under such circumstances, data collection is labor-intensive and may put a strain on limited resources. It is therefore essential to initially establish a carefully defined goal that considers how information that is generated will actually be employed to monitor and improve microbiology services.

QA data are usually collected to monitor and maintain a process at a targeted level of performance. For quality improvement purposes, however, information

II. MATERIALS AND RESOURCES *(continued)*

should be used for analysis of how a process is functioning in order to find the causes of expected or unexpected deficiencies. This knowledge is then used to implement adjustments that will prevent future deficiencies and thereby create sustained improvement.

The following outline delineates the steps that should be considered for development of a quality assessment and improvement procedure. Specific examples will follow.

1. Objectives
Objectives may be based on suggestions from the medical staff, a deficiency noted in the laboratory, or a generally accepted pattern of appropriate laboratory practice. General categories to be considered include specimen collection and transport, test selection, and utilization of results from microbiology examinations. During the planning stage, it may be useful to create a fishbone chart that explains specific aspects of the process and that may direct attention to specific problem-prone areas (Fig. 14.1–2). A proposal outlining an analysis of the process and a basis for undertaking the quality improvement procedure should be established.

2. Consensus
Quality improvement requires that all participants involved in the process comprehend the objectives and possess a commitment to improvement. Openness and communication are the key ingredients for developing constructive attitudes toward change and improvement. Most importantly, everyone must have an opportunity for input, agree on what is appropriate, and endorse the plan (7, 21, 38).

3. Quality indicators
Choose data elements that discriminate between a system that is operating appropriately and one that is faulty (28, 39). Table 14.1–3 lists examples of quality indicators appropriate for medical microbiology practice.

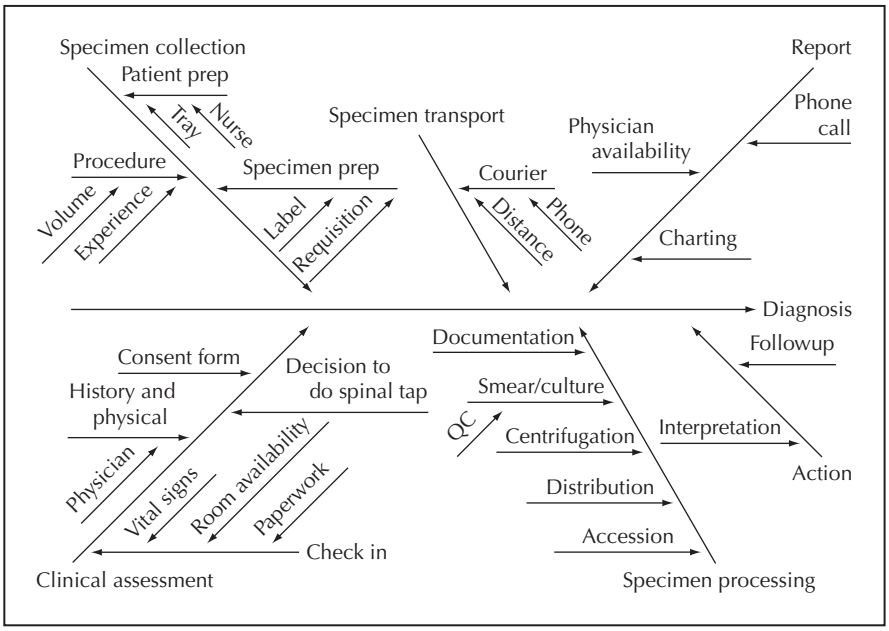

Figure 14.1–2 Fishbone diagram of bacterial meningitis diagnosis. A fishbone diagram displays important components of a process and their interrelationships. Examination of a process from this perspective helps demonstrate potential weaknesses and reveals how improvements in the process might be achieved.

Table 14.1–3 Examples of quality indicators for medical microbiology

Procedure or specimen	Objective	Indicator	Analysis
Blood culture	Appropriate use	Solitary or >3 collections/24 h	Determine prevalence and reason for solitary and excessive blood cultures.
	Adherence to aseptic collection and processing procedures	Contaminated cultures	Determine percentage of contaminants, rates overall, and rates per phlebotomist, ward, etc. Review procedures when high rate is identified.
Sputum	Appropriate collection	Specimens with >25 epithelial cells/low-power field (smear)	Determine percentage of specimens and rates overall and per specific location. Educational intervention when high rates are identified.
	Appropriate use	Solitary collections for acid-fast bacilli	Determine percentage of specimens and rate overall and per specific location. Determine reasons for solitary collections.
		Repeat cultures on same patient	Determine reasons for submission of multiple specimens.
	Accuracy of smear interpretation	Correlation of smear and culture results	Review discrepancies and reasons for misinterpretation of smear results.
Stool	Appropriate use	Ovum and parasite examination or bacterial cultures ordered after ≥3 hospital days	Determine percentage of positive results (should be very low or 0) and reasons for requests. Determine whether *Clostridium difficile* etiology was considered.
CSF	Appropriate collection	Sufficient volume for requested tests	Determine no. with insufficient volume; calculate rates overall and by service or location. Review procedures and reasons when high rate is identified.
		Contaminated cultures	Determine no. of contaminants; calculate rates overall and by service or location. Review on increasing trend (laboratory or collection deficiency?).
	Appropriate use	Orders for syphilis serology, cryptococcal antigen, or mycobacterial cultures with otherwise-normal CSF findings	Determine percentage of positive results and reasons for test selection. Develop algorithms for testing.
	Use of results	Therapeutic response to preliminary Gram smear report	Determine percentage of patients treated with antibiotics after positive Gram smear reported (should be 100% in unambiguous cases).
Urine	Appropriate collection	No. of cultures with (≥3 different) organisms	Determine percentage of specimens overall and per location. Determine reasons (delayed transport, collection problem, patient selection, etc.).
	Appropriate use	No. of cultures without concomitant urinalysis (for pyuria determination)	Determine percentage of specimens overall and per location. Determine reasons urinalysis was not ordered and whether urine culture is indicated.
		Repeated cultures on same patient	Determine reason for >2 specimens/week. Urinary tract infection suspected or confirmed?
Reporting errors	Accuracy of reports	Random review of worksheets with final charted results	Review all discrepancies. Determine reasons for clerical errors and their effects on patient outcome.
Susceptibility tests	Use of results	Correlation of antimicrobial therapy with susceptibility testing results	Record percentage of appropriate and discrepant treatments. Immediately notify physician of discrepancies.
	Antimicrobial agent use	Cumulative antimicrobial susceptibility rate	Publish data for empirical therapy decisions, and review them for endemic-resistance trends.

II. MATERIALS AND RESOURCES *(continued)*

4. Data analysis and strategies for improvement

Specifications for meeting QA goals (also referred to as thresholds) are used for defining acceptable performance and as grounds for initiating actions when an undesirable trend is identified. Thresholds are established on the basis of experience, appropriate medical requirements, and scientific data. Practice guidelines may also be considered (9, 37). In some circumstances, the goals are clear (e.g., no CSF specimen should ever be lost). Despite the theoretical value of aiming for a zero-defect process, such a goal may be unnecessarily restrictive and wasteful. For example, it may be inefficient to track down the cause of excessive urine cultures if such excess occurs infrequently and has no detrimental consequence. On the other hand, setting thresholds with too much tolerance can lead to a false sense of security or conceal potential avenues for improvement. Interinstitutional comparisons (referred to as "benchmarking") can also be utilized to help establish guidelines for setting thresholds (19). This is discussed in item III below. As an alternative to setting exact thresholds, it may be more valuable in some situations to analyze both the sources of error and their prevalence. In this manner, the most significant improvements are accomplished by addressing the most prevalent problems(s). This type of analysis is typically displayed as a Pareto chart (Fig. 14.1–3).

5. Documentation

Procedures, data, and actions must be documented. Data collection can be facilitated by using a log sheet that specifies the indicator, results, criteria for analyzing data (e.g., thresholds), and actions. *Documentation is not an end in itself.* Data are most valuable when they facilitate the understanding of how flaws in the system develop in order that appropriate adjustments can be made for improvement. Requiring that the monitor's quality improvement objective be specified helps direct attention toward the purpose of the objectives. Appendix 14.1–1 provides an example of how quality assessment data can be summarized and used to document quality improvement.

It is also important to recognize that QA endeavors are continuous and that improvement often occurs in small increments. Therefore, expectations for progress should not be set too high, nor should efforts abate when accomplishments appear to be meager in relationship to established goals (7).

E. Examples

It is not feasible to provide an exhaustive list of quality assessment and improvement procedures. These activities must be individualized based upon the needs and resources of each institution. Furthermore, these procedures should be modified as permanent solutions are attained and new problems evolve. In general, it is worthwhile to evaluate specimen collection and transport, test selection, and result utilization for each major category of microbiology test (tests can conveniently be stratified by specimen source, e.g., urine, blood, or respiratory). Four specific examples of basic QA procedures for specimen collection, turnaround time, clerical-error tracking, and utilization of antimicrobial susceptibility test results are described below. Use these examples only as a general guide for developing and implementing quality assessment and improvement procedures that pertain specifically to your individual laboratory and institutional needs.

Quality improvement procedures have special characteristics that do not conform easily to the guidelines recommended by the NCCLS for technical procedure manuals or by JCAHO for institutional quality assessment and improvement. A combination of these two formats was employed to create a generic laboratory quality assessment and improvement procedure that is described in Appendix 14.1–2. This may be used as a general blueprint requiring modification as applicable to the specific needs of each laboratory.

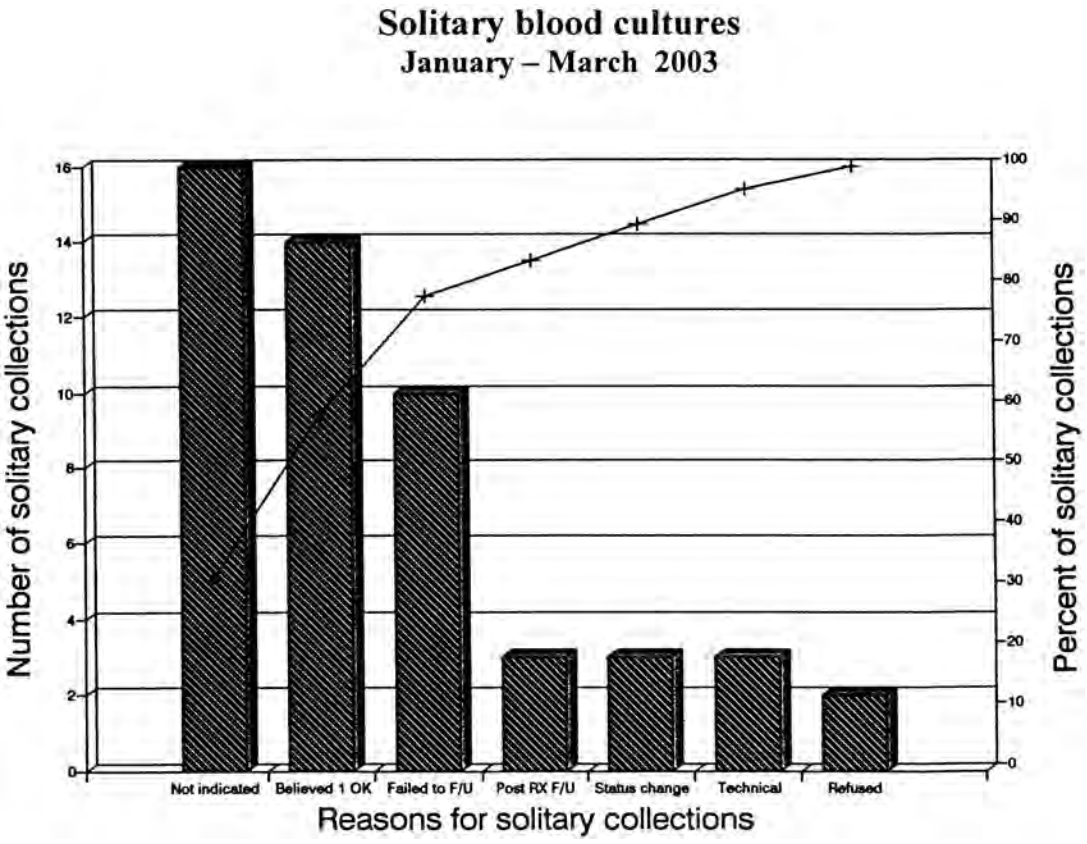

Figure 14.1–3 Pareto chart of solitary blood cultures. On the horizontal axis, the Pareto chart displays specific categories or groupings, beginning with the most prevalent type of event and continuing with other events in descending order of prevalence. The absolute numbers in each category are shown on the left vertical axis, and cumulative distributions (from 0 to 100%) are displayed on the right vertical axis. Making a change in the most prevalent category will have the most significant impact on overall improvement. Displaying quantitative relationships between groups in this way facilitates anticipation of the most likely sources of difficulty and sets priorities for improvement (32).

II. MATERIALS AND RESOURCES *(continued)*

1. Specimen collection and transportation (*see* Appendix 14.1–3)
   Examples of specimen collection and transportation indicators include transport times, sputum quality by cytologic examination, and presence of "contaminants" in blood, urine, and other specimens. Monitoring trends may be more effective when data are stratified by specific components of the process involved in specimen collection and transport. This makes it easier to detect factors in the process that may be responsible for longer-than-average specimen transport times, disproportionately large numbers of sputum samples with excessive epithelial cells, or contaminated cultures (Table 14.1–4).
2. Error tracking (*see* Appendix 14.1–4)
   An ongoing system that actively searches for errors in reported results by retrospective comparison of results on worksheets, instrument logs, etc., should be employed. Results should be placed in the patient's record (17). The clinical significance of errors discovered after the report is available should be classified and periodically reviewed to determine if modifications in procedures or policies are warranted.
3. Turnaround time (*see* Appendix 14.1–5)
   One of the first steps in developing a turnaround time procedure is the establishment of measurable goals (15) based on both the time constraints

Table 14.1–4 Example of results from sputum quality monitor

Ward	No. of specimens	
	Total	With ≥25 SEC/LPF (%)[a]
A	12	2 (17)
B	29	7 (24)
C	6	3 (50)[b]
D	71	18 (25)
E	33	4 (12)
F	9	7 (78)[b]
G	11	1 (9)
Total (average)	171	42 (25)

[a] SEC, squamous epithelial cells; LPF, low-power field.

[b] These units need more intensive instruction on specimen collection or indications for sputum culture.

II. MATERIALS AND RESOURCES *(continued)*

associated with performing the test and the realistic and medically relevant expectations of the physician. Turnaround time goals should always be developed by consensus of those involved in the process to ensure that the goals are realistic and that everyone understands how the system functions. Discussion also affords an opportunity for proffering suggestions to improve the process.

The effect of the turnaround time of laboratory tests on patient care is a function of numerous factors, many of which are external to laboratory processing. Tracking only internal laboratory turnaround times is simple but insufficient. Instead, the entire process must be examined. Whenever possible, record the times that specimens are collected as well as the times that results are appropriately interpreted *and used (not just reported).* For example, reporting "pleomorphic gram-negative bacilli with numerous inflammatory cells" for a CSF specimen would invoke a procedure to verify that the patient receives timely and appropriate antimicrobial therapy.

4. Monitoring and evaluating tests and use of test results (*see* Appendix 14.1–6)

The most important goal of quality improvement procedures for test utilization should be to optimize the contribution to patient care that laboratory services make while avoiding deleterious effects. To achieve these goals, laboratorians must ensure that testing is purposeful and efficient. Improvement in test utilization has traditionally been measured by the degree to which the volume of testing is reduced. However, an association between reduced testing and clinical outcome is difficult to document. An alternative argument might be that it is actually better to test somewhat excessively than to risk missing an important clinical finding (20). In some cases, however, certain tests that are costly or have limited value may be restricted (Table 14.1–5). In other cases, such as solitary blood cultures or single sputum cultures for mycobacteria, underutilization may be problematic (8, 31).

While restriction policies may reduce unnecessary testing (and workload), they provide little direct benefit to the patient. Ensuring that appropriate test orders are placed when indicated (as opposed to preventing unnecessary tests from being ordered when they are not indicated) and that results from such testing are being utilized optimally represent quality improvement objectives that directly benefit patients, especially if corrective action can be simultaneously linked to the monitoring and evaluation processes.

The clinical importance of microbiology examinations may not be adequately recognized when information about the patient's therapy and clinical

Table 14.1–5 Examples of indications for restricting microbiology testing

Routine stool bacterial culture and ovum and parasite examinations on specimens collected from patients hospitalized for >3 days (33, 41)
Clostridium difficile toxin testing on hospitalized inpatients who have not been treated with antimicrobial agents over the preceding month and who do not have significant diarrhea and/or abdominal pain (13)
CSF culture for mycobacteria on specimens with normal cell counts and glucose and protein (12) levels
Urine cultures for asymptomatic patients receiving antimicrobial agents (18)
In other than immunocompromised individuals, sputum cultures for mycobacteria from patients who have not had a tuberculin skin test and chest roentgenogram examination (34)
Venereal disease research laboratory and antigen testing of CSF before initial CSF findings are reviewed (1)
More than one specimen from same site by same method of collection received each day (except blood cultures, CSF, and feces) (3)
Bacterial culture of poor-quality specimens: mouth, bowel contents, perirectal abscess, decubiti, pilonidal abscess, lochia, Foley catheter tip, vomitus, placenta (vaginally delivered) (3)
Anti-hepatitis A virus (immunoglobulin M) testing in patients with normal liver enzymes (10)

II. MATERIALS AND RESOURCES *(continued)*

condition is unavailable. Nevertheless, the microbiology laboratory is the earliest source of critical diagnostic information that can directly benefit the patient. Any deficiency in the system can result in significant delays in therapeutic adjustments, i.e., even when all the necessary information is available to the "system," the patient does not immediately benefit if the data are not integrated in a timely and effective fashion.

Since the turnaround times for culture and susceptibility testing are long, antimicrobial therapy is initiated empirically until such time as laboratory results either support the therapeutic decision(s) or suggest alternative antimicrobial agent choices. Assuming that culture and susceptibility testing results are valid guides to therapeutic response, any further delays in applying this information can compromise the use of these tests in a number of ways. First, an extended lapse between the time test results are reported and the time they are evaluated by a physician can have serious consequences if such a lapse delays either recognition of a significant infection or modification of treatment. Physicians are reluctant to change therapy when a patient shows improvement, even when test results indicate that less expensive or less toxic agents might be prescribed. This reluctance to modify therapy becomes even stronger as the time between specimen collection and evaluation of results by the physician lengthens (27, 35, 36). Computerized systems that link pharmacy information with data from the microbiology laboratory are now available from manufacturers of automated antimicrobial susceptibility testing instruments. Reports generated by these systems can be used to identify patients who are receiving either inappropriate or less-than-ideal therapy. Application of these sources of information with effective intervention strategies may prove to be one of the most important quality improvement foci in medical microbiology.

III. EXTERNAL QUALITY ASSURANCE PROGRAMS

Q-Probes is a national interlaboratory QA program developed by the CAP for the assessment of QA practice (2). A series of quality assessment studies containing data collection methods, worksheets, and input forms are distributed throughout the year to >1,000 subscribers. Participants conduct each study for a specified time and return the results for comparative analysis. A separate data analysis is computed for each institution and is accompanied by a critique of the study's general findings and recommendations for implementing additional quality improvement procedures. The data generated by Q-Probes are particularly useful for comparing an institution's performance with that of others, a process referred to as benchmarking. For example, a survey of over 400 clinical laboratories conducted as part of the CAP Q-Probes QA program found that the median turnaround time for processing, examining, and reporting CSF Gram smears was 45 min (16). The target turnaround time that laboratories established for this procedure was <60 min for 45% of specimens, 60 min for 47%, and >60 min for 8%. The Q-Probes survey demonstrated that these goals were met, on average, 62% of the time; 15.3% of participants met the goal 100% of the time. Examples for other Q-Probes studies relevant to microbiology practice are shown in Table 14.1–6.

Table 14.1–6 QA issues examined by CAP interlaboratory Q-Probes program with application to microbiology in years 1989 to 1991

Blood culture utilization
Nosocomial infections
Cumulative antimicrobial susceptibility test results
Antimicrobial agent usage
Turnaround time
Error reporting
Sputum quality

IV. INFECTION CONTROL AND CUMULATIVE ANTIMICROBIAL SUSCEPTIBILITY REPORTS

Properly organized laboratory information can be used for identifying clusters of infections that might be caused by a breakdown in technique or by cross-infections (14, 23). Information systems, such as the Sunquest FlexiLab System, that compare current data with earlier records provide an ever more powerful method for detecting epidemiologically significant trends (Fig. 14.1–4) (24, 31). Perhaps less well recognized is the ease of estimating total or specific nosocomial-infection rates by simply calculating the ratio of positive cultures from patients who have been hospitalized for several days to the total number of hospital admissions or discharges (11, 29). It has become increasingly common and useful to express rates of infection as the number of infections per 1,000 patient days. This ratio permits intra- and extrainstitutional comparisons in any chosen period. Such rates are very accurate reflections of the real situation even when the numbers of patients involved differ. It also proves useful for purposes of providing in-service education to attending physicians, house officers, and nursing personnel. The laboratory should also track cumulative antimicrobial susceptibility patterns and make this information available to the medical staff, as well as to ancillary medical personnel on a need to know basis (25). Such information aids in the empirical selection of antimicrobial agents, formulary development by the pharmacy and therapeutics committee of the medical staff, and trend analysis of endemic resistance by infection control practitioners (Fig. 14.1–5: *see* p. 14.1.11 and 14.1.12 for an example of a cumulative susceptibility report published for the medical staff of Magnanimous Medical Center).

REFERENCES

1. **Albright, R. E., R. H. Christenson, R. L. Habig, T. P. Mears, and K. A. Schneider.** 1988. Cerebrospinal fluid (CSF) TRAP. A method to improve CSF laboratory efficiency. *Am. J. Clin. Pathol.* **90:**707–710.
2. **Bachner, P., and P. J. Howanitz.** 1991. Q-Probes: a tool for enhancing your lab's QA. *Med. Lab. Obs.* **23:**37–38, 40, 42.
3. **Bartlett, R. C.** 1981. How far to go—how fast to go, p. 12–44. *In* V. Lorian (ed.), *Significance of Medical Microbiology in the Care of Patients,* 2nd ed. Williams & Wilkins Co., Baltimore, MD.
4. **Batalden, P. B., and E. D. Buchanan.** 1989. Industrial models of quality improvement, p. 133–159. *In* N. Goldfield and D. B. Nash (ed.), *Providing Quality Care.* American College of Physicians, Philadelphia, PA.
5. **Berwick, D. M.** 1988. Measuring health care quality. *Pediatr. Rev.* **10:**11–16.
6. **Berwick, D. M.** 1989. Continuous improvement as an ideal in health care. *N. Engl. J. Med.* **320:**53–56.

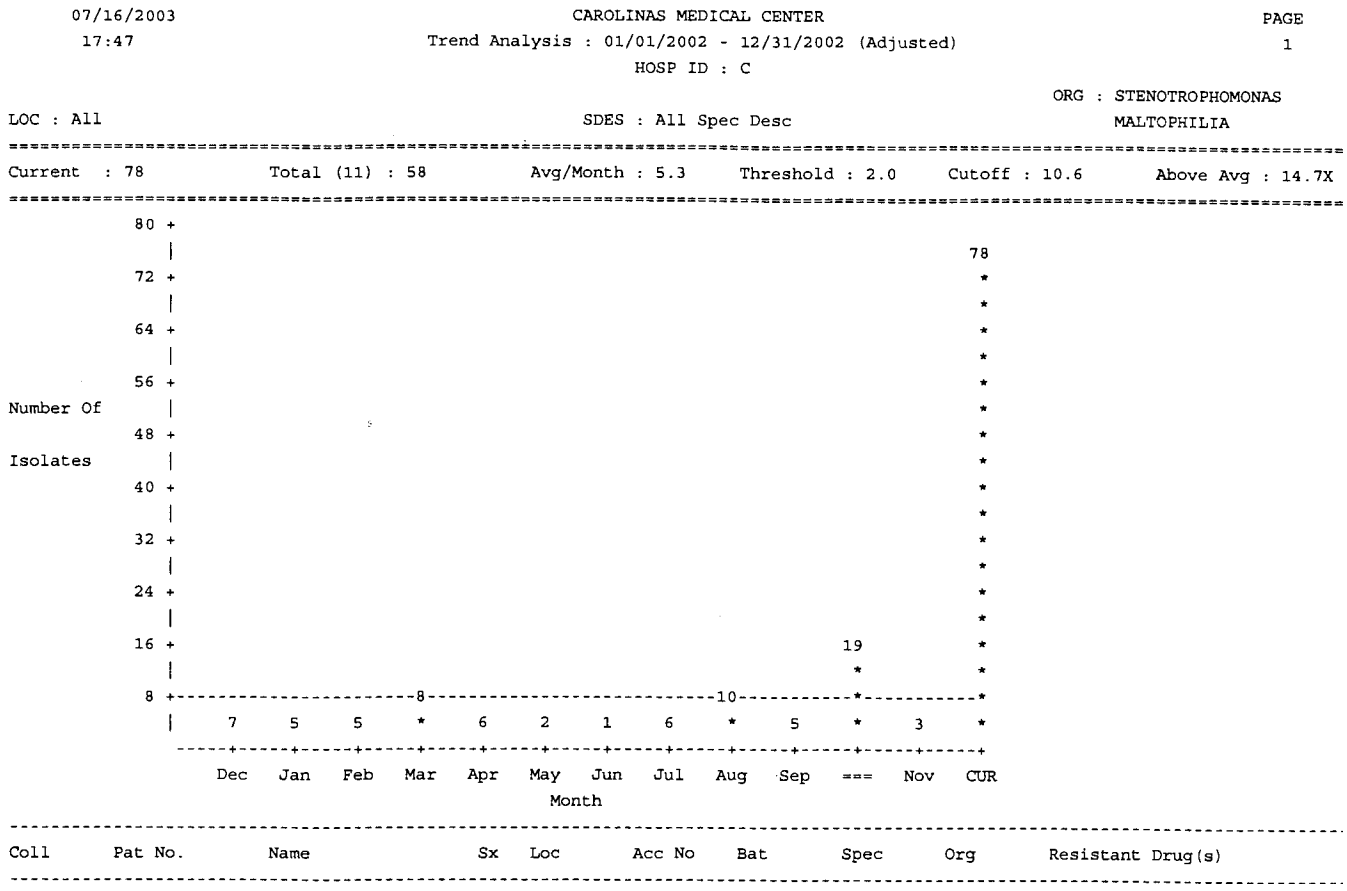

Figure 14.1–4 Computerized trend analysis of culture results for the identification of clusters that may have epidemiologic significance (courtesy of Misys Healthcare [Sunquest Information Systems], Tucson, AZ). Analysis of positive culture results may be employed to identify clusters of related infections that might signify an infection control problem. This requires analysis of positive-culture rates over an extended period. In this example from a commercial software program, a potential cluster is flagged as a positive-culture rate for a specific species, *Stenotrophomonas maltophilia*, recovered in respiratory cultures at more than twice the running monthly baseline. Although patient identifier information has been deleted from this example for confidentiality purposes, the report includes the following information on each patient from whom this organism was recovered in culture: collection date (in chronological order), history number, name, sex, location, sample accession number, specimen type, and antimicrobial agents to which the isolate was resistant. This information can be used to detect potential outbreaks at a very early stage.

REFERENCES *(continued)*

7. **Berwick, D. M., A. B. Godfrey, and J. Roessner.** 1991. *Curing Health Care. New Strategies for Quality Improvement. A Report on the National Demonstration Project on Quality Improvement in Health Care.* Jossey-Bass, San Francisco, CA.
8. **Braun, E., A. Louis-Charles, and C. Strand.** 1988. Evaluation of clinical microbiologic culture practices and sputum mycobacterial cultures, abstr. C191, p. 363. *In Abstracts of the Annual Meeting of the American Society for Microbiology 1988.*
9. **Brook, R. H.** 1989. Practice guidelines and practicing medicine. Are they compatible? *JAMA* **262:**3027–3030.
10. **Cervin, J. R., R. B. Schifman, R. P. Spark, and M. K. Micksin.** 1991. A strategy for improving utilization of the hepatitis A virus IgM antibody test. *Am. J. Clin. Pathol.* **96:**419.
11. **Costel, E. E., S. Mitchell, and A. B. Kaiser.** 1985. Abbreviated surveillance of nosocomial urinary tract infections: a new approach. *Infect. Control* **6:**11–13.
12. **Crowson, T. W., E. C. Rich, B. F. Woolfrey, and D. P. Connelly.** 1984. Overutilization of cultures of CSF for mycobacteria. *JAMA* **251:**70–72.

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[*] Use only for treatment of urinary tract infections. [**] Minocycline [a] Use 3 g q4h to 4 g q6h piperacillin or 3.375 g q4h or 4.5 g q6h Zosyn for treatment of *Pseudomonas aeruginosa* infections. [b] Use only when organisms are resistant to narrower-spectrum agents.	Aminoglycosides			Cephalosporins						Penicillins				Miscellaneous							
	Gentamicin	Tobramycin	Amikacin[b]	Cefazolin	Cefuroxime	Ceftriaxone	Cefotaxime (Neonates)	Ceftazidime (Neonates)	Cefepime[b]	Ampicillin	Ampicillin-sulbactam[b]	Piperacillin	Piperacillin-tazobactam[b]	Aztreonam	Clindamycin	Imipenem[b]	Levofloxacin	Metronidazole	Nitrofurantoin	Tetracyclines	SMX-TMP
Gram-negative species																					
Escherichia coli (*n* = 590)	97	98	100	93	97	99	99	99	99	62	91	67	98	98		100	96		99[*]	81	85 97
Klebsiella pneumoniae (*n* = 304)	93	93	100	84	79	93	93	92	93	0	89	77	93	92		100	91		59[*]	79	88 93
Klebsiella oxytoca (*n* = 51)	98	98	100	95	90	95	95	98	98	0	92	86	98	96		100	98		95[*]	100	9098
Proteus mirabilis (*n* = 76)	95	97	100	97	99	100	100	100	100	89	100	97	100	99		99	84		0	0	9295
Enterobacter aerogenes (*n* = 73)	95	96	100	0	67	86	86	81	100	0	16	75	93	88		100	95		32[*]	100	9595
Enterobacter cloacae (*n* = 155)	88	91	100	0	31	77	77	79	99	0	8	75	90	82		99	88		43[*]	82	8688
Citrobacter koseri (*n* = 28)	100	100	100	0	98	96	96	100	100	0	96	96	100	96		100	100		100[*]	100	10010
Citrobacter freundii (*n* = 25)	88	92	100	0	76	94	94	76	100	0	40	88	100	96		100	96		91[*]	100	9688
Serratia marcescens (*n* = 114)	99	97	100	0	6	94	94	96	99	0	13	95	99	96		98	99		0	18	10099
Acinetobacter baumannii (*n* = 73)	65	88	93					72	65		78	64		0		100	72			100[**]	6865
Pseudomonas aeruginosa (*n* = 496)	85	94	96					92	85			97	—[a]	70		87	6685		94	96	
Stenotrophomonas maltophilia (*n* = 62)	60	37	58					58	18			45				0	79			100[**]	9860
Anaerobes																					
Bacteroides fragilis (*n* = 31)											94				77			100			

These recommendations should be used as empirical guidelines only until specific antimicrobial susceptibility testing results are available. If additional or more specific information is required, contact the microbiology lab at XXX-XXX-XXXX or the microbiology director at XXX-XXX-XXXX.

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	Aminoglycosides		Cephalosporins				Penicillins				Miscellaneous											
	Gentamicin	Streptomycin	Cefazolin	Cefuroxime	Ceftriaxone	Cefotaxime (Neonates)	Ampicillin	Penicillin	Ampicillin-sulbactam	Nafcillin	Chloramphenicol	Clindamycin	Erythromycin (macrolides)	Imipenem	Levofloxacin	Linezolid	Minocycline	Nitrofurantoin	Rifampin	SMX-TMP	Synercid	Vancomycin
Gram-positive species																						
Staphylococcus aureus (MSSA) (*n* = 550)	98[c]		100	100	100	100	14	14	100	100		73	71	100	93	100	95	100[*]	99[**]	99		100
Staphylococcus aureus (MRSA) (*n* = 669)	72[c]		0	0	0	0	0	0	0	0		6	4	0	8	99	97	100[*]	92[**]	94		100
Coagulase-negative staphylococci (*n* = 282)	66[c]		23	23	23	23	6	6	23	23		64	30	23	41	97	81	100[*]	93[**]	65		100
Enterococcus faecalis (*n* = 77)	82[c]	75[c]	0	0	0	0	99		99						70[*]	100	40	96[*]			0	100
Enterococcus faecium (*n* = 42)	48[c]	57[c]	0	0	0	0					100				3	100	64	69[*]			88	194
Streptococcus anginosus (*milleri*) group (*n* = 44)	—[c]	—[c]				95		95				95	72		100							—[c]
Other viridans group streptococci (*n* = 19)	—[c]	—[c]			100	100		53				89	58		100							100
Streptococcus pneumoniae (nonmeningitis) (*n* = 239)					88	88		56				92	69		97		—[***]					100
Streptococcus pneumoniae (meningitis)					72	72		56									—[***]					100
Streptococcus pyogenes (*n* = 20)								100				100	85		100							100
Streptococcus agalactiae (*n* = 28)					100	100		100				88	72		100	100						100

[*] Drug should only be used for treatment of urinary tract infections.

[**] Do not use rifampin alone therapeutically.

[c] Use for synergistic purposes only.

[***] Nationwide, 22% of pneumococci are resistant to the tetracyclines.

- Clavulanic acid, ceftazidime, cefoxitin, and imipenem are all strong inducers of class C β-lactamases in many nosocomial gram-negative organisms.
- Antibiotics demonstrating in vitro activity against <70% of all isolates of a given bacterial species are shaded in the table to underscore less than optimal activity.
- 22% of pneumoccocci at Magnanimous Medical Center now display high-level resistance to penicillin (MIC ≥ 2.0 µg/ml), and 31% are macrolide resistant.
- In serious or deep-seated infections (such as pneumonia, meningitis, osteomyelitis, or sepsis) with *Enterobacter* spp., *Stenotrophomonas maltophilia, Serratia* spp., *Pseudomonas aeruginosa, Citrobacter* spp., *Morganella morganii, Providencia* spp., *Proteus vulgaris,* and *Acinetobacter baumannii,* treatment with two antibiotics (e.g., a β-lactam plus an aminoglycoside or a fluoroquinolone) should be considered to increase clinical efficacy and decrease the chances for the development of resistance.
- Deep-seated enterococcal infections (e.g., endocarditis) should be treated with ampicillin or vancomycin plus low-dose (e.g., peak 3 to 5 µg/ml) gentamicin or streptomycin.
- 55% of all *Staphylococcus aureus* isolates recovered from inpatients are now resistant to oxacillin and nafcillin (MRSA).

These recommendations should be used as empirical guidelines only until specific antimicrobial susceptibility testing results are available. If additional or more specific information is required, contact the microbiology lab at extension X-XXXX.

REFERENCES *(continued)*

13. **El-Gammal, A., V. Scotto, S. Malik, K. C. Casey, R. Cody, D. V. Alcid, and M. P. Weinstein.** 2000. Evaluation of the clinical usefulness of *C. difficile* testing in hospitalized patients with diarrhea. *Diagn. Microbiol. Infect. Dis.* **36:**169–173.
14. **Evans, R. S., R. A. Larsen, J. P. Burke, R. M. Gardner, F. A. Meier, J. A. Jacobson, M. T. Conti, J. T. Jacobson, and R. T. Hulse.** 1986. Computer surveillance of hospital-acquired infections and antibiotic use. *JAMA* **256:**1007–1011.
15. **Hilborne, L. H., R. K. Oye, J. E. McArdle, J. A. Repinski, and D. O. Rodgerson.** 1989. Use of specimen turnaround time as a component of laboratory quality. A comparison of clinical expectations with laboratory performance. *Am. J. Clin. Pathol.* **92:**613–618.
16. **Howanitz, P. J., and S. Steindel.** 1991. Intralaboratory performance and laboratorians' expectations for Stat turnaround times. *Arch. Pathol. Lab. Med.* **115:**977–983.
17. **Howanitz, P. J., K. Walker, and P. Bachner.** 1991. Detection of errors in clinical laboratory reports. *Am. J. Clin. Pathol.* **96:**431.
18. **Hyams, K. C.** 1987. Inappropriate urine cultures in hospitalized patients receiving antibiotic therapy. *Arch. Intern. Med.* **147:**48–49.
19. **Joint Commission on Accreditation of Healthcare Organizations.** 1992. Quality assessment and improvement, p. 137–143. *In Accreditation Manual for Hospitals, 1992.* Joint Commission on Accreditation of Healthcare Organizations, Chicago, IL.
20. **Kassirer, J. P.** 1989. Our stubborn quest for diagnostic certainty. A cause of excessive testing. *N. Engl. J. Med.* **320:**1489–1491.
21. **Kritchevsky, S. B., and B. P. Simmons.** 1991. Continuous quality improvement. Concepts and applications for patient care. *JAMA* **266:**1817–1823.
22. **Laffel, G., and D. Blumenthal.** 1989. The case for using industrial quality management science in health care organizations. *JAMA* **262:**2869–2873.
23. **Laxson, L. B., M. J. Blaser, and S. M. Parkhurst.** 1984. Surveillance for the detection of nosocomial infections and the potential for nosocomial outbreaks. *Am. J. Infect. Control* **12:**318–324.
24. **McGuckin, M. B., and E. Abrutyn.** 1979. A surveillance method for early detection of nosocomial outbreaks. *Am. J. Infect. Control* **7:**18–21.
25. **NCCLS.** 2002. *Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data.* Approved Guideline M39-A. NCCLS, Wayne, PA.
26. **Pedler, S. J., and A. J. Bint.** 1991. Survey of users' attitudes to their local microbiology laboratory. *J. Clin. Pathol.* **44:**6–9.
27. **Pestotnik, S. L., R. S. Evans, J. P. Burke, R. M. Gardner, and D. C. Classen.** 1990. Therapeutic antibiotic monitoring: surveillance using a computerized expert system. *Am. J. Med.* **88:**43–48.
28. **Pfaller, M. A.** 1992. The microbiology laboratory, p. 493–507. *In* R. P. Wenzel (ed.), *Assessing Quality Health Care.* Williams & Wilkins Co., Baltimore, MD.
29. **Ryan, K. J.** 1984. The computer in microbiology: future application in test performance and reporting. *Ann. N. Y. Acad. Sci.* **428:**243–250.
30. **Schifman, R. B.** 1990. Quality assurance goals in clinical pathology. *Arch. Pathol. Lab. Med.* **114:**1140–1144.
31. **Schifman, R. B., and R. A. Palmer.** 1985. Surveillance of nosocomial infections by computer analysis of positive culture rates. *J. Clin. Microbiol.* **21:**493–495.
32. **Schifman, R. B., C. Strand, E. Braun, A. Louis-Charles, R. P. Spark, and M. L. Fried.** 1991. Solitary blood cultures as a quality assurance indicator. *Qual. Assur. Util. Rev.* **4:**132–137.
33. **Siegel, D. L., P. H. Edelstein, and I. Nachamikin.** 1990. Inappropriate testing for diarrheal diseases in the hospital. *JAMA* **263:**979–982.
34. **Soo Hoo, G. W., D. L. Palmer, and R. L. Sopher.** 1984. Reducing tuberculosis detection costs. *Chest* **86:**860–862.
35. **Trenholme, G. M., R. L. Kaplan, P. H. Karakusis, T. Stine, J. Fuhrer, W. Landau, and S. Levin.** 1989. Clinical impact of rapid identification and susceptibility testing of bacterial blood culture isolates. *J. Clin. Microbiol.* **27:**1342–1345.
36. **VonSeggern, R. L.** 1987. Culture and antibiotic monitoring service in a community hospital. *Am. J. Hosp. Pharm.* **44:**1358–1362.
37. **Wachtel, T. J., and P. O'Sullivan.** 1990. Practice guidelines to reduce testing in the hospital. *J. Gen. Intern. Med.* **5:**335–341.
38. **Walton, M.** 1986. *The Deming Management Method.* Dodd, Mead & Co., New York, NY.
39. **Wenzel, R. P.** 1990. Quality assessment. An emerging component of hospital epidemiology. *Diagn. Microbiol. Infect. Dis.* **13:**197–204.
40. **Westgard, J. O., and P. L. Barry.** 1989. Beyond quality assurance: committing to quality improvement. *Lab. Med.* **20:**241–247.
41. **Yannelli, B., I. Gurevich, P. E. Schoch, and B. A. Cunha.** 1988. Yield of stool cultures, ova and parasite tests, and *Clostridium difficile* determinations in nosocomial diarrheas. *Am. J. Infect. Control* **16:**246–249.

APPENDIX 14.1–1

Example of Quality Assessment and Improvement Data Summary for Urine Specimen Examinations

Table 14.1–A1 Procedures for quality assessment of urine specimen examinations

Objective	Indicator	Standard	Threshold (%)
Improve test interpretation	Concomitant urinalysis	Number and % with concomitant urinalysis	>90
Improve test utilization	Duplicate cultures	No more than 1 culture/patient/day	100
Improve patient treatment (inpatients)	Positive culture with no treatment	Clinical pharmacy review of case and recommendations, when appropriate	100

Table 14.1–A2 Summary of data for urine specimen examinations

Mo	No. of urine cultures		
	Total (% with UA[a])	Duplicate (% duplicates)	Significant (% reviewed)
January	134 (92)	40 (10)	36 (91)
February	120 (84)	34 (12)	30 (100)
March	166 (98)	51 (10)	46 (100)
April	111 (94)	28 (0)	26 (100)
May	133 (95)	30 (3)	26 (100)
June	Discontinued	40 (0)	38 (100)
July			
Cumulative (2002)	1,521 (94)	321 (13)	279 (92)

[a]UA, urinalysis.

Review and action summary

January: Pharmacist was not available to review weekend laboratory reports. Three urinary tract infections (untreated for >24 h) on the urology service were identified. Chief of service was contacted, and physicians on this service were reminded about reviewing culture and susceptibility reports on computer terminal.

February: Ward unit clerks reminded to check with physician whenever urine culture ordered without urinalysis (UA). Reviewed duplicate urine cultures in January and February. Cause was determined to be combination of doctors' orders with addition of standing admission orders for urine cultures for all urology patients having a TURP (transurethral prostatic resection; seven of eight cases). This problem will be discussed with urology section for suggestions on how to avoid duplication.

March: Improvement in UAs noted. Assistant supervisor suggested automatic UA for all urine culture requests. Will discuss with laboratory director. Urology decided to remove standing urine culture orders from admission orders.

April: Data reviewed; no action. Laboratory director reports having support from medical staff about policy for automatic UA testing and will present idea to Clinical Executive Board. Duplicate urine cultures reduced after standing admission orders revised.

May: Data reviewed; no action. Medical Staff Executive Board approved automatic UA for all urine culture requests. Will implement in June and discontinue checking for concomitant UA. Staff asked for ideas on how to monitor urine cultures for appropriate indications.

June: Data reviewed. Concomitant UA monitor discontinued. Laboratory staff presented ideas for appropriate indication monitor and suggested review of cases with pyuria (by UA) but no pertinent microbiologic examinations (e.g., urine culture, *Chlamydia trachomatis* screen, etc.). This idea was presented to laboratory director.

APPENDIX 14.1–2

Outline for Quality Assessment and Improvement Procedure

I. OBJECTIVE
To describe the purpose of the quality improvement procedure.

II. PRINCIPLE
Describe the rationale for performing the quality improvement procedure, including how the procedure and data to be collected will meet the objective.

III. INDICATOR(S)
Describe the specific elements of data and source(s) of information.

IV. DATA COLLECTION
Describe in step-by-step fashion how the data will be collected and recorded. Refer to worksheets, computer databases, or other pertinent sources. Describe who is responsible for each aspect of data collection and the amount of data (by period of time, proportion of larger pool of data, etc.) to be obtained. If the plan is complex, an organizational flowchart may prove useful.

V. DATA ANALYSIS
As specifically as possible, explain how the data are to be analyzed and interpreted. This may include the establishment of thresholds for evoking action or examination of trends. In some instances, data are collected to define the source of a problem. In such a situation, describe how the data will be used to identify potential flaws. Describe any special calculations or data presentations in this section. A statement such as "data will be reviewed monthly" is not sufficient.

VI. ACTION
Precisely describe the actions that will be taken when analysis of the data suggests a problem. Describe arrangements for interdepartmental interactions and communications. Define any ongoing procedures and their documentation as they relate to preventing defects (e.g., periodic educational activities, solicited feedback, review of committee reports, incident reports, etc.).

VII. IMPROVEMENT
Describe how improvement will be defined and measured. Improvement may take the form of a trend or a change in a process. In some situations, improvement may be demonstrated by showing that a process will probably deteriorate without implementation of the plan.

VIII. DOCUMENTATION
Describe how the effectiveness of the quality improvement plan will be recorded and communicated. Documentation usually takes the form of periodic written summary reports to the supervisor, laboratory director, and/or appropriate laboratory or institutional committees or departments.

APPENDIX 14.1–3

Quality Assessment and Improvement of Collections of Expectorated Sputa

I. OBJECTIVE
To collect expectorated sputum specimens in a manner that will provide the best opportunity for generation of an accurate diagnostic result.

II. PRINCIPLE
The quality (probable diagnostic value) of expectorated sputum specimens can be measured by cytologic examination. Specimens with large numbers of squamous epithelial cells (SECs) are presumed to contain a large complement of oropharyngeal microbiota that can obscure or misrepresent microbiologic findings associated with lower respiratory tract infections. The quality of sputum specimens largely depends upon the conscientiousness displayed in the instruction of patients in how to collect samples. This task generally falls upon the nursing staff. It is useful to monitor the quality of sputum specimens by specific nursing unit in order to define areas that show proportionately large numbers of sputum samples with oropharyngeal contamination. Such a finding would lead to the conclusion that more-intensive educational activities, changes in collection procedures, or other corrective measures are warranted. Likewise, continued monitoring will be useful in demonstrating the success or failure of attempts to maintain good-quality sputum collections.

APPENDIX 14.1–3 *(continued)*

III. INDICATOR

The quantity of SECs observed per low-power field (LPF) in specimens collected from patients for whom microscopic examination of smears prepared from expectorated sputum are performed is used as a measure of the quality of sputum collections.

IV. PROCEDURE

A. After each sputum Gram smear is examined, record the results on the Sputum Gram Smear Worksheet (*see* Appendix 14.1–6, p. 14.1.22) by placing a mark in the section corresponding to the hospital unit and number ($\geq$25 or $<$25) of SECs per LPF.

B. At the end of each month, the supervisor will tally the results as described below and record them in the yearly Sputum Quality Data Summary report (*see* Appendix 14.1–6, p. 14.1.23).

V. DATA ANALYSIS

A. Calculations

Count the number of specimens with $\geq$25 or $<$25 SECs per LPF for each unit and as a grand total. Calculate the percentage of specimens for each unit with $\geq$25 SECs per LPF as follows: (number of specimens with $<$25 SECs/LPF) $\times$ 100/[(number of specimens with $\geq$25 SECS/LPF) + (number of specimens with $<$25 SECS/LPF)].

B. The threshold for action is any consecutive 2 months in which the proportion of sputum samples having $\geq$25 SECs per LPF exceeds 25% on any unit or any single month in which the percentage is $>$50%.

VI. ACTION

A. Perform targeted educational activities at each location for which thresholds for evaluation reveal excessive rates of unacceptable sputum specimen quality.

B. Provide cumulative data summaries to nursing service monthly.

C. Conduct global educational activities yearly by written communication to all nursing staff. Include summarized feedback of the surveillance data. Emphasize the following points.

1. Instruct patients to produce a specimen only from a deep cough.
2. Instruct patients to avoid, as much as possible, mixing the specimen with saliva or nasal secretions.
3. Ensure that patients understand the difference between spit (from the mouth) and sputum (from the chest).
4. Gargling with tap water immediately prior to specimen collection may improve the quality of the specimen.
5. The likelihood of obtaining a good quality specimen is reduced if the nurse simply hands a container to the patient with instructions to cough into it and then leaves the room.

D. Once each year, ask the nursing staff and the pulmonary section for suggestions as to how best to improve specimen collection.

VII. IMPROVEMENT

Improvement is demonstrated by documenting a decline in SEC counts after intervention by targeted educational activities, changes in procedures, or recommendations from nursing staff or other departments. Sustained improvement will be shown by maintenance of a low proportion of specimens with $\geq$25 SECs/LPF.

VIII. DOCUMENTATION

Discuss summarized reports, trend analysis, modification of criteria, problem identification, and problem resolution at the laboratory's monthly QA meeting. Report results to the nursing service QA committee.

Supplemental Reading

Bartlett, R. C. 1985. Cost containment in microbiology. *Clin. Lab Med.* **5:**761–792.

APPENDIX 14.1–4

Quality Assessment and Improvement in Result Reporting

I. OBJECTIVE

To monitor the quantity, causes, and clinical significance of clerical reporting errors and to reduce the adverse impact of reporting errors by (i) swift intervention when serious errors are recognized and (ii) understanding the reasons for reporting errors so that procedural adjustments can be implemented to prevent recurrences.

II. PRINCIPLE

One of the more important factors in communication of microbiology results to clinical personnel is the accuracy of the information. Reporting errors may result in adverse clinical consequences. It is therefore essential to actively seek out mistakes and investigate their roots. The resulting knowledge may identify reporting procedures that could be improved to prevent the recurrence of similar mistakes. Continued monitoring for errors will provide information to determine if corrective actions are successful in reducing the frequency and clinical severity of errors and if the microbiology section is able to maintain a very low incidence of errors. Sample forms are given in Appendix 14.1–6, p. 14.1.24, 14.1.25, and 14.1.26.

III. INDICATOR

A. Error: a verified and reported result that is made available for patient care and later discovered to contain erroneous information.

B. Sources of data

1. Random review: error is detected by correlation of final report with worksheet or instrument log.
2. Laboratory detection: error is detected by microbiologist or other laboratorian outside of the random-review process.
3. External detection: error is reported by physician, nurse, or other health care provider external to the laboratory.

IV. DATA COLLECTION PROCEDURES

A. Random review

1. Select five reports from all the final reports issued on the day of review. Reports with positive findings are preferred. Microbiologists should not review reports of cultures upon which they worked.
2. Compare the results on the worksheet and requisition form to the final results displayed.
3. Check for accuracy of the following.
   a. Requisition form
      Patient identification and date and time of receipt on the requisition match information on the final report. Routine and special processing requests detailed on the requisition have been accomplished or addressed appropriately.
   b. Results worksheet
      All smear, culture, and antimicrobial susceptibility testing results documented on the worksheet are accurately described on the final patient report.
   c. Technical error
      No mistakes have been made while processing specimens, performing procedures, or interpreting results. Errors detected by QC procedures have been investigated and corrected.
4. Total the number of reports reviewed with and without detectable errors.
5. On the Reported Error Description Log, describe in detail all detected errors and immediately provide this information to the microbiology supervisor.

B. Laboratory detection

Errors may be specifically identified within the laboratory when a QC problem that causes a test to be repeated is uncovered or when a data transmission problem is identified subsequent to issuance of a final report. In the error-reporting log, describe the type of errors that appeared in final reports, causing those reports to require correction. Notify the microbiology supervisor of such errors immediately.

C. External detection

In the error-reporting log, document errors reported in incident reports, oral communications, or others means by physicians, nurses, or others external to the laboratory. Report these to the microbiology supervisor immediately.

APPENDIX 14.1–4 *(continued)*

D. Classification of errors
   1. Errors are divided into three categories based on their impact on patient care.
      a. Category A error
         The primary provider has responded to the result by ordering another test, repeating the test, modifying treatment, or changing the diagnosis.
      b. Category B error
         A category B error is serious but unlikely to affect patient care. It is similar to a category A error, but the primary care provider has not yet been notified or has not acted upon the reported erroneous result.
      c. Category C error
         Category C errors are minor clerical errors resulting in "cosmetic" corrections to the report.
   2. The microbiology supervisor is responsible for determining the category of error (A, B, or C). Category A and B errors may necessitate chart review or discussions with nurses or physicians. Final classification as category A or B is made after review and verification by the laboratory director.

E. Calculations
   Every month, calculate the frequency of errors detected by random review as the total number of errors divided by the total number of cases reviewed.

V. DATA ANALYSIS THRESHOLD FOR EVALUATION
   A. All category A and B errors are sentinel events that must be brought to the attention of the laboratory director as soon as possible for evaluation, verification, and further action as necessary.
   B. Category C errors require no immediate action unless the random-review monthly rate exceeds 2%.

VI. ACTION
   A. Action on category A or B errors requiring correction should address the cause and severity of the problem. Contact the primary care physician directly by phone or in a written communication for all category A errors; such contact may also be necessary for some category B errors.
   B. Appropriate written documentation, including a review of findings after investigation of the problem and a discussion of circumstances related to the cause and effect of the error, should accompany documentation of each category A and B error. Develop a plan for corrective actions to prevent or reduce the recurrence of errors that are critical or repetitive in nature.
   C. Category C errors that exceed the threshold for action should prompt a review of the section's clerical and analytical procedures and the development of a plan to improve performance. Focus attention on the most common types of errors and the circumstances under which they occur. Solicit suggestions for improvement from those who are involved with reporting of the test results.

VII. IMPROVEMENT
   Improvement is measured by the extent to which the number of errors can be reduced and their recurrence limited. On a monthly basis, discuss prevention strategies for each category A error during the section meeting, and forward recommendations to the laboratory director for review and consideration.

VIII. DOCUMENTATION
   In section meeting minutes, document error-reporting data and identified sources of error that have been reviewed with the technologists and clerical support staff. Document all procedural changes that may have an impact on error reduction or prevention. Incorporate summarized error-reporting data, significant findings, plans of action, and resolution of problems into the laboratory service minutes.

APPENDIX 14.1–5

Quality Assessment and Improvement of CSF Gram Stain Turnaround Time

I. OBJECTIVE

To maintain the shortest possible interval between the time a CSF specimen is collected and the time the Gram stain results are utilized for patient management.

II. PRINCIPLE

Bacterial meningitis is a medical emergency requiring prompt diagnosis and treatment. A critical test for the initial stages of patient management is the Gram-stained smear examination of CSF. All components of the testing process, from specimen collection to final interpretation and use of CSF smear results, should be accomplished in a manner that minimizes delays. The times taken to complete various parts of this testing process are monitored to determine whether turnaround time goals are being met and to identify factors in the process that might further shorten total testing times.

III. INDICATORS

The intervals between all of the following components are determined.

A. Time of specimen collection (source, requisition slip)
B. Time of specimen arrival in the laboratory for accessioning (source, requisition slip or computer entry)
C. Time of specimen arrival in microbiology section (source, laboratory worksheet or computer entry)
D. Time of verbal report of results (source, laboratory worksheet or computer entry)
E. Time when pharmacy receives antimicrobial therapy order (source, pharmacy department)

IV. DATA COLLECTION

A. The time for each of the first four indicators is recorded on the worksheet after the CSF Gram stain smear result has been verbally reported to a medical caretaker.
B. If the Gram stain smear result shows evidence of bacterial or fungal infection (presence of microorganisms), call the pharmacy within 1 h of reporting results to determine whether orders for antimicrobial therapy have been received. If applicable, record the time the orders were received.

V. DATA ANALYSIS

A. Threshold for evaluation

Every month, the median values for each turnaround time indicator, the total turnaround time, and the percentage of occurrences in which the times exceeded goals are calculated on the CSF Gram Stain Turnaround Time Log Sheet (*see* Appendix 14.1–6, p. 14.1.27). Reviews of procedures are initiated whenever >10% of goals for any indicator are not met or when >10% of turnaround time information is missing. Any case with a turnaround time of twice the goal for any indicator should be flagged for review by the supervisor.

B. Turnaround time goals

1. 30 min

Transport time (time between specimen collection and specimen arrival in laboratory)

2. 15 min

Accession time (time between specimen arrival in laboratory and specimen arrival in microbiology section)

3. 45 min

Processing and reporting time (time between specimen arrival in microbiology section and smear preparation, examination, and result reporting)

4. 60 min

Test interpretation and response to results (if positive) (time between report of results and receipt of antimicrobial orders by pharmacy)

C. Antimicrobial therapy

If information regarding antimicrobial treatment was obtained, check that appropriate therapy (ceftriaxone or cefotaxime, ampicillin, or a combination of the cephalosporin and ampicillin for gram-negative organisms or gram-positive rods; high-dose penicillin or cefotaxime or ceftriaxone, with or without vancomycin, for gram-positive cocci) was initiated. Refer any questions about appropriate therapy to the supervisor or laboratory director. See below for instructions on how to proceed

APPENDIX 14.1–5 *(continued)*

when potentially inappropriate therapy is prescribed or when treatment is not initiated within 1 h of reporting of the Gram stain results.

VI. ACTION

A. Failure to meet turnaround time goals should prompt a review of applicable protocols and procedures in an effort to discover potential problems with the system that may result in unnecessary delays. If no problems are uncovered, consult with appropriate medical staff through the laboratory director, and consider revising goals. Alternatively, examine new procedures or other approaches for improving turnaround time.

B. Failure to initiate therapy within 1 h of reporting a CSF smear with microorganisms should immediately be brought to the attention of the laboratory director or on-call laboratory physician for case review and consultation with the patient's physician. Appropriate members of the medical staff and QA committee are responsible for investigating causes of delayed therapy and for making suggestions and modifying systems for improvement.

C. Review procedures and reinforce the importance of monitoring turnaround times with appropriate individuals when missing data are excessive.

D. Once per year, review stat CSF smear turnaround time goals to determine whether they might be reduced.

VII. IMPROVEMENT

Improvement is measured by the extent to which turnaround times can be reduced and goals met. Evaluate recommendations for improving turnaround times and the success or failure of procedural changes as they relate to reduction in turnaround time. Successful and timely therapy of patients with meningitis should be demonstrable by this procedure.

VIII. DOCUMENTATION

A. In monthly section meeting minutes, document turnaround times and the percentage of goals met. Document all changes in procedures or interactions with medical staff that have had an impact on CSF Gram smear turnaround times or turnaround time goals.

B. Include summarized data, significant findings, plans of action, and resolution of problems in the quarterly laboratory service minutes. For each therapeutic delay identified, the hospital's QA committee should document recommendations for improvements and procedural and/or policy modifications.

APPENDIX 14.1–6

Quality Assessment and Improvement of Antimicrobial Susceptibility Test Results

I. OBJECTIVE

To retrieve and analyze pharmacy and laboratory data automatically and to intervene appropriately when discrepancies are identified as a way of improving the treatment of patients with infections.

II. PRINCIPLE

An automated information system for monitoring antimicrobial agent usage is employed to link pharmacy and laboratory data. A report of these data is reviewed, and appropriate guidelines for detecting potential discrepancies between current therapy and antimicrobial susceptibility testing results are followed. In conjunction with the infectious-disease service, the laboratory identifies patients who may not be receiving appropriate therapy. Rapid transmission of these findings to the primary care physician improves the timeliness and appropriateness of antimicrobial treatment for patients with infections.

III. INDICATORS

A daily computer report contains a listing for each culture and associated antimicrobial susceptibility testing results, pertinent laboratory information, and current antimicrobial therapy. This report serves as the data source for indicators of appropriate and inappropriate therapy.

APPENDIX 14.1–6 *(continued)*

IV. DATA COLLECTION

A. After all final culture and antimicrobial susceptibility testing results are entered, generate a computer report listing the patient's antimicrobial therapy and microbiology results.

B. For each patient listed on the report, check results against the guidelines listed in item V below, and classify therapy as appropriate or inappropriate. Record the results of each analysis on the worksheet.

C. The supervisor is responsible for reviewing the worksheet and listing all cases with potentially inappropriate antimicrobial therapy for evaluation by an infectious-disease specialist.

D. Record the results of the infectious disease specialist's review, and return them to the microbiology supervisor.

E. Summarize the number of cases with appropriate therapy, the number of cases reviewed, and the outcomes of such reviews.

V. DATA ANALYSIS

A. Threshold for case review

1. Urine: >10,000 CFU/ ml or a single or predominant organism *with* documented pyuria (if urinalysis has been performed).
2. Blood or other usually sterile body fluid (CSF, joint, etc.): any positive result. Exclude cases with coagulase-negative staphylococci or diphtheroids, except for blood cultures when more than one culture is positive.
3. Sputum or bronchoalveolar lavage fluid: predominant organism with Gram stain and culture yielding a predominant morphotype and the Gram stain exhibiting many inflammatory cells with few or no epithelial cells present.
4. Wound, tissue, aspirate, or cutaneous specimen: *Staphylococcus aureus*-, β-hemolytic streptococci, or predominant organism with inflammatory cells seen on Gram smear.

B. Antimicrobial therapy

Patient either is not receiving antimicrobial therapy or is receiving an agent to which the organism is resistant.

VI. ACTION

A. It is typically the infectious-disease specialist's responsibility to review the cases and, if necessary, to contact the physician for recommendations regarding modifications in the patient's therapy. Record the results of all actions, and transmit them to the laboratory.

B. Twice per year and as needed, review changes in the guidelines and selection methods used for case review with the laboratory and medical staff.

VII. IMPROVEMENT

Since this is a concurrent review and intervention procedure, improvement is obtained through the procedure itself. Without this review process, delays in antimicrobial agent therapy or inappropriate therapy would occur.

VIII. DOCUMENTATION

In the monthly general laboratory minutes, record a summary of the case reviews. Document the number and proportion of patients receiving appropriate empirical therapy, as well as the results of intervention by infectious disease specialists. Document all changes in review procedures and review criteria. The infectious disease specialist should present recommendations for improvements in empirical therapy or formulary changes to the hospital's QA committee or the pharmacy and therapeutics committee of the medical staff, as appropriate.

Sample forms are shown on p. 14.1.28 and 14.1.29.

Sputum Gram Smear Worksheet

Month	Year

Unit	< 25 SEC/LPF																			Total
A																				
B																				
C																				
D																				
E																				
F																				
G																				
H																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Unit	> 25 SEC/LPF																			Total
A																				
B																				
C																				
D																				
E																				
F																				
G																				
H																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Grand total	Percent >25 SEC/LPF

Instructions:

For each sputum specimen, sequentially place a mark in the appropriate box corresponding to the nursing unit and quantity of SECs seen on Gram smear. At the end of the month, tally the results and calculate totals, grand totals, and % of specimens with >25 SEC/LPF for each unit and for all units.

Sputum Quality Data Summary

Year:

Unit		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total specimens	Total >25/LPF
A	Total specimens														
	Number >25/LPF														
	Percent >25/LPF														
B	Total specimens														
	Number >25/LPF														
	Percent >25/LPF														
C	Total specimens														
	Number >25/LPF														
	Percent >25/LPF														
D	Total specimens														
	Number >25/LPF														
	Percent >25/LPF														
E	Total specimens														
	Number >25/LPF														
	Percent >25/LPF														
F	Total specimens														
	Number >25/LPF														
	Percent >25/LPF														
G	Total specimens														
	Number >25/LPF														
	Percent >25/LPF														
H	Total specimens														
	Number >25/LPF														
	Percent >25/LPF														
													Grand total		

Yearly % >25/LPF %

Random Review for Errors Worksheet

Date:

		Clerical							Technical
		Requisition form			Results worksheet				
	Accession no.	Patient ID	Time received	Test(s) ordered	Gram smear	Culture results	Susceptibility results	Other	
1									
2									
3									
4									
5									
Total									

Error description(s):

Instructions:

Select 5 final reports and compare the results with information on the requisition form and the results worksheet. If no errors are detected, place a 0 in the appropriate box. Place a 1 in the appropriate box for each error identified and a 1 in the accession column for each case reviewed. Describe each error in detail, total all columns, and submit the completed worksheet to the supervisor promptly for review and corrective action.

Reported Error Description Log

Date	Accession no.	Detection			Error type		Classification		
		Random	Lab	External	Clerical	Technical	A	B	C

Error description and clinical impact:

Laboratory director's review and comment (A and B errors only):

Reported Error Summary

Review period:

	Error type	Clerical: Requisition form	Clerical: Results worksheet	Technical	Total errors: A	Total errors: B	Total errors: C	Percent of all randomly reviewed cases
Random review *n* =	A							- %
	B							- %
	C							- %
Lab detection	A							
	B							
	C							
External detection	A							
	B							
	C							
Total								Grand total
% of grand total		%	%	%	%	%	%	

Comments and summary of actions taken:

CSF Gram Stain Turnaround Time Log Sheet

Time:[a]	Acc. no. / Date	Acc. no. / Date	Acc. no. / Date	Acc. no. / Date	Acc. no. / Date	Acc. no. / Date	Acc. no. / Date	Acc. no. / Date	Acc. no. / Date	Acc. no. / Date	No data
Collected											
Lab arrival											
Micro arrival											
Results reported											
Patient treated[b]											

Circle all TATs that do not meet goal. Supervisor must immediately review any TAT that exceeds 2× goal.

TAT[c] calculation	Goal											Average
Transport: Lab arrival-collect	30											
Accession: Micro-lab arrival	15											
Processing: Report-arrival	45											
Treatment: Treatment-report	60											
Overall TAT: Report-collect	90											
Overall TAT: Treatment-collect[b]	120											

		Number evaluated	Goals met
Specific TAT	Transport:		
	Accession:		
	Processing:		
	Treatment:		
Overall TAT	Report-collect		
	Treatment-collect		

Review and comment:

[a]If time is not recorded, mark the box "ND" for no data.
[b]If microorganisms are seen on Gram smear, record the time that doctor ordered antimicrobial agent(s); otherwise, leave blank.
[c]TAT, turnaround time.

Culture and Antimicrobial Susceptibility/Therapy Review

Date:

Accession no.		Urine		Sputum		Blood	Other:	Therapy	Interpretation		
	Pyuria: Y N		Smear, culture, and susceptibility results						OK	Resistant	No Rx
Patient ID									Recommendation		
									None	ID rev	Sup rev

Accession no.		Urine		Sputum		Blood	Other:	Therapy	Interpretation		
	Pyuria: Y N		Smear, culture, and susceptibility results						OK	Resistant	No Rx
Patient ID									Recommendation		
									None	ID rev	Sup rev

Accession no.		Urine		Sputum		Blood	Other:	Therapy	Interpretation		
	Pyuria: Y N		Smear, culture, and susceptibility results						OK	Resistant	No Rx
Patient ID									Recommendation		
									None	ID rev	Sup rev

Accession no.		Urine		Sputum		Blood	Other:	Therapy	Interpretation		
	Pyuria: Y N		Smear, culture, and susceptibility results						OK	Resistant	No Rx
Patient ID									Recommendation		
									None	ID rev	Sup rev

Infectious Disease and Medical Microbiology Case Review

Date	Patient ID	Location	Specimen	Positive culture with:	
				No therapy	
				Resistant organism	
Notes:				Recommendation:	
				No action needed	
				Modify therapy	

Date	Patient ID	Location	Specimen	Positive culture with:	
				No therapy	
				Resistant organism	
Notes:				Recommendation	
				No action needed	
				Modify therapy	

Date	Patient ID	Location	Specimen	Positive culture with:	
				No therapy	
				Resistant organism	
Notes:				Recommendation	
				No action needed	
				Modify therapy	

Date	Patient ID	Location	Specimen	Positive culture with:	
				No therapy	
				Resistant organism	
Notes:				Recommendation	
				No action needed	
				Modify therapy	

Dr. _____

14.1.1 Process Improvement

I. PRINCIPLE

Using a scientific approach for process improvement requires using data to evaluate the current situation, analyze and improve processes, and track progress (FOCUS [Fig. 14.1.1–1]). In the context of the plan-do-check-act cycle (PDCA [Fig. 14.1.1–2]), data and analytical tools are used throughout the do, check, and act portions. This section will help in understanding what each tool reveals about the process being improved, when to use it, and how to use it. An overall outline of a typical process improvement project can be seen in Appendix 14.1.1–1. Specific examples related to the process improvement project outline from Appendix 14.1.1–1 can be seen in Appendix 14.1.1–2.

II. VARIATION

Within the process improvement cycle, variation represents the excess in the system that needs to be reduced. Variation is the extent to which a process differs from the norm or desired result. Variation exists in every process and always will. Both common-cause and special-cause variations are studied in process improvement. Common-cause variation is that inherent variance in the process that is a result of how the process is performed. Special-cause variation is that which can be attributed to a particular source. A process that displays little variation, or variation only under predictable circumstances, is considered under control. Simple monitoring techniques are required to confirm that a process is under control.

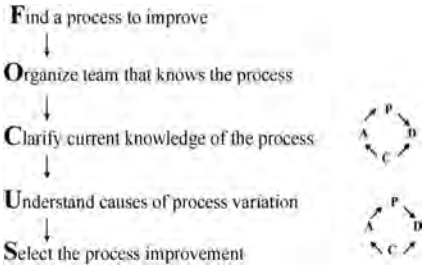

Find a process to improve
Organize team that knows the process
Clarify current knowledge of the process
Understand causes of process variation
Select the process improvement

Figure 14.1.1–1 Diagram of the FOCUS summary of process improvement.

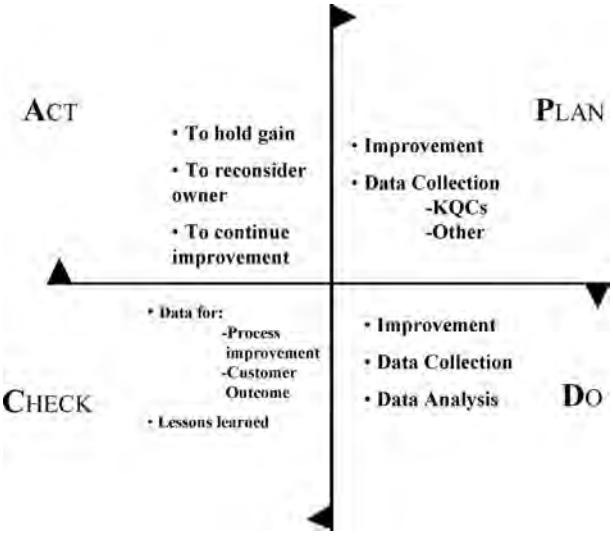


Figure 14.1.1–2 Strategy: diagram of the plan-do-check-act cycle for process improvement. (From M. Brassard, *The Memory Jogger: a Pocket Guide of Tools for Continual Improvement.* Goal/QPC, Salem, NH, 1998.)

III. PROCESS IMPROVEMENT (CONTINUOUS) VERSUS QUALITY ASSURANCE

Process improvement (continuous) focuses mostly on the internal sources of variation. QA usually emphasizes special-cause variation. Process improvement (continuous) follows a logical four-step sequence. Initially, the process is described and sources of variation are identified. Then an analysis (in-depth) that clarifies both the sources and extent of problems follows. Next, the team weighs the alternatives and makes decisions about how to reduce variation. In the end, the team will try one of the alternatives and measure its effect on the process. Once improvements are made, the organization will continue to monitor progress over time to ensure that the process remains in control.

IV. QUALITY IMPROVEMENT TOOLS

One of the most valuable techniques used in improving quality is the measurement of the process, both before and after the improvement efforts. A set of statistical methods agreed upon by most experts includes the following list: (i) flowcharts or diagrams, (ii) cause-and-effect diagrams, (iii) check sheets/self-coded data sheets, (iv) Pareto diagrams or charts, (v) histograms, (vi) run charts, and (vii) control charts. These tools are described below, as they are useful to the continuous process improvement system. When the process is described and the sources of variation are identified, key tools to use would be (i) flowcharts or diagrams and (ii) cause-and-effect diagrams. Flowcharts or diagrams are pictorial representations of how the system works. See Appendix 14.1.1–2 for an example of a flowchart. They track the steps that the "object" of a process goes through from start to finish. The steps in developing a flowchart include the following: (i) define the basic stages of the process, (ii) separate each stage into the steps needed to complete the process, (iii) verify the process by observation, and (iv) discuss the process representation with the project team and reach consensus on the underlying process.

One of the most useful purposes of a flow diagram is to allow the team to focus on the process flow. Surprisingly, the continuous-improvement team is likely to find that the existing process owners and users do not share a common understanding of how the current system works. In the end, after much discussion, with an accurate, shared representation of how the system works, the team is then able to consider how to improve it. Symbols are customarily used to draw flowcharts as follows (Appendix 14.1.1–2): (i) an activity is represented by a rectangle, (ii) a decision node by a diamond, (iii) a wait or an inventory by a triangle, (iv) a document by a symbol that looks like a rectangle with a curve on the bottom, (v) a file by a large circle, and (vi) a continuation of the flow to another sheet by a small circle.

Once agreement is reached on the representation of the current process, team members should ask questions about the process. Some examples include the following. (i) Who are the customers? How are they receiving the process? (ii) Are there opportunities for improvement? Do the data validate the performance gap issue? (iii) Do the team members represent all the process issues? (iv) What measurements are we currently collecting about this process? Are they the right ones for the issues raised? How might we go about adding the right measures?

The potential benefits of flowcharting are considerable. Staff members get to know the process much better. The results can be used as a training aid. People begin to take ownership of the process by participating in the activity, and most important, the possibilities for improvement become clear almost immediately.

Cause-and-effect diagrams, also called fishbone or Ishikawa diagrams, sort and interrelate the multiple causes of process variation, once the team has described the process. See Appendix 14.1.1–2 for an example of a cause-and-effect diagram. Step 1 starts by putting the identified performance gap in a box at the right and a big arrow pointing to the gap. Step 2 involves drawing angle arrows from the big arrow to represent main causes, namely, major classifications of causes such as labor,

IV. QUALITY IMPROVEMENT TOOLS *(continued)*

materials, and equipment. Step 3 adds arrows pointing to the angle arrows which are specific causes.

Process improvement requires performing in-depth analyses to clarify knowledge and present the results. The tools used at this stage include revisiting and updating the flowcharts stratifying for detail the cause-and-effect diagrams, using check sheets/self-coded data sheets to collect process data, using Pareto diagrams as a key tool to stratify causes, using frequency distributions (histograms) to help in presentation, and using run charts that are important to relate data temporally to changes. Appendix 14.1.1–2 contains a table (Linking Customers Chart) that reveals the requirements of some of the data at this stage.

A check sheet is a matrix in which an observer records the number (frequency) of events (Appendix 14.1.1–2). Check sheet data are then presented as a histogram, the simplest form of presentation of stratification. A histogram is a bar chart representing the frequency distribution of a set of data. A Pareto diagram is a vertical bar chart with the bars arranged from the longest first on the left and moving successively toward the shortest. Each bar represents the frequency of a specific cause of an error. The data come from a cause-and-effect diagram and a check sheet identifying the frequency of a quality failure due to a specified cause.

Representations of the frequency of causes of error or check sheet values are useful, but they cannot indicate trends or other characteristics of the phenomenon being observed that change over time. Another way of displaying the variation of a process is to look at its performance over time.

This allows the observer to see what the temporal behavior of the process is and establish the times of process performance changes so that they can be linked to the times of other possible related events. A process is considered under control if most of the observations are near the central line, if there are few to no points outside the upper and lower control limits (UCL and LCL, respectively; usually above the mean, plus or minus 3 standard deviations) and there are no runs (more than eight consecutive observations to one side of the mean).

Measures for run (and control) charts can be either attributes or variables. Attribute data arise from classification of data and counts. An example would be numbers of defects. Variables are measured directly. Charts often present the variable mean (X bar), process range (R), and/or standard deviation (S) for a specific process parameter. Following run charts of key parameters is the essence of continuous process improvement.

The team decides whether the error being studied at this point is common cause or special cause (see references for further descriptions of these terms). After changes are implemented, the gains must be maintained. A system of measuring and monitoring must be put into place to find out whether these improvements are actually being captured and whether the new process is under control. Control charts—similar to run charts—are the most helpful tool for this stage. Control charts are usually run charts with the additional notation indicating the control limits of plus or minus 3 standard deviations (commonly referred to as “3 sigma”). These control limits are referred to as the UCL, which is 3 standard deviations above the mean, and the LCL, which is 3 standard deviations below the mean. See an example in Appendix 14.1.1–2.

A process that is under control is not the team’s final objective. The next step is to return to the plan stage of the PDCA cycle (Fig. 14.1.1–2) and determine whether it would be worthwhile to try and improve the process further. Thus, continuous process improvement occurs.

SUPPLEMENTAL READING

The following are considered classic references about quality in health care.

Berwick, D. M. 1996. Quality comes home. *Ann. Intern. Med.* **125:**839–843.

Berwick, D. M. 1989. Continuous improvement as an ideal in health care. *N. Engl. J. Med.* **320:**63–67.

Deming, W. E. 1986. *Out of the Crisis.* Center for Advanced Engineering Study, Institute of Technology, Cambridge, MA.

Institute of Medicine. 2001. *Crossing the Quality Chasm: a New Health System for the 21st Century.* National Academy Press, Washington, DC.

Millenson, M. L. 1997. *Demanding Medical Excellence: Doctors and Accountability in the Information Age.* University of Chicago Press, Chicago, IL.

The following references discuss systems thinking and aims for improvement.

Baier, S. 1986. *Bed Number 10.* CRC Press, New York, NY.

Berwick, D. M. 1994. Eleven worthy aims for clinical leadership of health system reform. *JAMA* **272:**797–802.

Berwick, D. M. 1996. A primer on leading the improvement of systems. *BMJ* **312:**619–622.

Epstein, L., et al. 1996. Patients' perceptions of office medical practices: judging quality through the patients' eyes. *Am. J. Med. Qual.* **11:**73–80.

Gerteis, M., et al. 1993. *Through the Patient's Eyes: Understanding and Promoting Patient Centered Care.* Jossey-Bass, San Francisco, CA.

The Institute for Family-Centered Care. http://www.familycenteredcare.org.

Levinson, W., et al. 1997. Physician-patient communication: the relationship with malpractice claims among primary care physicians and surgeons. *JAMA* **277:**553–559.

Meterko, M. 1996. The evolution of customer feedback in healthcare. *Jt. Comm. J. Qual. Improv.* **22:**307–310.

Murray, M., and C. Tantou. 1998. Must patients wait? *Jt. Comm. J. Qual. Improv.* **24:**423–425.

Nolan, T. 1998. Understanding medical systems. *Ann. Intern. Med.* **128:**293–298.

Senge, P., et al. 1994. *The Fifth Discipline: the Art and Practice of the Learning Organization.* Doubleday, New York, NY.

The following references concern variation and measurement.

Balestracci, D. 1996. *Quality Improvement: Practical Applications for Medical Group Practices,* 2nd ed. Medical Group Management Association, Englewood, CO.

Carey, R. G., and R. C. Lloyd. 1997. *Measuring Quality Improvement in Health Care: a Guide to Statistical Process Control Applications.* ASQ Quality Press, Milwaukee, WI.

Pisek, P. 1992. Tutorial: introduction to control charts. *Qual. Manag. Health Care* **1**(1)**:**65–74.

Pisek, P. 1994. Tutorial: planning for data collection, part II—designing the study. *Qual. Manag. Health Care* **2**(4)**:**73–81.

Pisek, P. 1994. Tutorial: planning for data collection, part III—sample size. *Qual. Manag. Health Care* **3**(1)**:**78–92.

Wheeler, D. 1993. *Understanding Variation: Keys to Managing Chaos.* Statistical Process Controls, Inc., Knoxville, TN.

The following references concern psychology.

Bridges, W. 1991. *Managing Transitions: Making the Most of Change.* Addison-Wesley Publishing Company, New York, NY.

Kaplan, S. H., S. Greenfield, and J. E. Ware, Jr. 1989. Assessing the effects of physician-patient interactions on the outcomes of chronic disease. *Med. Care* **27**(3 Suppl.)**:**S110–S127.

Katzenbach, J., and D. Smith. 1993. *The Wisdom of Teams: Creating the High Performance Organization.* Harvard Business School Press, Boston, MA.

Kohn, A. 1993. *Punished by Rewards: the Trouble with Gold Stars, Incentive Plans, A's, Praise and Other Bribes.* Houghton Mifflin and Co., Boston, MA.

Kohn, A. 1993. *No Contest: the Case Against Competition.* Mariner Books, New York, NY.

Scholtes, P. 1996. *The Team Handbook: How To Use Teams To Improve Quality.* Oriel Inc., Madison, WI.

SUPPLEMENTAL READING
(continued)

The following references discuss PDSA and the model for improvement.

Berwick, D. M. 1998. Developing and testing change in delivery of care. *Ann. Intern. Med.* **128:**651–656.

Berwick, D. M. 1996. Harvesting knowledge from improvement. *JAMA* **275:**877–888.

Brassard, M. 1998. *The Memory Jogger: a Pocket Guide of Tools for Continual Improvement.* Goal/QPC, Salem, NH.

Brock, W., K. Nolan, and T. Nolan. 1998. Pragmatic science: accelerating the improvement of critical care. *New Horizons* **6:**61–68.

Kaluzny, A., and C. McLaughlin. 1999. *Continuous Improvement in Health Care: Implementation and Applications.* A.S.P.E.N. Publications, Silver Spring, MD.

Langley, G. J., K. M. Nolan, T. W. Nolan, C. L. Norman, and L. P. Provost. 1996. *The Improvement Guide: a Practical Approach to Enhancing Organizational Performance.* Jossey-Bass Publishers, San Francisco, CA.

Nolan, T. 1997. Accelerating the pace of improvement—an interview with Thomas Nolan. *Jt. Comm. J. Qual. Improv.* **23:**217–222.

The following are resources for improving safety.

Aspden, P., J. M. Corrigan, J. Wolcott, and S. M. Erickson (ed.). 2004. *Patient Safety: Achieving a New Standard for Care.* National Academy Press, Washington, DC. http://www.nap.edu.

Barach, P., and S. D. Small. 2000. Report and preventing medical mishaps: lessons from non-medical near miss reporting systems. *BMJ* **320:**759–763.

Best Practice Network. http://best4health.org.

Billings, C. E. 1998. Some hopes and concerns regarding medical event-reporting systems: lessons from the NASA Aviation Safety Reporting System. *Arch. Pathol. Lab. Med.* **122:**214–215.

Gaba, D. M. 2000. Structural and organizational issues in patient safety: a comparison of health care to other high hazard industries. *California Manag. Rev.* **43:**83–102.

Institute for Safe Medication Practices. http://www.ismp.org.

Kohn, L. T., J. M. Corrigan, and M. S. Donaldson (ed.). 2000. *To Err Is Human: Building a Safer Health System.* Committee on Quality of Health Care in America, Institute of Medicine, National Academy Press, Washington, DC. http://www.nap.edu.

Leape, L. 1994. Error in medicine. *JAMA* **272:**1851–1857.

National Patient Safety Foundation. http://www.npsf.org.

Reason, J. T. 1990. *Human Error*. Cambridge University Press, New York, NY.

Van Vuuren, W. 1999. Organizational failure: lessons from industry applied to the medical domain. *Safety Sci.* **33:**14–29.

Weeks, W. B., and J. P. Bagian. 2000. Developing a culture of safety in the Veteran's Health Administration. *Effective Clin. Pract.* **3:**270–276.

The following references discuss spreading successful change.

Gladwell, M. 2002. *The Tipping Point.* Little, Brown and Company, New York, NY.

Rogers, E. 1995. *Diffusion of Innovations.* The Free Press, New York, NY.

The following references address physician leadership of improvement.

Berwick, D. M., and T. W. Nolan. 1998. Physicians as leaders in improving health care: a new series in the Annals of Internal Medicine. *Ann. Intern. Med.* **128:**289–292.

Berwick, D. M. 1993. TQM: redefining doctoring. *Internist* **34**(3)**:**8–10.

The Institute for Healthcare Improvement. http://www.ihi.org.

The Institute for Healthcare Improvement. IHI Calls to Action series. http://www.ihi.org/conferences/callstoaction/audiotapes.asp.

James, B. Advanced Training Program in Health Care Delivery Improvement (ATP), Intermountain Health Care. http://www.ihc.com/xp/ihc/generalinfo/healthcaresystem/research/

deliveryresearch/educationprograms/.

Merry, M. D. 1993. Physician leadership for the 21st century. *Qual. Manag. Health Care* **1**(3)**:**31–41.

14.1.1

Appendix 14.1.1–1—Outline of a Typical Process Improvement Project

Project name
Project aim
Team
Diagnosis
Flowchart
Pareto chart
Cause-and-effect diagram linking customers' charts
Intervention—statement of plan
Tally or check sheet
Self-coded data sheet
Impact—statement of results
Unstable process
Stable process
  Run chart
  Control chart
Defects data
Holding the gains—ongoing monitoring
Future plans

Figure 14.1.1–A1 Outline of a typical process improvement project.

14.1.1.2 Appendix 14.1.1.2–1—Examples of Items in Process Improvement Project Outline

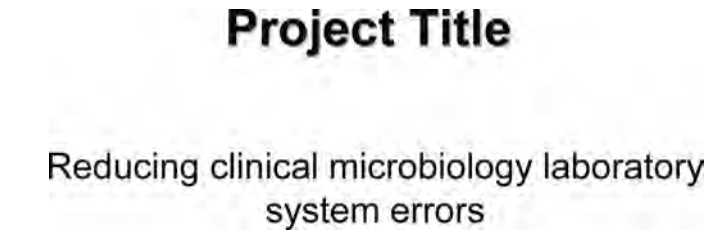


Figure 14.1.1.2–A1 The project title describes the process improvement project.

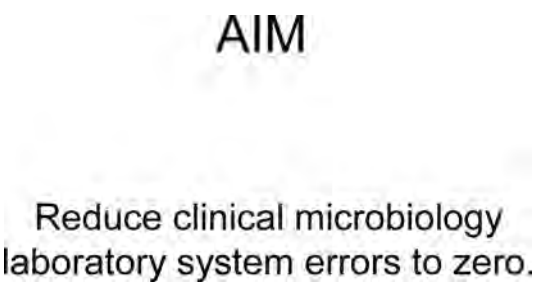


Figure 14.1.1.2–A2 The aim allows the team to focus on the goal of the process improvement project.

Figure 14.1.1.2–A3 The team is assembled to establish its purpose, process, and measures of team progress.

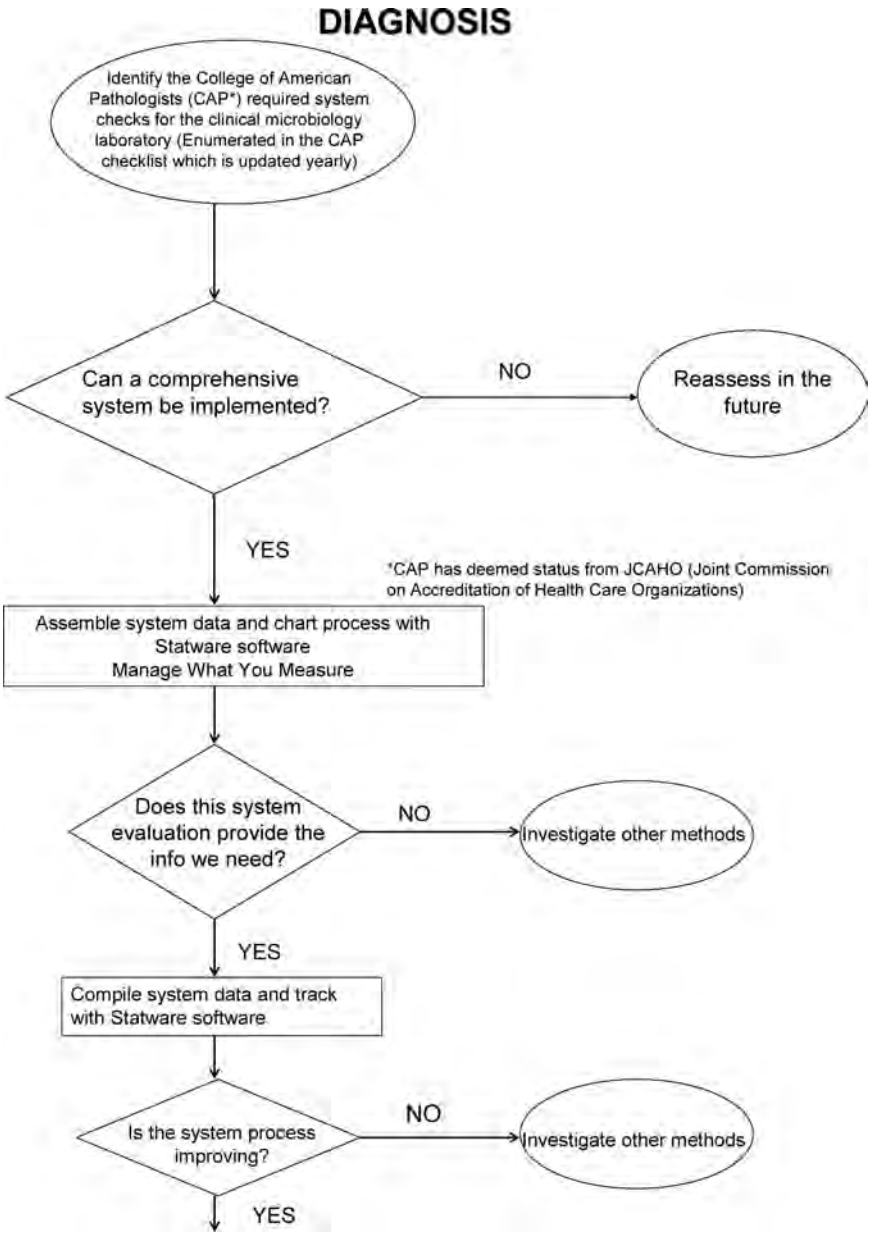
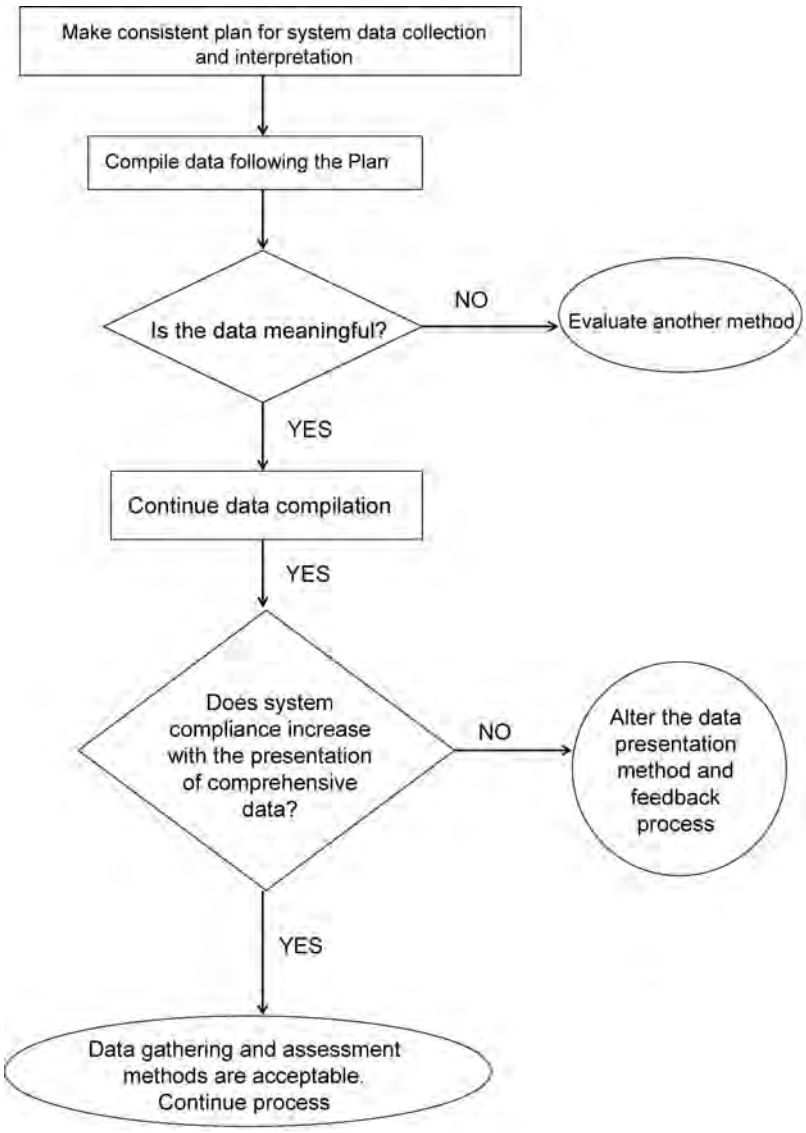

DIAGNOSIS
Identify the College of American Pathologists (CAP*) required system checks for the clinical microbiology laboratory (Enumerated in the CAP checklist which is updated yearly)
Can a comprehensive system be implemented?
NO
Reassess in the future
YES
*CAP has deemed status from JCAHO (Joint Commission on Accreditation of Health Care Organizations)
Assemble system data and chart process with Statware software
Manage What You Measure
Does this system evaluation provide the info we need?
NO
Investigate other methods
YES
Compile system data and track with Statware software
Is the system process improving?
NO
Investigate other methods
YES

Figure 14.1.1.2–A4 Flowchart of process.


Make consistent plan for system data collection and interpretation
Compile data following the Plan
Is the data meaningful?
NO
Evaluate another method
YES
Continue data compilation
YES
Does system compliance increase with the presentation of comprehensive data?
NO
Alter the data presentation method and feedback process
YES
Data gathering and assessment methods are acceptable. Continue process

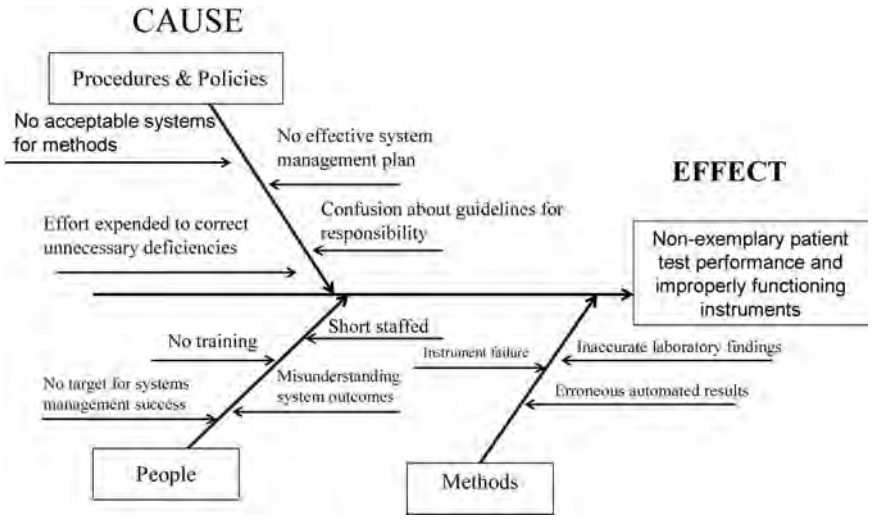


Figure 14.1.1.2–A5 The cause-and-effect fishbone diagram allows team members to find and cure causes, not symptoms. This allows a team to identify, explore, and graphically display, in increasing detail, all of the possible causes related to a problem or condition to discover its root causes(s).

Linking Process, Customers and Measurements

Key Process Factor	Step still necessary in the current environment?	Desired Outcome From Process Step	Customers of Process Step	Customers Expectations	Key Quality Characteristic Measurement to determine if outcome meets expectations
System management tracking plan implemented	YES	Comprehensive Data Overview is available	Patients Clinicians	Confidence in Microbiology results	Deficiencies/ opportunities = Percent errors
System management tracking plan reveals opportunities for improvement	YES	Excellence is achieved for overall microbiology methods and product outcome for patients and physicians	Patients Clinicians	Microbiology results are reliable and rapid due to good system management	Decrease in Quality Deficiencies/ opportunities = Decreased % Errors

Figure 14.1.1.2–A6 Data from a process are summarized and graphically presented.

Intervention
Statement of Plan

- On a monthly basis the team will analyze the System Deficiency Data
- Process improvement possibilities will be discussed, charted, and implemented after receiving input from the team.
- Process improvement trends will be analyzed with process improvement alterations made as needed to accomplish System Plan outcome goals.

Figure 14.1.1.2–A7 The intervention describes what action will be taken to improve the process.

Process Improvement Summary
2003

DEFICIENCY	MONTH	PROCESS ALTERATION REQUIRED?	RESULTING IMPROVEMENT ACTION
No daily QC recorded	Jan	NO	Tech cites personal error of clerical omission only. States that process is adequate
No Vitek Jr. maintenance	Jan	Yes	New training program developed and put into place.
No respiratory DFA QC	Feb	Yes	QC sheet moved to be more visible. Attached to shelf directly in view from microscope.
No Beta Lactamase QC	Feb	NO	Tech cites personal error of clerical omission only. States that process is adequate.
No Germ Tube QC	Feb	ALTERED	Federal Register guidelines no longer require weekly GT QC. Completed on a per-lot basis.
No filter cleaning – Bactec 2	Feb	Yes	Double check method instituted concerning QC records.
No Germ Tube QC	March	ALTERED	Federal Register guidelines no longer require weekly GT QC. Completed on a per-lot basis.
No timer check – Parasit Centrifuge	March	Yes	Double check method instituted concerning QC records.
No CO2 incubator fyrite check	May	Yes	Obtained new redundant equipment. Explained new process.
No Strep Grouping QC	July	Yes	Moved this item on checklist to top #1 position, placed also at bottom as double check with greater visibility.
No blood worksheet check	July	No	Tech cites personal error or omission only. States that process is adequate.
No timer check – American Rotator	August	Yes	Double check method instituted concerning QC records.
No mycology hood maintenance	October	No	CLA cites personal error only. States that process is adequate
No bacteriology scope maintenance	October	No	CLA cites personal error only. States that process is adequate
No fluorescent scope maintenance	October	No	CLA cites personal error only. States that process is adequate.
No inverted scope maintenance	October	No	CLA cites personal error only. States that process is adequate.
No mycology scope maintenance	October	No	CLA cites personal error only. States that process is adequate.
No mycology bench cleaning	October	Yes	No response from tech on improvement suggestions Checkoff sheet moved to be more visible
No mycology bench cleaning	October	Yes	No response from tech on improvement suggestions Checkoff sheet moved to be more visible
No mycology bench cleaning	October	Yes	Process error results in moving checkoff sheet to an "in the way" spot for much greater visibility
No blood cx worksheet check	Nov	No	Tech states that system and process are effective. Sites personal error only.
No weekly cytospin maintenance X2	Nov	YES	New daily maintenance procedure instituted
No monthly cleaning Centr. 3000	Dec	No	Reminder concerning dry erase board notes
No cytospin clip disinfect. Recorded	Dec	Yes	Add a month-end procedure to check monthly data
No gram stain QC recorded week 2	Dec	No	Reminder of reagent QC procedure
No blood worksheet check X2	Dec	No	No response from tech (same tech, both occurrences) on improvement suggestions indicates adequate process.

Figure 14.1.1.2–A8 The actions for process improvement are documented each month.

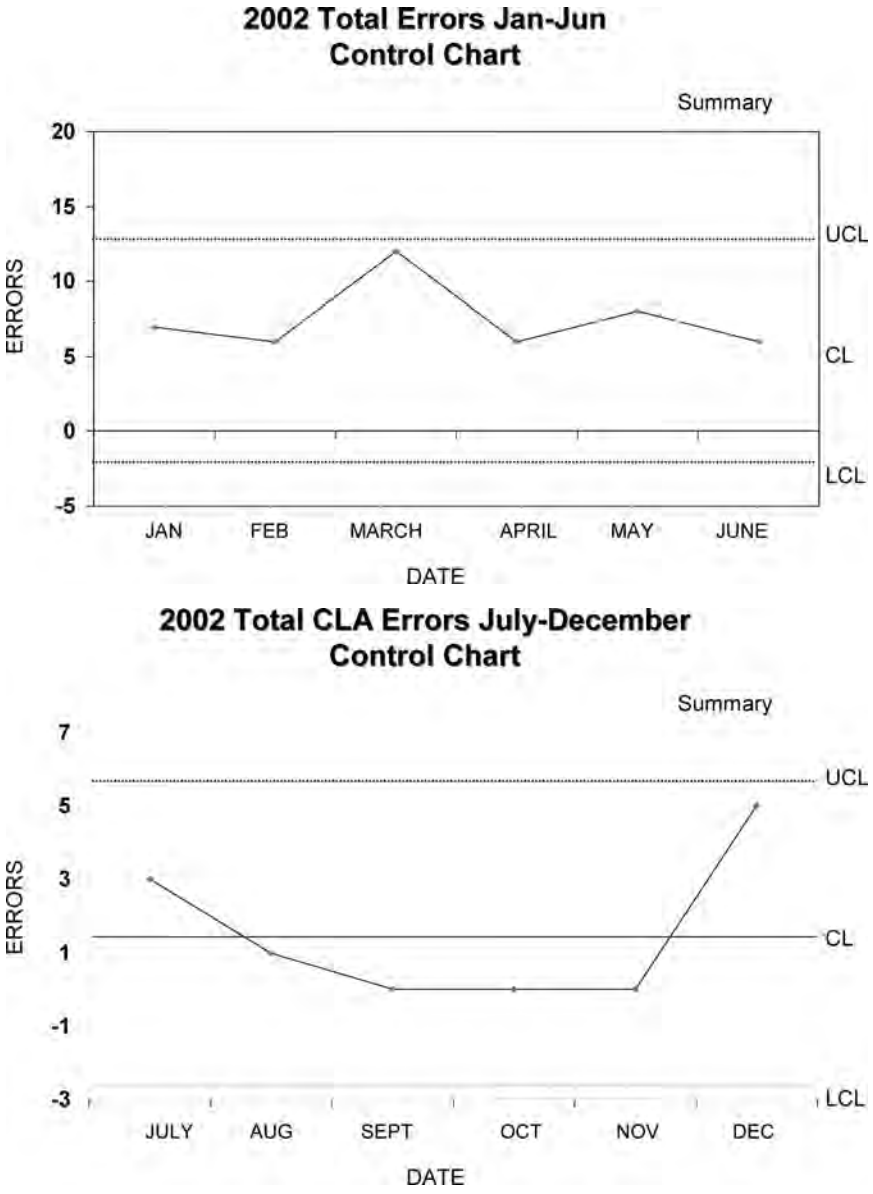


Figure 14.1.1.2–A9 The control chart is a graphic display of the data as the process change evolves over time *(continued).*

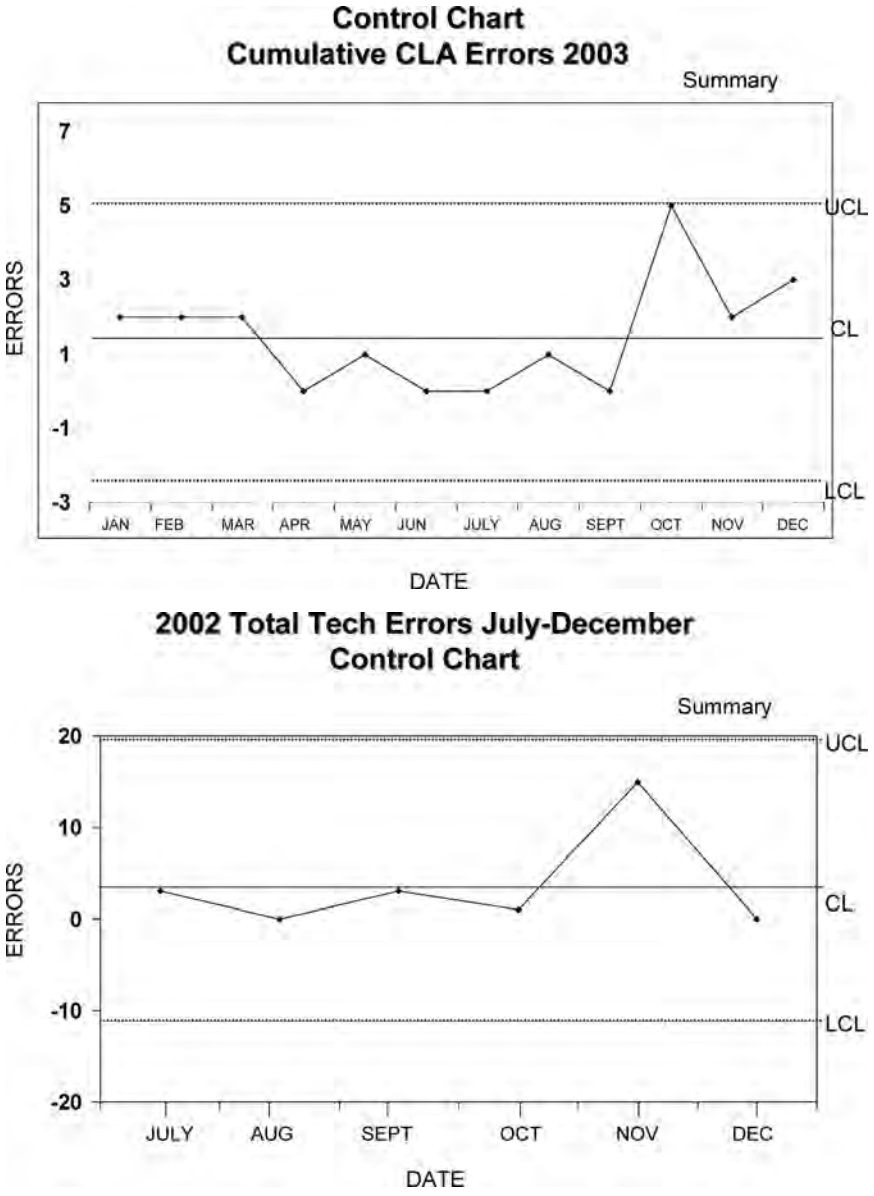


Figure 14.1.1.2–A9 *(continued)*

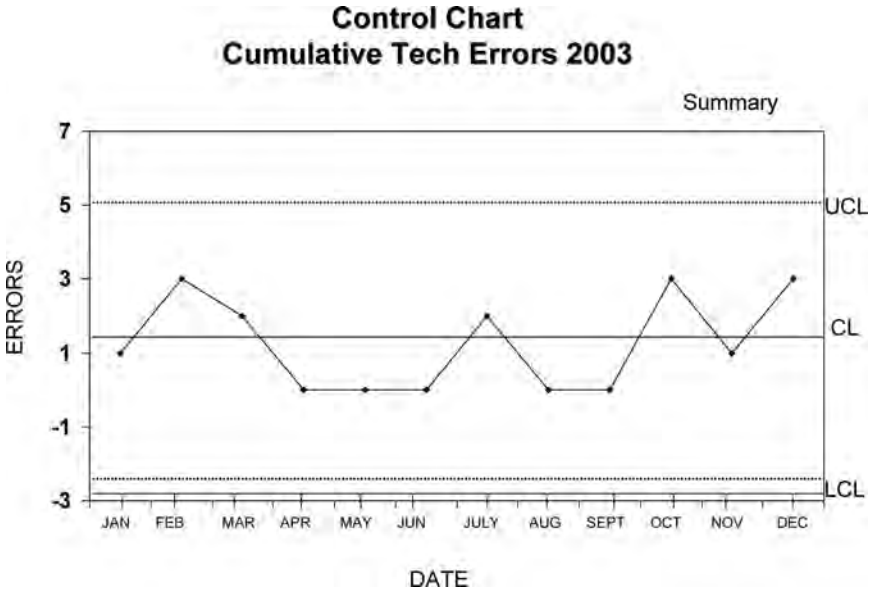


Figure 14.1.1.2–A9 *(continued)*

Impact

- A system monitoring method is in place to detect deficiencies in equipment maintenance and test procedures.
- Quality of patient test results is directly impacted by proper Microbiology System Assessment.
- An excellent system method makes compliance straightforward and effortless.
- Over a two year period, the error rate for systems in the clinical microbiology laboratory changed from seven to one by instituting process improvement techniques in a routine fashion.

Figure 14.1.1.2–A10 The impact statements describe the process change.

Holding the Gains

- Education for team members in system management requirements will continue as requirements change
- More specific feedback can pinpoint areas needing improvement as team members become increasingly familiar with the method.
- Team members with fundamental knowledge will make recommendations for making the goal easy to accomplish

Figure 14.1.1.2–A11 The process improvement is observed over time to maintain the change.

14.2 Quality Control

I. INTRODUCTION

QC programs ensure that information generated by a laboratory is accurate, reliable, and reproducible. This is accomplished by assessing the quality of the specimens; monitoring the performance of test procedures, reagents, media, instruments, and personnel; reviewing test results; and documenting the validity of the test methods.

II. SOURCES FOR QUALITY CONTROL GUIDELINES

The guidelines discussed in this procedure are based upon the sources listed in Appendix 14.2–1 and the References. Federal guidelines are considered minimum standards and are superseded by higher standards imposed by states or other certifying agencies.

III. GENERAL QUALITY CONTROL PARAMETERS

Table 14.2–1 lists QC parameters.

A. Specimen collection and transport

1. Include screening tests (e.g., quantitation of WBCs and squamous epithelial cells in sputum) among the criteria for acceptable specimens (*see* section 2).
2. Do not test unacceptable specimens unless it is impossible to re-collect them. When unacceptable specimens must be processed, include a disclaimer in the patient report indicating that the specimen was not acceptable but was processed at the specific request of the physician, whose name has been recorded in the test report.

B. Procedure manual

1. The CAP requires that laboratories maintain a copy of NCCLS document GP2-A4 (13) and that procedures be written in a format largely consistent with those guidelines.

   ◩ **NOTE:** All applicable procedures in this handbook comply with NCCLS GP2-A4.
2. Each test procedure in this handbook includes detailed instructions for the QC of that test procedure and, when appropriate, QC forms for recording of the results.

Table 14.2–1 QC parameters

QC parameter	Guidelines	Additional information
Specimen collection and transport	Provide instructions for collection and transport.	Procedures 2.1, 4.2, 8.2, and 9.2
	Establish criteria for acceptable specimens.	Procedure 2.1
	Establish rejection criteria for unacceptable specimens.	Procedure 2.1
Procedure manual	Write in NCCLS format. Group all procedures, when applicable, as preanalytical, analytical, and postanalytical	Reference 13
	Define test performance, tolerance limits, specimen acceptability, reagent preparation, QC, calculations, and reporting.	
	Review and initial annually.	Appendix 14.2–5, sample form
	Approve and date all changes.	Appendix 14.2–5, sample form
	Make available in work area.	
	Retain obsolete procedure for 2 years.	
Personnel	Employ sufficient numbers of qualified personnel for volume and complexity of work.	Reference 1; reference 2 in Appendix 14.2–1
	Document continuing-education activities.	
	Provide employees with written performance standards.	
	Evaluate employees annually.	
QC records	Record all QC results on QC forms or in computer.	Appendix 14.2–5, sample form
	Report all out-of-control results to supervisor, and note corrective action(s) taken on QC forms.	Appendix 14.2–5, sample form
	Hold monthly review of QC records with supervisor.	
	Retain QC records for a minimum of 2 years.	
Patient reports	Report results only to authorized personnel.	Reference 8
	Notify test requester of "panic" values immediately.	
	When rendering verbal reports, record name of individual notified, date, and time.	
	Provide normal ranges, when appropriate.	
	Correct errors in patient reports in a timely fashion.	
	Retain records for a minimum of 2 years. *Note:* Time may vary in different states.	
Referral specimens	Use only accredited or licensed reference laboratories.	
	Include the name of the reference laboratory actually performing the testing on the patients' reports.	
Proficiency testing	Participate in an appropriate-level external proficiency-testing program.	Appendix 14.2–2
	Consider internal proficiency-testing program.	
Instrument or equipment performance	Document function checks of equipment.	See procedures 15.4.3 and 15.4.4 for sample form.
	Perform as frequently as necessary to ensure proper function or as specified by manufacturers.	
	Document routine preventive maintenance.	
	Retain maintenance records for life of instrument.	
Commercially prepared media exempt from QC	Certain primary plating media are exempt from QC testing by user.	Reference 14
	Retain manufacturer's QC protocol.	
	Obtain written assurance that manufacturer follows NCCLS standards (e.g., package insert, label, protocol).	Appendix 14.2–5, sample form
	Inspect each shipment for cracked media or petri dishes, hemolysis, freezing, unequal filling, excessive bubbles, and contamination.	Appendix 14.2–5, sample form
	Document medium deficiencies and corrective action, and inform manufacturer.	
	Perform in-house QC testing until deficiency is corrected.	

(continued)

Table 14.2–1 QC parameters *(continued)*

QC parameter	Guidelines	Additional information
User prepared and nonexempt media	Record amount prepared, source, lot number, sterilization method, preparation date, pH, expiration date, and name of preparer.	Appendix 14.2–5, sample form
	Check medium for proper color, consistency, depth or slant, smoothness, hemolysis, excessive bubbles, and contamination.	
	Test medium with QC microorganisms of known physiological and biochemical properties.	Tables 14.2–2, 14.2–3, and 14.2–4 References 2, 3, 10, 12, 14, 15, and 17
Stains, reagents, and antisera	Label containers as to contents; concentration; storage requirements; dates prepared, received, and/or placed in service; and expiration date or shelf life.	References 1, 3, and 11
	If user prepared, record volume, source, and lot number.	
	Store according to manufacturer's recommendations.	
	Test reagents with positive and negative controls prior to use (with each batch, lot number, and shipment).	References 1, 3, 5, and 11 Tables 14.2–5 and 14.2–6
	Test Gram stain reagents with control organisms with each batch, lot number, and shipment and weekly thereafter.	
	Test methylene blue stain with QC organisms with each batch, lot number, and shipment and with each use. Check each batch, lot number, and shipment of antiserum when prepared or opened and once every 6 months thereafter with positive and negative controls.	
	Discard outdated material and reagents that fail performance standards.	
Commercial kits	Test each new batch, lot, and/or shipment. Follow manufacturers' recommendations for QC testing.	

III. GENERAL QUALITY CONTROL PARAMETERS *(continued)*

C. Personnel

1. Supervisory personnel should attend courses on laboratory administration (e.g., personnel management and cost accounting).

2. Regularly scheduled meetings to inform laboratory personnel of pertinent changes and to solicit their suggestions for improving the laboratory are highly advisable.

D. QC records

1. When a QC result is out of range:

a. Notify the supervisor.

b. Repeat the test.

c. If the test result remains out of the acceptable control range, repeat the test with new lots of reagents and/or a fresh QC isolate from stock.

d. Record all pertinent actions.

e. If QC testing results remain outside of accepted ranges, do not test patient specimens until the problem has been resolved.

2. Options for handling patient specimen results when test systems fail QC

a. Do not report patient results until the test is repeated and determined to be in control.

b. Do not report patient results until the test is repeated with an alternative test system or by a reference laboratory.

c. If erroneous patient results have been reported, contact the physician and submit a corrected report. Do not remove the erroneous result from the patient's chart.

3. QC records must be retained for a minimum of 2 years in an easily accessible location.

III. GENERAL QUALITY CONTROL PARAMETERS *(continued)*

E. Patient reports

1. The requester of the test should be notified immediately of any life-threatening situations indicated by test results (panic values) (*see* section 2 of this handbook).

2. Options for detection of errors on patient reports

a. Review the hard copies of all patient reports.

b. Use computer flags for unusual results (e.g., *Burkholderia pseudomallei, Vibrio cholerae,* vancomycin-resistant staphylococci, or linezolid-resistant *Staphylococcus aureus*).

3. When applicable, the laboratory must provide information that is useful for test interpretation (e.g., normal ranges, sensitivity, specificity, precision, linearity, predictive value, accuracy, and test interferences) (*see* Appendix 14.2–4).

4. Patient reports and laboratory records must be retained for a minimum of 2 years in an easily accessible location.

◩ **NOTE:** Times may vary in different states.

F. Referral specimens

1. Reference laboratories must be accredited and/or must meet city, county, or state standards.

2. Reference laboratories are used for tests that require a high level of expertise and that are infrequently performed.

G. Proficiency testing program

1. All accredited laboratories must participate in an external proficiency program that reflects the specialty (e.g., mycology) and level of expertise (e.g., reference laboratory) of the laboratory. A partial list of proficiency-testing services for hospital and office laboratories is presented in Appendix 14.2–2.

The 1988 Clinical Laboratories Improvement Amendments indicates that all laboratories must maintain an average score of 80% on proficiency tests (6).

2. Internal proficiency programs are used to monitor various aspects of laboratory work (e.g., uniformity of Gram stain interpretation and identification of parasites or fungi). Be careful to avoid reporting results of internal controls to outside health care personnel. Options for an internal proficiency program include the following.

a. Seed a simulated specimen with an unknown organism, and label it as an autopsy specimen or as from a fictitious patient.

b. Reprocess specimens for analysis by different personnel.

c. Send a portion of the specimen to a reference laboratory for comparison.

IV. SPECIFIC QUALITY PARAMETERS

Table 14.2–1 lists QC parameters.

A. Instrument and equipment performance

1. Perform preventive maintenance at least as frequently as recommended by the manufacturer. See manufacturer's instructions.

2. The manufacturer, an independent service company, or the in-house biomedical engineering service can perform preventive maintenance.

Include QC information on reagent container and in QC records.

B. Media

1. Commercially prepared media (exempt from QC by the user)

a. The primary isolation media listed in NCCLS M22-A2 (14) are exempt from QC testing by the user (except for chocolate agar, *Campylobacter* media, and media for the selective isolation of pathogenic *Neisseria* spp.) provided that the manufacturer follows NCCLS criteria for QC. The man-

IV. SPECIFIC QUALITY PARAMETERS *(continued)*

ufacturer must indicate by a label, package insert, technical manual, or other document that the media conform to NCCLS standards. The laboratory must retain a copy of this documentation.

b. Test nonexempt media with the QC organisms and methods listed in NCCLS M22-A2 (minimum criteria).

c. Maintain a record of vendor, lot number, date received, acceptable QC, and date put into service for all media shipments.

2. User- or commercially prepared media requiring QC testing

a. Test each new numbered lot of dehydrated medium, reagent, or ingredient in parallel with an approved lot before it is released for use. Maintain a record of the amount of medium prepared, source, lot number, sterilization method, preparation date, pH, date of initial use, expiration date, and name of preparer.

b. Check for contamination by incubating a 5% sample for batches of 100 or less or 10 randomly selected units from larger batches (3). Incubate the medium for 48 h at the temperature at which it will be used and then for an additional 48 h at room temperature.

c. QC organisms should be derived from well-characterized strains. Reference strains are available from the American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852-1776 [(301) 881-2600]; the Mycobacterial Culture Collection, National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson St., Denver, CO 80206 [(303) 398-1339]; commercial sources; and proficiency-testing programs.

d. Performance standards for commonly used media and medium-based tests are presented in Tables 14.2–2 to 14.2–4.

e. The QC method should conform to NCCLS M22-A2 recommendations.

3. Guidelines for establishing expiration dating (16)

a. The performance and expiration dating of culture media are affected by numerous factors that may vary from laboratory to laboratory. *It is imperative that each laboratory establish its own stability testing of all products that it produces.*

b. Factors adversely affecting the expiration dating and performance of prepared media

(1) Overheating of base ingredients
(2) Using blood >5 days old
(3) Adding blood or heat-labile ingredients at >50°C
(4) Using improper storage temperatures
◩ **NOTE:** Do not store media in a frost-free refrigerator.
(5) Underfilling of medium container
(6) Sealing plates in storage bags before they have cooled and gelled properly
(7) Exposing medium to light
(8) Alternating the storage temperature of sealed media from room temperature (22 to 25°C) to 2 to 8°C
(9) Using a small fill volume (<1.0 ml) for tubed media
(10) Using loosely capped tubed media
(11) Storing plates in open or improperly sealed bags (e.g., at setup or work benches) (Unsealed bags result in a variable loss of medium volume depending upon the temperature and humidity of the room.)

Table 14.2–2 Performance standards for bacteriological media

Medium	pH (±0.2)	Method of inoculation[a]	Length of incubation	Control organism[b]	ATCC[c] no.	Expected results[d]
Acetamide agar	6.5	C	1–4 days	*Pseudomonas aeruginosa*	27853	Pink to red
				Stenotrophomonas maltophilia	13637	No color change
Acetate agar	6.7	C	1–2 days	*Escherichia coli*	25922	Growth; ± blue
				Shigella flexneri	12022	No growth or color change
Andrades fermentation	7.4	C	1–7 days			
Control (no carbohydrate added)				*Klebsiella pneumoniae*	13883	No color change or yellow
Adonitol				*Enterobacter aerogenes*	13048	Pink to red
				E. coli	25922	No color change or yellow
Arabinose				*E. aerogenes*	13048	Pink to red
				Serratia marcescens	43861	No color change or yellow
Cellobiose				*K. pneumoniae*	13883	Pink to red
				Morganella morganii	25830	No color change or yellow
Dulcitol				*Citrobacter freundii*	8090	Pink to red
				S. marcescens	43681	No color change or yellow
Glucose				*E. coli*	43861	Pink to red
				Moraxella osloensis	10973	No color change or yellow
Inositol				*E. aerogenes*	13048	Pink to red
				E. coli	25922	No color change or yellow
Lactose				*E. coli*	25922	Pink to red
				Proteus mirabilis	12453	No color change or yellow
Maltose				*E. coli*	25922	Pink to red
				P. mirabilis	12453	No color change or yellow
Mannitol				*E. coli*	25922	Pink to red
				P. mirabilis	12453	No color change or yellow
Melibiose				*E. aerogenes*	13048	Pink to red
				S. marcescens	43861	No color change or yellow
Raffinose				*E. aerogenes*	13048	Pink to red
				S. marcescens	43861	No color change or yellow
Rhamnose				*E. aerogenes*	13048	Pink to red
				S. marcescens	43861	No color change or yellow
Salicin				*E. aerogenes*	13048	Pink to red
				Salmonella sp.	35664	No color change or yellow
Sorbitol				*E. coli*	25922	Pink to red
				P. mirabilis	12453	No color change or yellow
Sucrose				*E. aerogenes*	13048	Pink to red
				Salmonella sp.	35664	No color change or yellow
Trehalose				*K. pneumoniae*	13883	Pink to red
				M. morganii	25830	No color change or yellow

(continued)

Table 14.2–2 Performance standards for bacteriological media *(continued)*

Medium	pH (±0.2)	Method of inoculation[a]	Length of incubation	Control organism[b]	ATCC[c] no.	Expected results[d]
Xylose				*K. pneumoniae*	13883	Pink to red
				M. morganii	25830	No color change or yellow
Bacteroides bile esculin agar	7.0	A, B	2–3 days	*Bacteroides fragilis*	25285	Growth; blackens agar
				Clostridium perfringens	13124	Inhibition
β-Hemolytic *Streptococcus* agar	7.3	A, B	1 day	*Streptococcus pneumoniae*	6305	Growth; α-hemolysis
				Streptococcus pyogenes	19615	Growth; β-hemolysis
				Staphylococcus aureus	25923	Inhibition
				E. coli	25922	Inhibition
Blood agar base[e]	7.4	A	1 day	*S. pneumoniae*	6305	Growth; α-hemolysis
				S. pyogenes	19615	Growth; β-hemolysis
				S. aureus	25923	Growth
				E. coli	25922	Growth
Blood culture[e]		A	1–7 days			
Aerobic (vented) bottle				*P. aeruginosa*	27853	Growth
				S. pneumoniae	6305	Growth
				Haemophilus influenzae	10211	Growth
Anaerobic (unvented) bottle				*S. pneumoniae*	6305	Growth
				B. fragilis	25285	Growth
Biphasic bottle				*Candida albicans*	10231	Growth
				S. pyogenes	19615	Growth
Bordet-Gengou agar	6.7	A, B	1–4 days	*Bordetella bronchiseptica*	10580	Growth
				Bordetella pertussis	12742	Growth
				S. aureus	25923	Inhibition
Brucella anaerobic agar	7.0		2–3 days			
Brucella agar[e]		A		*Porphyromonas levii*	29147	Growth
				Peptostreptococcus anaerobius	27337	Growth
				Fusobacterium nucleatum	25586	Growth
				C. perfringens	13124	Growth; β-hemolysis
				B. fragilis	25285	Growth
Brucella agar with neomycin		A, B		*C. perfringens*	13124	Growth; β-hemolysis
				B. fragilis	25285	Growth
				P. mirabilis	12453	Inhibition
Laked blood agar		A, B	1–2 days	*C. perfringens*	13124	Inhibition
				B. fragilis	25285	Growth
				E. coli	25922	Inhibition
				S. aureus	25923	Inhibition
Campylobacter agars		A, B	2–3 days	*Campylobacter jejuni*	33291	Growth
				Staphylococcus epidermidis	12228	Inhibition
				E. coli	25922	Inhibition
				P. mirabilis	12453	Inhibition
				P. aeruginosa	27853	Inhibition on some selective media but growth on Campy, BAP, Skirrow, THIO
				C. albicans	10231	Inhibition on some selective media but growth on Campy, Skirrow, Preston
Cetrimide agar	7.2	A, B	1 day	*P. aeruginosa*	27853	Growth; fluorescein pigment
				E. coli	25922	Inhibition
				S. maltophilia	13637	Inhibition

Table 14.2–2 *(continued)*

Medium	pH (±0.2)	Method of inoculation[a]	Length of incubation	Control organism[b]	ATCC[c] no.	Expected results[d]
Chocolate agar	7.2		1–3 days			
Chocolate agar		A		*Neisseria gonorrhoeae*	43069 or 43070	Growth
				H. influenzae	10211	Growth
Modified Thayer-Martin, Martin-Lewis, modified Martin-Lewis		A, B		*N. gonorrhoeae*	43069	Growth
				Neisseria meningitidis	13090	Growth
				S. epidermidis	12228	Inhibition
				E. coli	25922	Inhibition
				P. mirabilis	43071	Inhibition
				C. albicans	60193	Inhibition
				Neisseria sicca	9913	Inhibition
Clostridium difficile agar	7.4	A, B	2 days	*Clostridium difficile*	9689	Growth
				C. perfringens	13124	Inhibition
				B. fragilis	25285	Inhibition
				E. coli	25922	Inhibition
Columbia agar base[e]	7.3		1 day			
5% Sheep blood		A		*S. pyogenes*	19615	Growth; β-hemolysis
Colistin-nalidixic acid[e]		A, B		*S. pneumoniae*	6305	Growth; α-hemolysis
				S. aureus	25923	Growth
				P. mirabilis	12453	Inhibition
Cooked meat[e]	7.2	A	2 days	*C. perfringens*	13124	Growth; proteolysis
				B. fragilis	25285	Growth
Cystine lactose electrolyte-deficient agar (CLED agar)	7.3	A	1 day	*S. aureus*	25923	Growth; yellow
				E. coli	25922	Growth; yellow
				Proteus vulgaris	8427	Growth; blue-green
Cystine tryptic agar	7.3	D	1–2 days			
Cysteine proteose peptone agar	7.5	D	1–2 days			
Control (no carbohydrate added)				*N. gonorrhoeae*	43069	No color change or deep red
Glucose				*N. gonorrhoeae*	43069	Yellow
				Moraxella catarrhalis	25240	No color or deep red
Lactose				*Neisseria lactamica*	23971	Yellow
				N. gonorrhoeae	43069	No color or deep red
Maltose				*N. meningitidis*	13090	Yellow
				N. gonorrhoeae	43069	No color or deep red
Mannitol				*Enterococcus faecalis*	29212	Yellow
				S. pyogenes	19615	No color or deep red
Sorbitol				*E. faecalis*	29212	Yellow
				Streptococcus bovis	9808	No color or deep red
Sucrose				*N. sicca*	9913	Yellow
				N. gonorrhoeae	43069	No color or deep red
Decarboxylase (Moeller) broth	6.0	C	4 days			
Control (no amino acid added)				*K. pneumoniae*	13883	Yellow or no change
				Enterobacter cloacae	13047	Purple to yellow-purple
Arginine				*K. pneumoniae*	13883	Yellow or no change
Lysine				*K. pneumoniae*	13883	Purple to yellow-purple
				E. cloacae	13047	Yellow or no change
Ornithine				*E. cloacae*	13047	Purple to yellow-purple
				K. pneumoniae	13883	Yellow or no change

(continued)

Table 14.2–2 Performance standards for bacteriological media *(continued)*

Medium	pH (±0.2)	Method of inoculation[a]	Length of incubation	Control organism[b]	ATCC[c] no.	Expected results[d]
Deoxycholate agar[e]	7.3	A, B	1–2 days	*Salmonella enterica* serotype Typhimurium	14028	Growth; colorless
				S. flexneri	12022	Growth; colorless
				E. coli	25922	Growth; pink to red
				E. faecalis	29212	inhibition
Egg yolk agar	7.6	A	1–2 days	*C. perfringens*	13124	Lecithinase +
				Clostridium sporogenes	3584	Lipase +
Eosin methylene blue[e]	7.2	A	1 day	*S. enterica* serotype Typhimurium	14028	Growth; colorless
				E. faecalis	29212	Inhibition (partial)
				E. coli	25922	Growth; blue-black with metallic sheen
Esculin						
Bile esculin agar[e]	6.6	A, B	1–3 days	*E. coli*	25922	Inhibition
Bile esculin azide agar	7.1	A, B	1–2 days	*E. faecalis*	29212	Growth; blackens agar
				S. pyogenes	19615	Inhibition
Esculin agar	7.0	A	1–3 days	*K. pneumoniae*	13883	Growth; blackens agar
				E. coli	25922	Growth
Gardnerella agar	7.3	A, B	1–2 days	*Gardnerella vaginalis*	14018	Growth; β-hemolysis
				P. mirabilis	12453	Inhibition
Gelatin	6.8	C	2–14 days	*S. marcescens*	43861	Medium liquefied
				E. coli	25922	Medium remains solid
Gram-negative broth[e]	7.0	A, B	2–8 days	*S. enterica* serotype Typhimurium	14028	Growth on subculture
				Shigella sonnei	9290	Growth on subculture
				E. coli	25922	Inhibited
Heart infusion fermentation base	7.5		3–5 days			
Base control (no carbohydrate added)				*K. pneumoniae*	13883	No color change
Adonitol				*E. aerogenes*	13048	Yellow
				E. coli	25922	No color change
Arabinose				*E. aerogenes*	13038	Yellow
				S. marcescens	43861	No color change
Glucose				*E. coli*	25922	Yellow; gas
				M. osloensis	10973	No color change; no gas
Inositol				*E. aerogenes*	13048	Yellow
				E. coli	25922	No color change
Inulin				*S. pneumoniae*	6305	Yellow
				S. bovis	9809	No color change
Lactose				*E. coli*	25922	Yellow
				P. mirabilis	12453	No color change
Maltose				*E. coli*	25922	Yellow
				P. mirabilis	12453	No color change
Mannitol				*E. coli*	25922	Yellow
				P. mirabilis	12453	No color change
Melibiose				*E. aerogenes*	13048	Yellow
				S. marcescens	43861	No color change
Raffinose				*E. aerogenes*	13048	Yellow
				S. marcescens	43861	No color change
Rhamnose				*E. aerogenes*	13048	Yellow
				S. marcescens	43861	No color change

Table 14.2–2 *(continued)*

Medium	pH (±0.2)	Method of inoculation[a]	Length of incubation	Control organism[b]	ATCC[c] no.	Expected results[d]
Salicin				*E. aerogenes*	13048	Yellow
				Salmonella sp.	35664	No color change
Sorbitol				*E. coli*	25922	Yellow
				P. mirabilis	12453	No color change
Sucrose				*E. aerogenes*	13048	Yellow
				Salmonella sp.	35664	No color change
Trehalose				*E. coli*	25922	Yellow
				Edwardsiella tarda	15947	No color change
Hektoen enteric agar[e]	7.0	A, B	1–2 days	*S. enterica* serotype Typhimurium	14028	Growth; blue-green colonies ± black centers
				S. flexneri	12022	Growth; green with blue-green centers
				E. coli	25922	Partial inhibition; yellow colonies
Hippurate broth	7.3	C	2 days	*Streptococcus agalactiae*	12386	Precipitate that does not dissolve with shaking
				S. pyogenes	19615	Precipitate that dissolves with shaking
Legionella[e] agars	6.9–7.0	A, B	4 days	*Legionella pneumophila*	33152	Growth
				Legionella bozemanii	33217	Growth
				S. aureus	25923	Inhibition
				E. coli	25922	Inhibition
Loeffler	7.1	C	1 day	*Corynebacterium diphtheriae*	13812	Growth
				Corynebacterium pseudodiphtheriticum	10700	Growth
Lysine iron agar	6.7	D	1 day	*P. mirabilis*	12453	Red slant; H_2S; acid butt
				S. enterica serotype Typhimurium	14028	Alkaline slant; H_2S; alkaline butt
				S. flexneri	12022	Alkaline slant; acid butt
MacConkey MAC[e]	7.1	A, B	1–2 days	*P. mirabilis*	12453	Colorless colony; inhibition of swarming
				E. coli	25922	Red colony
				S. enterica serotype Typhimurium	14028	Colorless colony
				E. faecalis	29212	Inhibition
MacConkey-sorbitol agar	7.1	A, B	1–2 days	*E. coli* O157:H7	43888	Colorless colony
				E. coli	25922	Red colony
				E. faecalis	29212	Inhibition
Malonate broth	6.7	C	1–2 days	*E. coli*	25922	Green or yellow
				K. pneumoniae	13883	Blue to Prussian blue
Mannitol salt agar[e]	7.4	A, B	1–2 days	*S. aureus*	25923	Colonies have yellow zones
				S. epidermidis	12228	Colonies have red zones
				P. mirabilis	12453	Inhibition
Methyl red–Voges-Proskauer	6.9	C	1–5 days	*E. coli*	25922	MR +; VP −
				K. pneumoniae	13883	MR −; VP +
Motility-indole-lysine	6.6	D	1 day	*E. coli*	25922	Mot +; Ind +; Lys −
				K. pneumoniae	13883	Mot −; Ind −; Lys +

(continued)

Table 14.2–2 Performance standards for bacteriological media *(continued)*

Medium	pH (±0.2)	Method of inoculation[a]	Length of incubation	Control organism[b]	ATCC[c] no.	Expected results[d]
Motility-indole-ornithine	6.5	D	1 day	*E. coli*	25922	Mot +; Ind +; Orn +
				K. pneumoniae	13883	Mot −; Ind −; Orn −
Motility test	7.3	D	1 day	*E. coli*	25922	Positive
				K. pneumoniae	13883	Negative
New York City agar	7.2	A, B	1–2 days	*N. gonorrhoeae*	43069	Growth
				N. meningitidis	13090	Growth
				N. sicca	9913	Inhibition
				S. epidermidis	12228	Inhibition
				P. mirabilis	43071	Inhibition
				C. albicans	60193	Inhibition
				E. coli	25922	Inhibition
Nitrate reduction (agar or broth)	7.0	C	1–5 days	*P. mirabilis*	12452	Positive ± gas
				P. aeruginosa	27253	Positive + gas
				Acinetobacter baumannii	15308	Negative
Nutrient base[e]						
Broth	6.9	A	1 day	*S. aureus*	25923	Growth
				E. coli	25922	Growth
Agar	6.8	A	1 day	*S. aureus*	25923	Growth
				E. coli	25922	Growth
Oxidative-fermentative						
Hugh-Leifson	6.8	D	1–5 days			
King	7.3	D	1–5 days			
Control (no carbohydrate added)				*P. aeruginosa*	27853	No change
				K. pneumoniae	13883	No change
				M. osloensis	10973	No change
Fructose				*S. maltophilia*	13637	Oxidation
				K. pneumoniae	13883	Oxidation; fermentation
				M. osloensis	10973	No change
Glucose				*P. aeruginosa*	27853	Oxidation
				K. pneumoniae	13883	Oxidation; fermentation
				M. osloensis	10973	No change
Lactose				*Burkholderia cepacia*	25416	Oxidation
				K. pneumoniae	13883	Oxidation; fermentation
				M. osloensis	10973	No change
Maltose				*S. maltophilia*	13637	Oxidation
				K. pneumoniae	13883	Oxidation; fermentation
				M. osloensis	10973	No change
Mannitol				*B. cepacia*	25416	Oxidation
				K. pneumoniae	13883	Oxidation; fermentation
				M. osloensis	10973	No change
Sucrose				*S. maltophilia*	13637	Oxidation
				K. pneumoniae	13883	Oxidation; fermentation
				M. osloensis	10973	No change
Xylose				*B. cepacia*	25416	Oxidation
				K. pneumoniae	13883	Oxidation; fermentation
				M. osloensis	10973	No change
Phenol red						
Broth	7.4	C	3–5 days			
Agar	7.4	D	1 day			
Adonitol				*E. aerogenes*	13048	Yellow
				E. coli	25922	Red or no change

Table 14.2–2 *(continued)*

Medium	pH (±0.2)	Method of inoculation[a]	Length of incubation	Control organism[b]	ATCC[c] no.	Expected results[d]
Arabinose				*E. aerogenes*	13048	Yellow
				S. marcescens	43861	Red or no change
Arabitol				*K. pneumoniae*	13883	Yellow
				M. morganii	25830	Red or no change
Dulcitol				*C. freundii*	8090	Yellow
				S. marcescens	43861	Red or no change
Glucose				*E. coli*	25922	Yellow
				M. osloensis	10973	Red or no change
Inositol				*E. aerogenes*	13048	Yellow
				E. coli	25922	Red or no change
Lactose				*E. coli*	25922	Yellow
				P. mirabilis	12453	Red or no change
Raffinose				*E. aerogenes*	13048	Yellow
				S. marcescens	43861	Red or no change
Rhamnose				*E. aerogenes*	13048	Yellow
				S. marcescens	43861	Red or no change
Sorbitol				*E. coli*	25922	Yellow
				P. mirabilis	12453	Red or no change
Trehalose				*K. pneumoniae*	13883	Yellow
				M. morganii	25830	Red or no change
Phenylalanine agar	7.4	A	1 day	*P. mirabilis*	12453	Positive
				E. coli	25922	Negative
Phenylethyl alcohol agar[e]	7.5	A, B	1 day	*S. pneumoniae*	6305	Growth; α-hemolysis
				S. aureus	25923	Growth
				P. mirabilis	12453	Inhibition
				S. pyogenes	19615	Growth; β-hemolysis
Pigment production agar	7.5	C	1 day			
Fluorescein				*M. osloensis*	10973	No pigment
				Pseudomonas fluorescens	13525	Yellow-green
Pyocyanin				*M. osloensis*	10973	No pigment
				P. aeruginosa	27853	Blue-green
Potassium tellurite cystine agar	7.4	A, B	1–2 days	*C. diphtheriae*	13812	Growth; black colonies
				S. epidermidis	12228	Inhibition
Burkholderia cepacia agar	7.1	A, B	1–3 days	*B. cepacia*	25416	Growth
				P. aeruginosa	27853	Inhibition
				E. coli	25922	Inhibition
Purple broth	6.8	C	3–5 days			
Control (no carbohydrate added)				*K. pneumoniae*	13883	No change
				E. aerogenes	13048	No change
Glucose				*E. coli*	25922	Yellow
				M. osloensis	10973	No change
Lactose				*E. coli*	25922	Yellow
				P. mirabilis	12453	No change
Maltose				*E. coli*	25922	Yellow
				P. mirabilis	12453	No change
Raffinose				*E. aerogenes*	13048	Yellow
				S. marcescens	43861	No change
Rhamnose				*E. aerogenes*	13048	Yellow
				S. marcescens	43861	No change
Sucrose				*E. aerogenes*	13048	Yellow
				Salmonella sp.	35664	No change
Pyruvate broth	7.3	C	3–5 days	*E. faecalis*	29212	Yellow
				S. bovis	9809	No change or yellow-green

(continued)

Table 14.2–2 Performance standards for bacteriological media *(continued)*

Medium	pH (±0.2)	Method of inoculation[a]	Length of incubation	Control organism[b]	ATCC[c] no.	Expected results[d]
Regan-Lowe pertussis						
Agar	7.4	A, B	7 days	*B. pertussis*	12742	Growth
				S. aureus	25923	Inhibition
Transport	7.4	E	2 days	*E. coli*	25922	Inhibition
Salmonella-shigella agar[e]	7.0	A, B	1 day	*S. enterica* serotype Typhimurium	14028	Colorless colonies ± black centers
				S. flexneri	12022	Colorless colonies
				E. coli	25922	Inhibition or pink colonies
				E. faecalis	29212	Inhibition
Salt tolerance (6.5% NaCl)	7.3	C	1 day	*E. faecalis*	29212	Growth
				S. bovis	9809	No growth
Simmons citrate agar	6.0	C	1–2 days	*K. pneumoniae*	13883	Growth ± blue
				E. coli	25922	No growth or color change
Starch hydrolysis agar or broth	7.3	C	1–5 days	*S. bovis*	9809	Positive
				E. faecalis	29212	Negative
Streptococcus group B selective broth	7.6	B, C	1 day	*S. agalactiae*	12386	Growth
				E. coli	25922	Inhibition
Streptococcus selective (tryptic soy), sheep blood + neomycin, and sheep blood + trimethoprim-sulfamethoxazole	7.3	A, B	1 day	*S. pneumoniae*	6305	Growth; α-hemolysis
				S. pyogenes	19615	Growth; β-hemolysis
				S. aureus	25923	Inhibition
				P. mirabilis	12453	Inhibition
TCBS (Thiosulfate-citrate-bile salts agar)	8.6	A, B	1 day	*Vibrio alginolyticus*	17749	Yellow colony
				Vibrio parahaemolyticus	17802	Green colony
				E. coli	25922	Inhibition
Thioglycolate[e] broth (± indicator)	7.0	C	3–7 days	*S. aureus*	25923	Growth
				Clostridium novyi A	7659	Growth
				C. perfringens	13124	Growth
				P. levii	29147	Growth
				Bacteroides vulgatus	8482	Growth
Todd-Hewitt broth	7.8	C	4–18 h	*S. pyogenes*	19615	Growth
				E. faecalis	29212	Growth
Transport media						
Amies or Stuarts ± charcoal	7.3	E	6–8 h	*S. pyogenes*	19615	Growth on subculture
				N. gonorrhoeae	43069	Growth on subculture
Cary-Blair or enteric pathogen	8.4	E	1 day	*C. jejuni*	33291	Growth on subculture
				S. flexneri	12022	Growth on subculture
				Yersinia enterocolitica	9610	Growth on subculture
Triple-sugar iron agar	7.3	D	1 day	*S. enterica* serotype Typhimurium	14028	Alkaline slant; acid butt; H_2S +; ± gas
				E. coli	25922	Acid slant; acid butt; no H_2S; gas
				P. aeruginosa	27853	Alkaline slant and butt; no H_2S; no gas
Tryptic soy (anaerobic)						
Blood agar[e]	7.4	A	2 days	*F. nucleatum*	25586	Growth
				P. levii	29147	Growth
				P. anaerobius	27337	Growth
				C. perfringens	13124	Growth; β-hemolysis
				B. fragilis	25285	Growth

Table 14.2–2 *(continued)*

Medium	pH (±0.2)	Method of inoculation[a]	Length of incubation	Control organism[b]	ATCC[c] no.	Expected results[d]
Phenylethyl alcohol	7.3	A, B	2 days	*C. perfringens*	13124	Growth; β-hemolysis
				B. fragilis	25285	Growth
				P. mirabilis	12453	Inhibition
Tryptic soy (aerobic)						
Plain agar or broth[e]	7.3	A, C	1 day	*S. pyogenes*	19615	Growth
				E. coli	25922	Growth
Sheep blood agar[e]	7.5	A	1 day	*S. pneumoniae*	6305	Growth; α-hemolysis
				S. pyogenes	19615	Growth; β-hemolysis
				S. aureus	25923	Growth
				E. coli	25922	Growth
Tryptone	7.0	C	1 day	*E. coli*	25922	Positive for indole
				K. pneumoniae	13883	Negative for indole
Urea broth or agar	6.8	C	1 day	*P. mirabilis*	12453	Pink to red
				E. coli	25922	No change or pale yellow
Vaginalis agar	7.3	A	2–3 days	*G. vaginalis*	14018	Growth; β-hemolysis
				S. pneumoniae	6305	Growth; α-hemolysis
Wilkins-Chalgren anaerobic[e]						
Agar	7.2	A	1–2 days	*F. nucleatum*	25586	Growth
Broth	7.1	C		*P. levii*	29147	Growth
				P. anaerobius	27337	Growth
				B. fragilis	25285	Growth
				C. perfringens	13124	Growth
Xylose lysine deoxycholate agar[e] (XLD)	7.4	A, B	1–2 days	*S. enterica* serotype Typhimurium	14928	Red colony with black center
				S. flexneri	12022	Red colony
				E. coli	25922	Partial inhibition; yellow colony
				E. faecalis	29212	Inhibition
Yersinia selective agar[e]; CIN agar (cefsulodin-irgasan-novobiocin agar)	7.4	A, B	1 day	*Y. enterocolitica*	27853	Red "bull's eye" center with translucent border
				P. aeruginosa	25922	Inhibition
				E. coli	12453	Inhibition
				P. mirabilis	29212	Inhibition
				E. faecalis		Inhibition

[a] Abbreviations are as follows. A, for testing nutritive properties. Dilute standardized cell suspension (0.5 McFarland standard) 1:100 (0.1 ml of suspension plus 9.9 ml of saline). Inoculate each medium with 10 μl of dilution. If isolated colonies are not obtained, use 10-fold-lighter inoculum. B, for testing selective properties. Dilute standardized cell suspension (0.5 McFarland standard) 1:10 (0.1 ml of suspension plus 0.9 ml of saline). Inoculate each medium with 10 μl of dilution. C, for testing tubed media. Inoculate medium with 10 μl of the standardized suspension (0.5 McFarland standard). Adjust the inoculum if heavier or lighter growth is required. D, for testing biochemical media. Inoculate each medium according to the procedure for the routine use of the medium. E, for testing transport media. Place a sterile swab in the standardized suspension (0.5 McFarland standard), squeeze out excess fluid on the wall of the tube, and submerge the swab in the transport medium. Hold at room temperature, and plate on the appropriate medium.

[b] Organisms recommended for QC testing of media (2, 7–12, 14, 16, 17). Although American Type Culture Collection strains are listed, any organism that will yield an identical result is acceptable.

[c] ATCC, American Type Culture Collection.

[d] MR, methyl red; VP, Voges-Proskauer; Mot, motility; Ind, indole; Lys, lysine; Orn, ornithine; +, positive; −, negative; oxidation, color change in open tube; fermentation, color change in overlaid tube.

[e] Exempt from QC by user (14). Applies only to commercially prepared media.

Table 14.2–3 Performance standards for mycobacterial media and tests

Medium[a]	pH (±0.2)	Length of incubation	Control organism[b]	ATCC[c] no.	Expected results
Arylsulfatase agar	7.0	3 days, 14 days	*Mycobacterium fortuitum*	6841	Positive; pink to red
			Mycobacterium intracellulare	13950	Negative (no color change)
Catalase test		2 weeks	*Mycobacterium tuberculosis*	25177	Negative (no bubbles)
68°C			*M. fortuitum*	6841	Positive (bubbles)
Semiquantitative		2 weeks	*M. fortuitum*	6841	Positive (>45-mm column of bubbles)
			M. intracellulare	13950	Negative (<45-mm column of bubbles)
Dubos broth	6.6	4–8 weeks	*M. tuberculosis*	25177	Growth
			M. intracellulare	13950	Growth
Iron uptake		4 weeks	*M. fortuitum*	6841	Positive (rusty-brown colonies)
			Mycobacterium chelonae	35751	Negative (colonies not rusty brown)
Kirchner	7.3	4–8 weeks	*M. tuberculosis*	25177	Growth
			M. intracellulare	13950	Growth
Lowenstein-Jensen[d]	6.9	4–8 weeks	*M. tuberculosis*	25177	Growth
			M. intracellulare	13950	Growth
			Mycobacterium kansasii	12478	Growth
			Mycobacterium scrofulaceum	19981	Growth
			M. fortuitum	6841	Growth
			Escherichia coli	25922	Inhibition
MAC (without crystal violet)	7.4	5 days, 11 days	*M. fortuitum*	6841	Positive (growth)
			Mycobacterium phlei	11758	Negative (no growth)
Middlebrook[d] (selective)	6.6 6.9	4–8 weeks	*M. tuberculosis*	25177	Growth
			M. intracellulare	13950	Growth
			M. scrofulaceum	19981	Growth
			M. kansasii	12478	Growth
			M. fortuitum	6841	Growth
			E. coli	25922	Inhibition
Niacin		4–6 weeks	*M. tuberculosis*	25177	Positive (yellow)
			M. intracellulare	13950	Negative (no color change)
Nitrate reduction		3–4 weeks	*M. kansasii*	12478	Positive (pink to red)
			M. intracellulare	13950	Negative (no color change)
Pigment production		2 weeks	*M. kansasii*	12478	Positive (yellow after light exposure)
			M. intracellulare	13950	Negative (no color change)
Pyrazinamidase	6.6	4 days	*M. intracellulare*	13950	Positive (pink band in agar)
			M. kansasii	12478	Negative (no band in agar)
Salt tolerance		4 weeks	*M. fortuitum*	6841	Positive (growth)
			Mycobacterium gordonae	14470	Negative (no growth)
Tellurite reduction		7 days	*M. intracellulare*	13950	Positive (black precipitate)
			M. tuberculosis	25177	Negative (no black precipitate)
Thiophen-2-carboxylic acid hydrazide susceptibility		3 weeks	*Mycobacterium bovis*	35734	No growth
			M. tuberculosis	25177	Growth
Tween hydrolysis		1, 5, 10 days	*M. kansasii*	12478	Positive (pink to red)
			M. intracellulare	13950	Negative (no color change)
Urease broth		1–3 days	*M. kansasii*	12478	Positive (pink to red)
			M. intracellulare	13950	Negative (no color change)

[a] Inoculate primary plating media according to method A or B (detailed in Table 14.2–2, footnote *a*). Inoculate biochemical test media according to the procedure for each test.

[b] Organisms recommended for QC testing of media and tests (2, 7–12, 14, 15, 17). Although American Type Culture Collection strains are listed, any organism that will yield an identical result is acceptable.

[c] ATCC, American Type Culture Collection.

[d] Exempt from QC by user (14). Applies only to commercially prepared media.

Table 14.2–4 Performance standards for mycologic media

Medium[a]	pH (±0.2)	Length of incubation	Control organism[b]	ATCC[c] no.	Expected results
Ascospore agar	6.5	1–4 weeks	*Saccharomyces cerevisae*	9763	Ascospores
			Candida albicans	10231	No ascospores
BHI	7.4	1–3 days			
Plain[d]			*C. albicans*	60193	Growth
			Trichophyton mentagrophytes	9533	Growth
+ Sheep blood[d]			*T. mentagrophytes*	9533	Growth
			C. albicans	60193	Growth
			C. albicans	10231	Growth
+ Sheep blood + antimicrobial agent[d]			*T. mentagrophytes*	9533	Growth
			Escherichia coli	25922	Inhibition
			Aspergillus niger	16404	Inhibition in presence of cycloheximide
Birdseed (niger) agar	6.5	1–14 days	*Cryptococcus neoformans*	32045	Growth; brown pigment
			Cryptococcus laurentii	18803	Growth; no pigment
Bromcresol green agar	6.1	3 days	*C. albicans*	10231	Growth; pale yellow-green
			Candida krusei	6258	Growth; pale yellow-green; dry
			E. coli	25922	Inhibition
Casein agar	7.0	1–3 weeks	*Nocardia brasiliensis*	19296	Hydrolysis
			Nocardia asteroides	19247	No hydrolysis
Cornmeal agar					
± Polysorbate (Tween) 80	5.8	3 days	*C. albicans*	10231	Growth; chlamydospores
			Candida pseudotropicalis	8553	Growth; no chlamydospores
+ Glucose	5.8	1–4 weeks	*Trichophyton rubrum*	28188	Growth; red
			C. albicans	10231	Growth
Czapek-Dox agar	7.3	1–2 weeks	*A. niger*	16404	Growth
			N. asteroides	19247	Growth
Dermatophyte test[d]	5.5	7 days	*T. mentagrophytes*	9533	Growth; red
			C. albicans	10231	Reduced growth
			A. niger	16404	Inhibition
			E. coli	25922	Inhibition
Fungus selection agar[d]	6.9	4 weeks	*T. mentagrophytes*	9533	Growth
			C. albicans	10231	Growth
			A. niger	16404	Inhibition
			E. coli	25922	Inhibition
Inhibitory mold agar[d]	6.7	4 weeks	*A. niger*	16404	Growth
			C. albicans	10231	Growth
			E. coli	25922	Inhibition
			T. mentagrophytes	9533	Growth
Nickerson-bismuth sulfite-glucose-glycine-yeast agar	6.8	3 days	*C. albicans*	10231	Growth; colonies smooth; dark brown
			C. krusei	6258	Growth; colonies flat; dark brown; surrounded by halo
			E. coli	25922	Inhibition
Potato dextrose agar[d]	5.6	4 weeks	*C. albicans*	60193	Growth
			T. mentagrophytes	9533	Growth
Rice extract agar	5.8	1–2 days	*C. albicans*	10231	Growth; chlamydospores
			C. krusei	6258	Growth; chlamydospores absent
Sabouraud[d]	5.6	4 weeks	*C. albicans*	60193	Growth
Emmons modification[d]	6.9		*T. mentagrophytes*	9533	Growth
			E. coli	25922	Inhibition with antimicrobial agents
			A. niger	16404	Inhibition with cycloheximide; growth with gentamicin or chloramphenicol
Trichophyton agar	6.8	2–4 weeks	*T. mentagrophytes*	9533	Growth
			Trichophyton equinum		Inhibition
+ Nicotinic acid			*T. mentagrophytes*	9533	Growth
			Trichophyton tonsurans	28942	Inhibition

(continued)

Table 14.2–4 Performance standards for mycological media *(continued)*

Medium[a]	pH (±0.2)	Length of incubation	Control organism[b]	ATCC[c] no.	Expected results
Tyrosine agar	7.0	1–3 weeks	*N. brasiliensis*	19296	Hydrolysis
			N. asteroides	19247	No hydrolysis
Urea dextrose agar	6.8	4 days	*T. mentagrophytes*	9533	Pink to red
			Trichophyton rubrum	28188	No color
Xanthine agar	7.0	1–3 weeks	*Nocardia otitidiscaviarum*	14629	Hydrolysis
			N. asteroides	19247	No hydrolysis
Yeast carbohydrate assimilation	5.6	3 days			
Control (no carbohydrate added)			*C. albicans*	10231	No growth
			C. laurentii	18803	No growth
Cellobiose			*C. laurentii*	18803	Growth
			C. krusei	6258	No growth
Galactitol			*C. laurentii*	18803	Growth
			C. krusei	6258	No growth
Galactose			*C. laurentii*	18803	Growth
			C. krusei	6258	No growth
Glucose			*C. laurentii*	18803	Growth
Inositol			*C. laurentii*	18803	Growth
			C. krusei	6258	No growth
Lactose			*C. laurentii*	18803	Growth
			C. krusei	6258	No growth
Maltose			*C. laurentii*	18803	Growth
			C. krusei	6258	No growth
Melibiose			*C. laurentii*	18803	Growth
			C. krusei	6258	No growth
Raffinose			*C. laurentii*	18803	Growth
			C. krusei	6258	No growth
Trehalose			*C. laurentii*	18803	Growth
			C. krusei	6258	No growth
Xylose			*C. laurentii*	18803	Growth
			C. krusei	6258	No growth
Yeast carbohydrate fermentation broth	7.1	3 days			
Control (no carbohydrate added)			*C. albicans*	10231	Negative; no gas
			C. krusei	6258	Negative; no gas
Cellobiose			*Candida lusitaniae*	34449	Positive; gas
			C. albicans	10231	Negative; no gas
Galactose			*Candida kefyr*	2512	Positive; gas
			C. laurentii	18803	Negative; no gas
Glucose			*C. albicans*	10231	Positive; gas
			C. laurentii	18803	Negative; no gas
Lactose			*C. kefyr*	2512	Positive; gas
			C. laurentii	18803	Negative; no gas
Maltose			*C. albicans*	10231	Positive; gas
			C. kefyr	2512	Negative; no gas
Sucrose			*C. kefyr*	2512	Positive; gas
			C. krusei	6258	Negative; no gas
Trehalose			*Candida tropicalis*	13803	Positive; gas
			C. laurentii	18803	Negative; no gas

[a] Media can be inoculated directly from working slants. The inoculum is not standardized.

[b] Organisms recommended for QC of media and test (2, 7–12, 14, 15, 17). Although American Type Culture Collection strains are listed, any fungus that yields identical results is acceptable.

[c] ATCC, American Type Culture Collection.

[d] Exempt from QC by user (14). Applies only to commercial prepared media.

IV. SPECIFIC QUALITY PARAMETERS *(continued)*

c. Expiration-dating guidelines for plated media sealed in plastic (polyethylene or similar film), 10 plates per bag, at 2 to 8°C (unpublished data)

(1) Up to 20 weeks
Plain medium with a heavy fill (25 to 40 g); e.g., Sabouraud dextrose agar

(2) Up to 16 weeks
(a) Plain medium with standard fill (18 to 20 g); e.g., TSA
(b) Nonselective sheep blood agar
(c) Medium containing aseptically added supplements; heavy fill
(d) Enteric medium
(e) Selective and nonselective chocolate media
(f) Mueller-Hinton agar in 150-mm-diameter dishes

(3) Up to 12 weeks
(a) Medium with aseptically added supplements; standard fill
(b) Blood agar other than sheep blood
(c) Anaerobic sheep blood agar
(d) Selective sheep blood agar

(4) Up to 8 weeks
(a) Brucella sheep blood agar
(b) Rabbit blood agar
(c) Azide blood agar
(d) Campylobacter blood agar with antimicrobial agents

d. Expiration dating of tubed or bottled media (unpublished data)

◩ **NOTE:** Tubed and bottled media with tight caps may be stored at room temperature or at 2 to 8°C, depending on the presence of heat-labile components or dyes, tendency of media to darken at higher temperatures, or other factors.

(1) Up to 18 months
(a) Broth media with no aseptically added supplements; stored at 2 to 25°C; e.g., TSB or BHI
(b) Solid media with no aseptically added supplements; e.g., triple sugar iron agar (2 to 8°C) or nutrient agar (2 to 25°C)

(2) Up to 12 months
(a) Broth media with aseptically added supplements; 25°C; e.g., Middlebrook 7H9 broth
(b) Semisolid media with or without aseptically added supplements; 2 to 8°C; e.g., cystine tryptic agar or motility-nitrate medium
(c) Solid media with aseptically added supplements but no blood or serum; 2 to 8°C; e.g., chocolate agar slant or Middlebrook 7H10 agar

(3) Up to 6 months
Solid medium containing blood or serum and aseptically added supplements; e.g., BHI agar with sheep blood

4. Test procedure

a. Inoculum

(1) Suspend three to five isolated colonies of the test strain in 1 to 2 ml of soybean-casein digest medium, incubate for 4 to 5 h at 35°C, and adjust the turbidity to match that of a 0.5 McFarland standard.

(2) Alternatively, use a thawed stock culture suspension adjusted to a 0.5 McFarland standard.

(3) For mycobacteria, emulsify the growth in filter-sterilized diluent containing 2 g of bovine serum albumin and 0.2 ml of polysorbate (Tween) 80 per liter of deionized water (2). Add 8 to 12 sterile glass beads and vortex vigorously for 10 min. Let stand for 3 h to allow

IV. SPECIFIC QUALITY PARAMETERS *(continued)*

large particles to settle, and transfer the supernatant to sterile vials. Adjust the cell suspension to match a 0.5 McFarland standard and freeze. These vials may be stored for up to 6 months.

b. Inoculate media as described in Table 14.2–2 footnotes. The method is dependent on whether the medium is nutritive, selective, or diagnostic.

c. Incubate all test media under conditions normally used for media inoculated with clinical specimens.

d. Document all results on medium QC forms. See Appendix 14.2–5 and the specific procedures for examples of these forms.

e. Working control strains (14)

(1) Primary working control
Up to two serial subcultures of the primary control may be prepared.

(2) Secondary working control
Prepared from primary working control or its subculture and stored at 2 to 8° C or room temperature (22 to 25° C) for up to 4 weeks. Up to two serial subcultures of the secondary working control may be prepared. These subcultures are the tertiary controls.

(3) Inoculate medium to be tested from frozen stock vials or from primary, secondary, or tertiary working controls.

(4) Replace primary working control after two serial subcultures.

Include QC information on reagent container and in QC records.

C. Stains, reagents, and antisera (Tables 14.2–5 and 14.2–6)

1. Antisera are tested according to the manufacturer's recommendations in parallel with the current lot before use and every 6 months thereafter. The recommended frequency of testing varies with the accreditation organization (1).

2. The CAP accreditation program allows laboratories to use certain expensive reagents that are out of date. The laboratory must have a written policy specifying such reagents, the circumstances when extended usage applies, the special control procedures to be implemented, and the person authorizing usage (4).

Include QC information on reagent container and in QC records.

D. Commercial kits

1. Perform QC on each new shipment and each lot of the product according to the manufacturer's recommendation.

2. Components of a kit may not be interchanged with those of another kit of a different lot number unless specified by the manufacturer.

E. QC of mycology procedures
See section 8 of this handbook.

F. QC of parasitology procedures
See section 9 of this handbook.

G. QC of virology procedures
See section 10 of this handbook.

H. QC of antimicrobial susceptibility testing
See section 5 of this handbook.

Table 14.2–5 Performance standards for stains

Stain	Control organism[a]	ATCC[b] no.	Expected results	Frequency of testing
Acid-fast				
Fluorochrome	*Mycobacterium tuberculosis*	25177	Yellow-green fluorescing bacilli	Each lot and each day of use
	Escherichia coli	25922	No fluorescing bacilli	
Modified (*Nocardia*)	*Nocardia asteroides* *Streptomyces* sp.	19247	Pink-red bacilli Blue bacilli	Each lot and each day of use
Ziehl-Neelsen	*M. tuberculosis* *E. coli*	25177 25922	Pink-red bacilli Blue bacilli	Each lot and each day of use
Acridine orange	*E. coli* *Staphylococcus aureus*	25922 25923	Fluorescent bacilli Fluorescent cocci	Each lot and each day of use
Calcofluor white	*Candida albicans* *E. coli*	60193 25922	Fluorescent yeast cells No fluorescence	Each batch, lot number, and shipment when prepared or opened
Flagellum	*Proteus mirabilis* *Klebsiella pneumoniae*	12453 13883	Petritrichous flagella No flagella	Each lot and each use
Giemsa	Thin-film blood smear		Distinct staining of WBCs and RBCs	Each lot and each week of use
Gram	*E. coli* *S. aureus*	25922 25923	Gram-negative bacilli Gram-positive cocci	Each lot and each week of use
Iodine solution	Formalin-treated specimen with cysts		Visible cyst nuclei	Each lot
Methenamine-silver nitrate	Tissue containing fungus or *Pneumocystis jiroveci*		Gray-black fungus or *P. jiroveci* cysts	Each use
Periodic acid-Schiff	Specimen containing fungus		Magenta fungus; light-pink background	Each use
Spore	*Bacillus* sp.		Spores stain one color and bacillus stains with counterstain	Each lot and each week of use
Trichrome	Smear of polyvinyl alcohol-fixed specimen with protozoan		Nuclear components clearly stained	Each lot and each week of use

[a] Organisms recommended for QC testing of stains (2, 7–12, 15, 17). Although American Type Culture Collection strains are listed, any organism that yields identical results is acceptable.

[b] ATCC, American Type Culture Collection.

Table 14.2–6 Performance standards for reagents used for bacteria

Organism and reagent	Control organism[a]	ATCC[b] no.	Expected results	Frequency of testing
Aerobic bacteria				
Aminolevulinic acid, porphyrin test	*Haemophilus parainfluenzae*	7901	Positive; red fluorescence	Each batch, lot number, and shipment
	Haemophilus influenzae	43065	Negative; no fluorescence	
Bacitracin disk	*Streptococcus pyogenes*	19615	Zone of inhibition	Each batch, lot number, and shipment
	Streptococcus agalactiae	12386	No zone of inhibition	
β-Lactamase disks	*Staphylococcus aureus*	25923	Positive; red	Each lot and each day of use (other than cefinase)
	H. influenzae	10211	Negative; no color change	Each batch, lot number, and shipment (cefinase)
Campy-Pak envelope	*Campylobacter jejuni*	33291	Growth after 48 h	Each batch, lot number, and shipment
Catalase (3% H_2O_2)	*S. aureus*	25923	Positive (bubbles)	Each batch, lot number, and shipment
	S. pyogenes	19615	Negative (no bubbles)	
Coagulase	*S. aureus*	25923	Positive (any clotting)	Each batch, lot number, and shipment
	Staphylococcus epidermidis	12228	Negative (no clotting)	
Deoxycholate (10%) (bile solubility test)	*Streptococcus pneumoniae*	6305	Positive (lysis)	Each batch, lot number, and shipment
	Enterococcus faecalis	29212	Negative (no lysis)	
Ferric chloride (10%)	*Proteus vulgaris*	33420	Positive (green)	Each batch, lot number, and shipment
	Escherichia coli	25922	Negative (no color change)	
Indole (spot, Kovacs, or Ehrlich)	*E. coli*	25922	Positive (color depends on reagent)	Each batch, lot number, and shipment
	Pseudomonas aeruginosa	27853	Negative (no color change)	
Lead acetate strips (H_2S)	*P. vulgaris*	33420	Positive (brown)	Each batch, lot number, and shipment
	Klebsiella pneumoniae	13883	Negative (no color change)	
Methyl red	*E. coli*	25922	Positive (red)	Each batch, lot number, and shipment
	K. pneumoniae	13883	Negative (no color change)	
Ninhydrin	*S. agalactiae*	12386	Positive (purple)	Each batch, lot number, and shipment
	S. pyogenes	19615	Negative (no color change)	
Nitrate reagents	*E. coli*	25922	Positive (red)	Each batch, lot number, and shipment
	Acinetobacter baumannii	19606	Negative (no color change)	
ONPG[c]	*E. coli*	25922	Positive (yellow)	Each batch, lot number, and shipment
	Proteus mirabilis	29245	Negative (no color change)	
Optochin disk (10 mm)	*S. pneumoniae*	6305	Zone of inhibition ≥ 16 mm	Each batch, lot number, and shipment
	E. faecalis	29212	No zone of inhibition	
Oxidase	*P. aeruginosa*	27853	Positive (red to blue-black)	Each batch, lot number, and shipment
	E. coli	25922	Negative (no color change)	
Voges-Proskauer	*Enterobacter cloacae*	13047	Positive (red)	Each batch, lot number, and shipment
	E. coli	25922	Negative (no color change)	
X, V, and XV disks or strips	*H. influenzae*	10211	Growth around XV and only between X and V when closely spaced	Each batch, lot number, and shipment
Anaerobic bacteria disk tests				
Bile	*Bacteroides vulgatus*	8482	No zone of inhibition	Each batch, lot number, and shipment
	Fusobacterium nucleatum	25586	Zone of inhibition	
Colistin (10 μg)	*B. vulgatus*	8482	Resistant (<10-mm-diameter zone)	Each batch, lot number, and shipment
	F. nucleatum	25586	Susceptible (≥10-mm-diameter zone)	
Kanamycin (1,000 μg)	*B. vulgatus*	8482	Resistant (<10-mm-diameter zone)	Each batch, lot number, and shipment
	F. nucleatum	25586	Susceptible (≥10-mm-diameter zone)	

Table 14.2–6 *(continued)*

Organism and reagent	Control organism[a]	ATCC[b] no.	Expected results	Frequency of testing
Sodium polyanetholsulfonate	*Peptostreptococcus anaerobius*	27337	Zone of inhibition	Each batch, lot number, and shipment
Vancomycin (5 μg)	*B. vulgatus*	8482	Resistant (<10-mm-diameter zone)	Each batch, lot number, and shipment
	Clostridium perfringens	13124	Susceptible (≥10-mm-diameter zone)	
Ferric ammonium citrate (1%)	*Bacteroides fragilis*	25285	Positive (brown)	Each batch, lot number, and shipment
	Porphyromonas asaccharolytica	25260	Negative (no color change)	
GasPak envelope	*Clostridium novyi*	19402	Growth in 48 h	Each batch, lot number, and shipment
Indole (Ehrlich)	*P. asaccharolytica*	25260	Positive (red)	Each batch, lot number, and shipment
	B. fragilis	25285	Negative (no color change)	
Nagler's test	*C. perfringens* (+ antitoxin)	13124	Positive (precipitin formed upon addition of antitoxin)	Each batch, lot number, and shipment
	C. perfringens (− antitoxin)	13124	Negative (no precipitin formed upon addition of antitoxin)	
Nitrate	*C. perfringens*	13124	Positive (red)	Each batch, lot number, and shipment
	B. fragilis	25285	Negative (no color)	

[a] Organisms recommended for QC testing of reagent (2, 7–12, 15, 17). Although American Type Culture Collection strains are listed, any organism that yields identical results is acceptable.

[b] ATCC, American Type Culture Collection.

[c] ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

V. MAINTENANCE OF STOCK BACTERIOLOGY CULTURES

Include QC information on reagent container and in QC records.

A. Long-term storage (≥1 year) (3, 14)

1. Lyophilization

Obtain strains from commercial sources or prepare in-house. May be stored for years.

2. Frozen

a. Preparation of storage media

(1) Dispense sterile defibrinated sheep blood, rabbit blood, or soybean-casein digest broth containing glycerol (15% [vol/vol]) into plastic or glass vials. An alternative storage medium is sterile skim milk.

(2) The soybean-casein digest broth may be stored at 4°C for up to 6 months.

(3) Label vials with the date frozen and the organism code. Maintain an alphabetical listing of QC organisms with codes for the sources of the isolates.

b. Inoculation of storage medium

(1) From overnight growth on an appropriate nonselective medium prepare a dense suspension of cells. Vortex well.

(2) *Prepare enough vials to provide stock strains for 1 year.* Keep two vials as permanent stocks. Label these vials as permanent stocks.

(3) Freeze at −50° C or below. At −50°C, strains may be kept for 1 year. At −70°C or in liquid nitrogen, strains may be kept indefinitely. As a general rule, replace nonfastidious organisms every 5 years and fastidious organisms every 3 years.

V. MAINTENANCE OF STOCK BACTERIOLOGY CULTURES *(continued)*

c. Retrieval of frozen stock strains
(1) Remove vial and thaw contents rapidly in warm water. Use contents to initiate test, or subculture to a solid medium. Do not refreeze vial.
(2) Alternatively, remove vial and remove a portion of the frozen bacterial suspension with a wooden stick or loop. Subculture to a solid medium. Return vial immediately to freezer. Strains should be subcultured twice before being used as controls.
(3) Check strains annually for viability.

3. Alternative methods
Prepare soybean-casein digest deeps. Stab the deep with a nonfastidious QC strain, incubate overnight, and seal tube with Parafilm. Store at room temperature for 1 to 2 years.

B. Short-term storage (<1 year) (3, 14)

1. Media (available from commercial sources)
a. Cystine-tryptic agar without carbohydrate
Inject 1 ml of sterile horse serum into cystine-tryptic agar deeps for staphylococci, streptococci, and fastidious organisms.
b. Cooked meat without glucose (for anaerobes and facultative anaerobes)
Store at room temperature.
c. Soybean-casein digest deeps (for fastidious organisms)
d. Blood agar and chocolate agar slants (for fastidious streptococci and *Haemophilus* spp.)
Store for up to 2 weeks.

2. Inoculation of storage media
a. Label each tube with strain code and date of inoculation.
b. Inoculate with overnight growth of QC strain.
c. Incubate overnight, cap, and store at room temperature or 5°C.

3. Retrieval of stock strains
a. Remove a portion of the contents.
b. Subculture to a solid medium and incubate overnight.
c. Prepare new stock as needed or at 6 months.

VI. MAINTENANCE OF STOCK MYCOLOGY CULTURES

Include QC information on reagent container and in QC records.

A. Long-term storage (≥1 year) (3, 11)

1. Water culture method (simplest and most reliable)
a. Add 2 to 3 ml of sterile water to an actively sporulating culture on a potato dextrose agar slant.
b. Gently tease surface of growth to dislodge conidia without disrupting the medium.
c. Remove the suspension, and dispense it into sterile screw-cap vials.
d. Label vials with QC strain code and date of preparation.
e. Store vials tightly capped at room temperature. Organisms remain viable for years.
f. To retrieve stock organism, shake well and inoculate a drop to potato dextrose medium.

2. Sterile mineral oil overlay (sloppy and easily contaminated)
a. Cover the entire slant of an actively sporulating culture on potato dextrose agar with water-free sterile mineral oil.
b. Cap the tube tightly, and store at room temperature.
c. To retrieve stock organism, flame mouth of tube, obtain portion of growth (avoid aerial strands) with sterile inoculating wire, drain oil, and inoculate Sabouraud broth or slant. Incubate at 30°C until growth occurs.

VI. MAINTENANCE OF STOCK MYCOLOGY CULTURES *(continued)*

3. Frozen culture
   - **a.** Freeze an actively sporulating culture on potato dextrose agar at −70° C. Tube must be high-quality glass or it may break.
   - **b.** To retrieve stock organism, remove from freezer, immediately tease some of the growth from the slant, and inoculate potato dextrose agar.
   - **c.** Return stock to freezer. If the slant thaws, prepare a new culture.

B. Short-term storage (<6 months) (3, 11)

1. Store an actively sporulating culture on potato dextrose agar at 4°C for up to 6 months. Fastidious organisms require more frequent subculturing.
2. Frequent subculturing results in atypical or nonviable organisms.

VII. MAINTENANCE OF STOCK MYCOBACTERIOLOGY CULTURES

Include QC information on reagent container and in QC records.

A. Long-term storage (≥1 year) (3, 14)

1. Frozen
   - **a.** Grow isolate in Middlebrook broth, and dispense in vials labeled with organism's code and date of freezing. Place in a −70° C freezer.
   - **b.** Alternatively, suspend growth in skim milk or brucella broth plus 15% glycerol. Place in −70°C freezer.
   - **c.** To retrieve strain, thaw rapidly in a 37°C water bath. The stock suspension may be thawed and refrozen many times without loss of viability.
2. Refrigerated
   - **a.** Grow isolate on egg-based medium slants.
   - **b.** Store at 4°C for up to 1 year.

B. Short-term storage (<6 months) (3, 14)

1. Grow isolate on egg-based medium slants.
2. Store at room temperature in the dark for up to 6 months.

VIII. MAINTENANCE OF STOCK VIROLOGY, *CHLAMYDIA,* AND *CHLAMYDOPHILA* CULTURES

Include QC information on reagent container and in QC records.

Long-term storage of *Chlamydia* and *Chlamydophila* cultures (*see* section 8 of this handbook)

A. Pass a stock strain in cell culture until the monolayer is 80 to 100% infected.
B. Add 2 ml of sucrose phosphate glutamate to five infected tubes, and sonicate or vortex with glass beads for 2 min.
C. Pool the contents of the vials, and dispense 0.02 ml into small vials.
D. Freeze at −70°C.
E. To retrieve organism, rapidly thaw a vial.

IX. SELECTION OF TEST METHODS

See Appendix 14.2–3.

X. VALIDATION OF TEST METHOD

See Appendix 14.2–4.

REFERENCES

1. **August, M. J., J. A. Hindler, T. W. Huber, and D. L. Sewell.** 1990. *Cumitech 3A, Quality Control and Quality Assurance Practices in Clinical Microbiology.* Coordinating ed., A. S. Weissfeld. American Society for Microbiology, Washington, DC.
2. **Becton Dickinson Microbiology Systems.** 1991. *Quality Control and Product Information Manual for Prepared (Plated) and Tubed Media.* Becton Dickinson Microbiology Systems, Cockeyville, MD.
3. **Blazevic, D. J., C. T. Hall, and M. E. Williams.** 1976. *Cumitech 3, Practical Quality Control Procedures for the Clinical Microbiology Laboratory.* Coordinating ed., A. Balows. American Society for Microbiology, Washington, DC.
4. **Commission on Laboratory Accreditation.** 1990. *Inspection Checklist. Diagnostic Immunology and Syphilis Serology.* CAP, Northfield, IL.

REFERENCES *(continued)*

5. **Department of Health and Human Services.** 2003. Medicare, Medicaid, and CLIA programs; laboratory requirements relating to quality systems and certain personnel qualifications; final rule. *Fed. Regist.* **68:**3640–3714.
6. **Health Care Financing Administration.** 1988. Medicare, Medicaid, and CLIA programs; revision of the clinical laboratory regulations for the Medicare, Medicaid, and Clinical Laboratories Improvement Act of 1967 programs. *Fed. Regist.* **53:**29590–29632.
7. **Kirsop, B. E., and J. S. Snell.** 1984. *Maintenance of Microorganisms.* Academic Press, Inc., San Diego, CA.
8. **Kost, G. J.** 1990. Critical limits for urgent clinician notification at US medical center. *JAMA* **263:**704–707.
9. **McFaddin, J. F.** 1985. *Media for Isolation, Cultivation, Identification, and Maintenance of Medical Bacteria,* vol. 1. Williams & Wilkins Co., Baltimore, MD.
10. **Miller, J. M.** 1987. *Quality Control in Microbiology.* Centers for Disease Control, Atlanta, GA.
11. **Miller, J. M.** 1991. Quality control of media, reagents, and stains, p. 1203–1225. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), *Manual of Clinical Microbiology,* 5th ed. American Society for Microbiology, Washington, DC.
12. **Miller, J. M., and B. B. Wentworth (ed.).** 1985. *Methods for Quality Control in Diagnostic Microbiology.* American Public Health Association, Washington, DC.
13. **NCCLS.** 2002. *Clinical Laboratory Technical Procedure Manuals.* Approved Guideline–4th ed. NCCLS document GP2-A4. NCCLS, Wayne, PA.
14. **NCCLS.** 1996. *Quality Assurance for Commercially Prepared Microbiological Culture Media,* 2nd ed. Approved standard. NCCLS Document M22-A2. NCCLS, Wayne, PA.
15. **Remel.** 1990. *Remel Technical Manual.* Remel, Lenexa, KS.
16. **Weissfeld, A. S., and R. C. Bartlett.** 1987. Quality Control, p. 35. *In* B. J. Howard, J. Klaas, S. J. Rubin, A. S. Weissfeld, and R. C. Tilton (ed.), *Clinical and Pathogenic Microbiology.* C. V. Mosby Co., St. Louis, MO.
17. **Wicklund, G. D., and B. K. Horton.** 1990. *Manual of Product Technical Data Sheets.* PML Microbiologicals, Tualatin, OR.

APPENDIX 14.2–1

Sources for QC Guidelines

I. FEDERAL
- A. Clinical Laboratories Improvement Act of 1967 (1)
- B. Clinical Laboratories Improvement Amendments of 1988 (2, 3)
- C. Health Care Financing Administration
  Office of Survey and Certification
  6325 Security Blvd.
  Baltimore, MD 21207
  (301) 966-6821

II. STATE
- A. Contact the individual state accreditation or licensure agency
- B. JCAHO
  1 Renaissance Blvd.
  Oak Brook Terrace
  Chicago, IL 60181
  (708) 916-5600

III. ACCREDITATION OR CERTIFICATION SERVICES
CAP
325 Waukegan Rd.
Northfield, IL 60093-2750
(708) 446-8800

References

1. **Federal Register.** 1968. Clinical Laboratories Improvement Act of 1967. *Fed. Regist.* **33:**15297–15303.
2. **Federal Care Financing Administration.** 1988. Medicare, Medicaid, and CLIA programs; revision of the clinical laboratory regulations for the Medicare, Medicaid, and Clinical Laboratories Improvement Act of 1967 programs. *Fed. Regist.* **53:**29590–29632.
3. **Health Care Financing Administration.** 1990. Medicare, Medicaid, and CLIA programs; revision of laboratory regulations; final rule with request for comments. *Fed. Regist.* **55:**19538–19610.

APPENDIX 14.2–2

Proficiency Testing Services for Hospital and Office Laboratories

For hospital and office laboratories

CAP
325 Waukegan Rd.
Northfield, IL 60093-2750
(312) 446-8800

American Association of Bioanalysts
205 West Levee St.
Brownsville, TX 78520
(800) 338-2746

American Academy of Family Physicians
8880 Ward Pkwy.
Kansas City, MO 64114
(800) 274-2237

Wisconsin Proficiency Testing Program
State Laboratory of Hygiene
465 Henry Mall
Madison, WI 53706
(608) 262-0027

For office laboratories only

American Society of Internal Medicine
Medical Laboratory Evaluation
1101 Vermont Ave. N.W. Suite 500
Washington, DC 20005
(800) 338-2746

APPENDIX 14.2–3

Selection of Test Method (1)

I. OBJECTIVE

To identify, from the numerous tests available, the procedure that will be the best predictor of the presence or absence of disease. The final selection of the best test procedure is based upon consideration of the following factors applied to each unique situation.

II. FACTORS FOR CONSIDERATION

A. Clinical need for test
   1. Test volume
   2. Requirements of test requester (e.g., screening or diagnostic)
   3. Turnaround time (e.g., stat or routine)

B. Seriousness and treatability of disease

C. Prevalence of disease

D. Economic, social, and psychological consequences of a false test result

E. Range of available test options (i.e., different test methods)

F. Comparison of test methods (e.g., accuracy, precision, sensitivity, specificity, and predictive value)

G. Test costs (e.g., reagents, labor, overhead)

H. Specimen and requisition requirements

I. Availability of technically skilled personnel to perform tests

J. Physical-plant requirement (e.g., space and utilities)

Reference

1. **McPherson, B. S., and C. A. Needham.** 1987. Method evaluation and test selection, p. 27–33. *In* B. B. Wentworth (ed.), *Diagnostic Procedures for Bacterial Infections,* 7th ed. American Public Health Association, Washington, DC.

APPENDIX 14.2–4

Validation of the Test Method

I. INTRODUCTION

The laboratory must document the performance and comparability of a new test method before it is used routinely (3). The 1988 Clinical Laboratories Improvement Amendments does not dictate specific threshold values that determine the acceptability of a test method. Rather, *each laboratory is expected to document the performance of the test and make this information available to the clinician for use in the interpretation of a test result.* The procedure for the evaluation of a test method should include, where applicable, the precision, linearity, accuracy, sensitivity, specificity, and predictive value of a test and reference ranges for the patient population. In general, precision and linearity are not determined for culture methods. When noncultural methods are assessed, precision checks may be performed on control samples to determine the mean and standard deviation of replicate determinations. Linearity may be determined through the use of serial dilutions of external control materials or by utilizing extremely elevated patient samples. Interference checks may be accomplished by reviewing the literature or by the addition of recognized interfering substances to samples.

II. COMPARABILITY OF TEST METHODS

A. Methods of assessing test comparability
   1. Test patient samples in parallel with an established test.
   2. Test specimens with known potencies.
   3. Compare test results with the actual diagnosis by chart review.

B. Statistical methods used for evaluation of test results
   1. Linear regression analysis
   2. Coefficient of variation
   3. Mean and standard deviation
   4. Predictive value, sensitivity, specificity, and efficiency
   5. Confidence intervals (CIs)

III. DETERMINATION OF THE SENSITIVITY, SPECIFICITY, PREDICTIVE VALUE, AND EFFICIENCY OF A TEST (1, 4)

A. Necessary conditions
   1. The test provides a negative or positive result.
   2. The true patient status is known.

B. Arrange data in a two-way frequency table (Table 14.2–A1).

C. Calculate statistical parameters.
   1. Sensitivity is the frequency of positive tests in patients with the disease (percentage of true positives among all positive patients): sensitivity $= [a/(a + c)] \times 100$, where a is the number of true negatives and c is the number of false negatives.
   2. Specificity is the frequency of a negative test in patients not infected (percentage of true negatives among all negative patients): sensitivity $= [d/(b + d)] \times 100$, where d is the number of true negatives and b is the number of false positives.
   3. Predictive value of a positive test (PVP) is the probability that a positive result indicates that a patient has the disease (percentage of true positives among all positive tests): PVP $= [a/(a + b)] \times 100$.
   4. Predictive value of a negative test (PVN) is the probability that a negative result indicates that the patient is not infected (percentage of true negatives among all negative tests): PVN $= [d/(c + d)] \times 100$.

Table 14.2–A1 Format for a positive-negative diagnostic test

New test result	Possible interpretation when disease[a]:	
	Exists	Does not exist
Positive	a (true positive)	b (false positive)
Negative	c (false negative)	d (true negative)

[a] True positive, number of diseased patients correctly identified by test; false positive, number of nondiseased patients incorrectly identified by test; true negative, number of nondiseased patients correctly identified by test; false negative, number of diseased patients incorrectly identified by test.

APPENDIX 14.2–4 *(continued)*

5. Efficiency, or accuracy, is the frequency with which patients are correctly identified by the test result: efficiency = $[(a + d)/(a + b + c + d)] \times 100$.

IV. EFFECT ON STATISTICAL PARAMETERS $[(a + c)/(a + b + c + d)]$ OF DISEASE IN THE POPULATION (5)

A. The sensitivity and specificity of a test are independent of the prevalence of the disease in a population.

B. PVP, PVN, and efficiency are dependent on the sensitivity and specificity of the test and on the prevalence of the disease.

C. Example (Table 14.2–A2)

Assume that two populations of 100 patients each are tested with two tests with different sensitivities and specificities. The prevalence of disease is 50% in population A and 5% in population B. This comparison highlights several interactions.

1. Increasing the prevalence of the disease increases the number of positive and false-negative results, which increases the PVP but decreases the PVN.
2. Decreasing the prevalence of the disease increases the number of true negatives and false-positive results, which increases the PVN but decreases the PVP.
3. The PVP and PVN are affected more by the prevalence of the disease than by the sensitivity and specificity of the test.
4. In populations with a low prevalence of disease, negative test results are better predictors of the disease state than positive test results.
   a. A test with high sensitivity is used to screen a low-prevalence population. In this situation, a negative test accurately reflects the absence of disease.
   b. A second test method with high specificity is used to evaluate the positive specimens. This greatly improves the PVP.

V. USEFULNESS OF CIs FOR THE COMPARISON OF TEST METHODS (2, 6)

A. The CI is the interval containing the unknown true value of the parameter with a known probability.

B. CIs are used to indicate the precision (or imprecision) of the statistical parameter of the test as the population varies.

C. CIs are affected by the sample size and the level of confidence (e.g., 90, 95, or 99%).

D. The larger the CI, the less precise the estimate of sensitivity, specificity, PVP, and PVN.

E. CIs can be found in statistical tables or calculated (Table 14.2–A3)

Table 14.2–A2 Effects of sensitivity, specificity, and disease prevalence on test parameters

Parameter or result	Population A		Population B	
	Test 1	Test 2	Test 1	Test 2
Prevalence (%)	50	50	5	5
Sensitivity	0.7	0.9	0.7	0.9
Specificity	0.9	0.7	0.9	0.7
True positive (no.)	35	45	4	5
False positive (no.)	5	15	9	28
True negative (no.)	45	35	86	67
False negative (no.)	15	5	1	0
PVP	0.88	0.75	0.31	0.15
PVN	0.75	0.88	0.99	1.00
Efficiency	0.80	0.80	0.90	0.72

APPENDIX 14.2–4 *(continued)*

Table 14.2–A3 Determination of CI[a]

Test	Sample size (no. of patients)	Sensitivity (%)	95% CI (%)[b]
A	50	80	68–92
B	50	90	82–98

[a] Standard error (SE) = $[p(1 - p)]/n$, where p is the proportion of subjects with a parameter (e.g., sensitivity) in a sample size n. SE = $[0.8 \times (1 - 0.8)]/50$; SE = 0.06.

[b] Calculation of CI with 95% confidence level. 95% CI = $p - (1.96 \times \text{SE})$ to $p + (1.96 \times \text{SE})$; 95% CI = $0.8 - (1.96 \times 0.06) = 68$; 95% CI = $0.8 + (1.96 \times 0.06) = 92$. Normal distribution values for confidence levels are 1.65 for 90%, 1.96 for 95%, and 2.58 for 99%.

References

1. **Galen, R. S., and S. R. Gambino.** 1975. *Beyond Normality: the Predictive Value and Efficiency of Medical Diagnosis.* John Wiley & Sons, Inc., New York, NY.
2. **Gardner, M. J., and D. G. Altmann.** 1989. *Statistics with Confidence: Confidence Intervals and Statistical Guidelines.* British Medical Journal Publishers, London, United Kingdom.
3. **Health Care Financing Administration.** 1988. Medicare, Medicaid, and CLIA programs; revision of the clinical laboratory regulations for the Medicare, Medicaid, and Clinical Laboratories Improvement Act of 1967 programs. *Fed. Regist.* **53:**29590–29632.
4. **Ilstrup, D. M.** 1990. Statistical methods in microbiology. *Clin. Microbiol. Rev.* **3:**219–226.
5. **McPherson, B. S., and C. A. Needham.** 1987. Method evaluation and test selection, p. 27–33. *In* B. B. Wentworth (ed.), *Diagnostic Procedures for Bacterial Infections*, 7th ed. American Public Health Association, Washington, DC.
6. **Washington, J. A.** 1990. Confidence intervals. *Clin. Microbiol. Newsl.* **12:**109–110.

APPENDIX 14.2–5

Sample QC Forms (*see* p. 14.2.30 to 14.2.34)

Microbiology Laboratory Procedure Manual

A. Urine specimens

Date prepared: By:

Date accepted: By:

Reviewed and updated	Date: _____	By: _____
	_____	_____
	_____	_____
	_____	_____
	_____	_____
	_____	_____

REAGENT: COAGULASE (BBL rabbit plasma)

Year _____

CONTROL ORGANISMS – Expected results

(+) *Staphylococcus aureus* ATCC 25923

Slide: clumping when organisms is added to plasma

Tube: clotting when incubated for 3 hours or overnight

(−) *Staphylococcus epidermidis* ATCC 12228

Slide: no clumping when organism added to plasma

Tube: no clotting overnight

FREQUENCY: As prepared

Date	Tech	Plasma lot no. Prep. date	Expiration date	*Staphylococcus aureus* Slide +	Tube +	*Staphylococcus epidermidis* Slide neg.	Tube neg.	Comments/corrective action

	INCUBATION	ORGANISMS (ATCC)	
TRYPTICASE SOY AGAR	O_2, 24 h	*Streptococcus pyogenes* (19615)	Growth, β-hemolysis
WITH 5% SHEEP BLOOD		*Streptococcus pneumoniae* (6305)	Growth, α-hemolysis
	CO_2, 24 h	*Staphylococcus aureus* (25923)	Growth
		Escherichia coli (25922)	Growth

Place label from one bag of representative media received here

Cracked plate _____

Cracked agar _____

Unequal filling _____

Hemolysis _____

Freezing _____

Bubbles/pits _____

Contamination _____

Date received Initials

_____

Comment

Medium Preparation QC

Year _____

Date	Tech	Medium prepared	Manufacturer of powder	Lot no. of powder	Additives	Lot no. of additives	Quantity made	Form (tubes/plates, etc.)	pH (if needed)

Medium QC

Year _____

Media

Physical appearance

Acceptable =

Sterility:

Acceptable =

Performance

Acceptable =

References:

Date rec'd	Tech	Date checked	Source	Lot no.	Expiration date	Appearance[a]		Sterility[a]		Performance[a]		Comments/corrective action
						A	NA	A	NA	A	NA	

[a]A, acceptable; NA, not acceptable.

14.3 Laboratory Records

I. INTRODUCTION

"If it hasn't been recorded, it hasn't been performed." This sentence summarizes the absolute requirement for detailed and accurate record retention for all aspects of the clinical microbiology laboratory. Records serve at least four purposes. (i) They document what has transpired without recourse to memory (they provide a paper or electronic audit trail), (ii) they serve as a point of reference for developing the facts regarding any incident, (iii) they assist in the recognition of trends and the resolution of problems, and (iv) they establish the laboratory's credibility. The standards for record keeping and the length of time records must be maintained are established by the agencies presented in Appendix 14.3–1. Federal guidelines should be considered minimum standards and are superseded by the standards established by the states or other certification agencies.

II. FORMAT

A. Style

Most records are paper forms that are manually completed by personnel or that may involve specialized computer software programs that generate hard copies as necessary.

B. Organization

Most records are organized and stored in a chronological, numerical, or alphabetical system. Whichever approach is most efficient for the specific needs of the laboratory is acceptable.

III. RECORD RETENTION

A. Length of retention

Suggested guidelines for the retention of laboratory records are presented in Table 14.3–1. As a general guideline, records must be retained for a minimum of 2 years. Exceptions include instrument records, which are retained for the life of the instrument, and personnel and safety records, which must be held for a minimum of 5 years.

B. Accessibility

Records must be readily accessible for review.

IV. ESSENTIAL LABORATORY RECORDS

A. Accession list (*see* Appendix 14.3–2, p. 14.3.7)

1. Principle

The accession list provides a record of all specimens received by the laboratory.

2. Requisite information

- **a.** Patient name
- **b.** Patient identifying number (social security, hospital, or laboratory accession number)
- **c.** Specimen source and type
- **d.** Test(s) requested

Table 14.3–1 Suggested guidelines for minimal time of retention for laboratory records

Record	Retention period (yr)	Comments
Accession list	2	May also function as a work list.
Requisition	2	May be combined with test result form.
Work card	2	Store with requisition and patient test result(s).
Test report	2	Includes all preliminary reports. Place in patient's hospital record. Identical legally reproduced copy is retained by laboratory.
QC/QA	2	Store originals of manuals and test procedures that have been modified or replaced for 2 years.
Instrument maintenance	2	Store records in close proximity to instrument and assure access by instrument users.
Incident report	2	
Safety	5	
Reportable disease	2	*Note:* Reportable disease requirements may vary by state.
Personnel	5	

IV. ESSENTIAL LABORATORY RECORDS *(continued)*

e. Collection date and time
f. Date and time of receipt and processing of specimen in microbiology laboratory

3. Specimen acceptability
a. Document reason(s) for the rejection of a specimen (e.g., improper labeling, collection, or specimen transport).
b. Document specimen disposition (e.g., "Specimen grossly and microscopically resembles saliva; please resubmit").

B. Requisition (*see* Appendix 14.3–2, p. 14.3.8)

1. Principle

The requisition is a record of the test(s) on the specimen requested by the patient's physician.

2. Requisite information
a. Patient name
b. Patient identifying number (e.g., social security, hospital, or laboratory accession number)
c. Specimen source and type
d. Laboratory test(s) requested
e. Date and time of specimen collection
f. Date and time of receipt and processing of the specimen in the microbiology laboratory
g. Physician's name
h. Patient's location

3. Additional useful information
a. Pertinent clinical information (e.g., diagnosis, antimicrobial therapy, etc.)
b. Contact information for patient's physician (e.g., telephone number, pager number, or cellular telephone number)

C. Work card or LIS electronic specimen worksheet (*see* Appendix 14.3–2, p. 14.3.9 through 14.3.11)

1. Principle

A work card or unique laboratory accession number with associated electronic worksheet is assigned to each specimen so that procedures performed on the specimen, notes made by microbiology laboratory personnel, results obtained, and interactions between physicians and microbiology staff can be

IV. ESSENTIAL LABORATORY RECORDS *(continued)*

recorded. A properly completed work card or laboratory information system (LIS) specimen worksheet can be used to reconstruct and assess the accuracy of the final report.

2. Requisite information
- **a.** Patient name
- **b.** Patient identifying number (e.g., social security, hospital, or laboratory accession number)
- **c.** Source and type of specimen
- **d.** Test(s) requested
- **e.** Initials of laboratory personnel performing procedures
- **f.** Procedure, date performed, and (when applicable) medium, reagent, or stain used
- **g.** Preliminary and final results (e.g., Gram stain results, description and quantification of growth, or antimicrobial susceptibility testing results)
- **h.** Specific details of verbal or telephone discussions with health care personnel
  - (1) Time and date of telephone call
  - (2) Nature of call
  - (3) Name of individual initiating the call
  - (4) Name of person receiving the information

D. Test reports (*see* Appendix 14.3–2, p. 14.3.12)

1. Principle

Test reports convey laboratory data to the requesting physician. The report becomes a permanent component of the patient's hospital record.

2. Requisite information
- **a.** Patient's name and location
- **b.** Patient identifying number (e.g., social security, hospital, or laboratory accession number)
- **c.** Name of physician ordering the test
- **d.** Type and source of specimen
- **e.** Test(s) requested
- **f.** Time and date of specimen receipt in the microbiology laboratory
- **g.** Time and date of test completion
- **h.** Name(s) of laboratory personnel completing the test(s)

3. Types of reports
- **a.** Telephone report: to report information that is critical for care of the patient. Examples include positive microscopic smears, antigen detection tests, growth from normally sterile body sites (e.g., CSF, blood, or other usually sterile body fluids), and detection of causative agents of highly infectious diseases (e.g., positive smear or growth in culture of mycobacteria).
- **b.** Preliminary report: to report status of test within 24 to 48 h after specimen receipt.
- **c.** Final report: to convey results after test completion. Results should be reviewed for erroneous information prior to placement in the patient's medical record.
- **d.** Corrected report
  - (1) Contact the physician and explain that an erroneous test result was reported.
  - (2) Reissue a report clearly indicating that it represents a corrected report.
  - (3) Do not remove the original erroneous report from the patient's chart.

4. Characteristics of reports
- **a.** Whenever possible, include interpretive statements (e.g., gram-positive lancet-shaped diplococci resembling *Streptococcus pneumoniae*).

IV. ESSENTIAL LABORATORY RECORDS *(continued)*

b. Report Gram stain and bacterial growth results in a semiquantitative manner (e.g., 1+, 2+, 3+, 4+, few, moderate, or many).
c. Include statements regarding any recognized improper storage, collection, or handling.
d. Include normal range values with numerical data.
e. Report all isolates recovered from normally sterile body sites.
f. Report only relevant antimicrobial susceptibility testing results (e.g., do not report the results for nitrofurantoin for isolates recovered from other than urinary tract specimens).
g. Report negative results for applicable tests (e.g., "no *Salmonella, Shigella, Campylobacter* spp., or *E. coli* O157:H7 recovered").

E. QC and QA (*see* Appendix 14.3–2, p. 14.3.13 through 14.3.15)

See procedures 14.1 and 14.2 above and Appendix 14.3–2 for examples of records.

1. Principle

The records of control values for procedures, tests, and equipment are used to detect and identify problems and trends within the laboratory. QA reports assist in the identification of problems and trends associated with the application of laboratory data by the end user.

2. Requisite information
a. Name of test, procedure, or equipment
b. Date
c. Name of person(s) performing the test or recording the data
d. Tolerance limits (acceptable range of values)
e. Control result
f. Lot number(s) of reagent(s) used
g. Expiration date(s) of reagent(s) used
h. Corrective action(s)
i. Name of person reviewing results

3. Types of QC records
a. Media
b. Tests and procedures
c. Proficiency tests
d. Temperature records for water baths, incubators, refrigerators, etc.
e. Reviews of procedure manuals
f. Equipment preventive maintenance, troubleshooting, and repair records. These records should be kept in proximity to the instrument.
g. Equipment function checks

4. Review of records
a. *All QC records must be reviewed and signed at least monthly by the supervisor or director of the laboratory.*
b. When results exceed predefined tolerance limits, identify the problem and document on the form any corrective action(s) taken

F. Incident (problem) reports (*see* Appendix 14.3–2, p. 14.3.16)

1. Principle

Incident reports document problems related to the performance of care givers (e.g., collection, labeling, or transport of specimens; complaints; uncooperative behavior) and microbiology laboratory personnel (e.g., specimen processing, failure to follow procedures, safety violations). Equally as important, the incident reports may be used to document commendable performance.

2. Requisite information
a. Identification of the problem
b. Date
c. Name(s) of individual(s) involved

IV. ESSENTIAL LABORATORY RECORDS *(continued)*

d. Corrective action(s) taken
e. Name(s) of reviewer(s)
f. Date(s) of review(s)

G. Safety records (*see* Appendix 14.3–2, p. 14.3.17)

1. Principle

Safety records provide documentation that all employees with a potential occupational exposure to hazardous chemicals or potentially infectious material have participated in an appropriate training program at the time of hire and annually thereafter.

2. Requisite information

a. Dates of training sessions
b. Contents or summary of training sessions
c. Name(s) of individual(s) conducting training
d. Name(s) of person(s) attending training

H. Reportable-disease record (*see* Appendix 14.3–2, p. 14.3.18)

1. Principle

The reportable-disease record reports cases of infectious diseases as required by municipal, county, and/or state public health departments. The report may be based upon a clinical syndrome, isolation of a microbial species, or a positive serologic test.

2. Requisite information

a. Demographics of patient (as available)
(1) Name
(2) Address
(3) Telephone number
(4) Social security number
(5) Occupation
b. Disease
c. Test result(s)
d. Name and address of patient's physician

I. Personnel record (*see* Appendix 14.3–2, p. 14.3.19)

1. Principle

The personnel record includes information on ongoing personnel training, as well as the levels of certification and training of microbiology laboratory personnel.

2. Requisite information

a. Employee name
b. Past training and education
(1) College
(2) Specialized laboratory training in certified programs
(3) Certifying agency or agencies
(4) Certificate and/or registration number(s)
(5) Experience
c. On-the-job training
(1) Document tasks and procedures that the person is trained to perform.
(2) Explain nature of training.
d. Continuing-education training
Document courses attended by the employee.
e. Performance appraisal(s)

SUPPLEMENTAL READING

Federal Register. 2003. Medicare, Medicaid, and CLIA Programs; laboratory requirements relating to quality systems and certain personnel qualifications; final rule. *Fed. Regist.* **68:**13640–13714.

APPENDIX 14.3–1

Requirements for Laboratory Records

I. FEDERAL
   A. Clinical Laboratory Improvement Act of 1967 (1)
   B. Clinical Laboratory Improvement Amendments of 1988 and 1990 (2, 3)
   C. Department of Labor, OSHA (4)

II. STATE
   Contact the individual state accreditation or licensure agency.

III. ACCREDITATION OR CERTIFICATION SERVICES
   CAP
   325 Waukegan Rd.
   Northfield, IL 60093-2750
   (708) 446-8800

   Health Care Financing Administration
   Office of Survey and Certification
   6325 Security Blvd.
   Baltimore, MD 21207
   (301) 966-6821

   JCAHO
   1 Renaissance Blvd.
   Oak Brook Terrace
   Chicago, IL 60181
   (708) 916-5600

References

1. **Federal Register.** 1968. Clinical Laboratories Improvement Act of 1967. *Fed. Regist.* **33:**15297–15303.
2. **Health Care Financing Administration.** 1988. Medicare, Medicaid, and CLIA programs; revision of the clinical laboratory regulations for the Medicare, Medicaid, and Clinical Laboratories Improvement Act of 1967 programs. *Fed. Regist.* **53:**29590–29632.
3. **Health Care Financing Administration.** 1990. Medicare, Medicaid and CLIA programs; revision of laboratory regulations; final rule with request for comments. *Fed. Regist.* **55:**19538–19610.
4. **Occupational Safety and Health Administration.** 1989. Occupational exposure to bloodborne pathogens: proposed rule and notice of hearing. *Fed. Regist.* **54:**23134–23139.

APPENDIX 14.3–2

Examples of Laboratory Forms and Records (*see* p. 14.3.7 to 14.3.19). For abbreviations used on p. 14.3.9 and 14.3.10, refer to the following document: **NCCLS.** 2003. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically.* Approved standard M7-A6, 6th ed., p. 68–70. NCCLS, Wayne, PA.

March 16, 2003 15:37

Laboratory Service Microbiology Accessions (March 16, 2003–March 16, 2003)

= Not a MMC patient

Count	History no.	Patient	Accession no.	Date	Location	Specimen	Test
1)	000465854	Jones, Robert	S41321	03/16	EMAJ	Urine	C&S
2)	000547485	Smith, Pamela	S12321	03/16	KLAB	Stool	C&S
3)	000756544	Taylor, Stephen	S54656	03/16	PIC	Blood	C&S
4)	000545855	Redford, Patricia	S55554	03/16	MIC	CSF	C&S Gram stain
5)	00087452	Reznyk, John	S54327	03/16	CDHU	BAL	AFB cult Gram stain
6)	00035412	Jackson, Mary	S43234	03/16	TDC	Stool	*C. difficile* toxin
7)	00054142	Howell, James	S13579	03/16	PAC	Lung biopsy	Fungal culture Calcofluor white Gram stain C&S
8)	000474121	Jenkins, Joan	S24233	03/16	6TC	BAL	CMV culture Viral culture

MICROBIOLOGY ACCESSION LOG

Date/Time	Patient name (History #)	Accession #	Specimen	Test	Comments

MICROBIOLOGY REQUISITION

Incomplete information will result in delay in processing.

ALL ITEMS (1–10) MUST BE COMPLETED.

For questions or additional test availability call (704) 355-3227.

---

1. Examination requested:

☐ Bacterial C&S
☐ Gram stain
☐ Anaerobe C&S
☐ GC culture
☐ AFB C&S
☐ AFB (smear only)
☐ *C. difficile* toxin assay
☐ Occult blood

☐ Fungal culture and smear (specify)_____
☐ Fungal susceptibility
☐ Cryptococcal antigen
☐ Ova and parasite exam (complete)
☐ *Cryptosporidium/Giardia* screen
☐ Other

---

2. Specimen

☐ Aspirate (specify)_____
☐ BAL
☐ Blood (fungal and AFB require special media; contact Microbiology)
☐ Catheter (specify type)_____
☐ CSF
☐ Drainage (specify)_____
☐ Fluid (peritoneal)
☐ Fluid (pleural)
☐ Fluid (synovial)
☐ Fluid, other (specify)_____
☐ Sputum (expectorated)
☐ Sputum (induced)
☐ Stool
☐ Tissue (specify)_____
☐ Tracheal aspirate
☐ Urine (clean catch)
☐ Urine (indwelling catheter)
☐ Urine (other)
☐ Other (specify)_____

---

3. Patient location _____
4. Physician _____
5. Clinic number _____
6. Collection date and time _____
7. Clinical diagnosis/information _____
8. Antimicrobial therapy _____
9. Patient name (last, first) _____
10. Patient history number _____

(Patient name, history number, and demographics may be stamped at the bottom of this form.)

Culture Worksheet

BA	
MAC	
CHOC	
THIO	
ANA BA	
KV BHI MTM CNA	

Gram positive					Gram negative					Urine				
:1	:2	:3	:4	:	:1	:2	:3	:4	:	:1	:2	:3	:4	:
AM					AN					AN				
CZ					AM					AM				
LVX					ATM					ATM				
CC					CZ					CZ				
E					FEP					CRO				
TE					CRO					LEV				
P					LVX					GM				
SXT					GM					IPM				
VA					IPM					PIP				
LNZ					PIP					TE				
OX					SXT					NN				
SYN					SAM					SXT				

NGI _____ NG3 _____ No growth after _____ days

_____ Usual microbiota

Tech/date _____

Blood Culture Worksheet

BOTTLE

Date	:	Time	:	Results	:	Notified	:	By
	:		:		:		:	
	:		:		:		:	
	:		:		:		:	
	:		:		:		:	
	:		:		:		:	
	:		:		:		:	

	Gram positive					Gram negative				
	1	2	3	4		1	2	3	4	
AM					AN					
CZ					AM					
LVX					ATM					
CC					CZ					
E					CAZ					
OX					CRO					
P					CIP					
SXT					GM					
VA					IPM					
LNZ					PIP					
TE					SXT					
SYN					SAM					

Organism(s)

Bottles _____

_____ Sets _____

_____ Day _____

_____

_____ Date/tech _____

Mycology Worksheet

SAB

BHI

IMA

MYCO

OTHER

Slide cultures

Corn meal Potato dextrose

Week 1

Week 2

Week 3

Week 4

Biochemicals	Week: 1	2	3	4
DTM (7 days)				
Trichophyton #1				
Trichophyton #2				
Trichophyton #3				
Trichophyton #4				
Trichophyton #5				
CM w/dextrose				
Urea (4 days)				
Rice				
CHROMagar				
Casein				
Tyrosine				
Xanthine				
Starch				
Gelatin				
Urea				
Lactose				
Xylose				

Tech/date _____

PATIENT, ANONYMOUS 123-45-6789 Age: 45

----MICROBIOLOGY REPORT----

Accession: T2565 Received: March 27, 2003 16:16
Sample: Tissue biopsy Collection date: March 27, 2003 15:16
Site/specimen: Left lung/right lower lobe biopsy
Provider: Unknown
Comment on specimen: TEST PATIENT—DISREGARD
Test(s) ordered: CULTURE & SUSCEPTIBILITY—COMPLETED: MARCH 29, 2003
*BACTERIOLOGY FINAL REPORT ⟶ MARCH 29, 2003 TECH CODE: 365
GRAM STAIN: 3+ WBC
2+ GRAM-NEGATIVE RODS
CULTURE RESULTS: HEAVY GROWTH *KLEBSIELLA PNEUMONIAE*
ANTIMICROBIAL SUSCEPTIBILITY TESTING RESULTS: ("*" indicates display is suppressed)
KLEBSIELLA PNEUMONIAE (ESBL-producing organism; contact precautions required)

AMIKACIN	S*
AMPICILLIN	R
AZTREONAM	R
CEFAZOLIN	R
CEFEPIME	R
CEFTRIAXONE	R
LEVOFLOXACIN	R
GENTAMICIN	I
IMIPENEM	S
TETRACYCLINE	R
TOBRAMYCIN	S
TRIMETHOPRIM/S	R
AMPICILLIN/SUL	R
PIPERACILLIN	R

INTERPRETATION: I—The "intermediate" category includes isolates for which the antimicrobial agent MICs approach usually attainable blood and tissue levels and for which clinical response rates may be lower than those for susceptible isolates. The intermediate category implies clinical applicability in body sites where drugs are physiologically concentrated (e.g., most β-lactams in urine) or when a high dosage of drug can be used (e.g., most β-lactams). The intermediate category also includes a buffer zone that helps to prevent minor, uncontrolled technical factors from causing major discrepancies in interpretations, especially for antimicrobial agents with narrow pharmacotoxicity margins.

Quality Control Worksheet

REAGENT: COAGULASE (BBL rabbit plasma)

Year _____

CONTROL ORGANISMS—Expected results

(+) *Staphylococcus aureus* ATCC 25923

Slide: clumping when organism is added to plasma

Tube: clotting when incubated for 3 h or overnight

(−) *Staphylococcus epidermidis* ATTC 12228

Slide: no clumping when organism is added to plasma

Tube: no clotting overnight

FREQUENCY: As prepared

Date	Tech	Plasma lot # and Prep. date	Expiration date	*S. aureus*		*S. epidermidis*		Comments/corrective action
				Slide pos.	Tube pos.	Slide neg.	Tube neg.	

Microbiology Laboratory Procedure Manual

A. Urine specimens

Date prepared: By:

Date accepted: By:

Reviewed and updated	Date	_____	By	_____
		_____		_____
		_____		_____
		_____		_____
		_____		_____
		_____		_____
		_____		_____
		_____		_____
		_____		_____
		_____		_____

Medium Preparation QC

Year _____

Date	Tech	Medium prepared	Powder manufacturer	Powder lot #	Additive(s)	Lot # (s) of additive(s)	Quantity made	Form: tubes/plates, etc.	pH (if needed)

Incident Report

Problem/issue

Resolution of the problem/issue

Corrective action taken to prevent a similar problem

Comments

Prepared by: Date:

Reviewed by: Date:

Microbiology Laboratory Safety Checklist

SAFETY OFFICER:

EMPLOYEE: Date and initial each content area upon employment and annually thereafter.

Area	Objective(s)	Employee
1. General safety	Laboratory safety	
2. Biological safety		
a. Introduction	Risk factors associated with hospital-acquired infections	
	Standard precautions	
	Levels of precautions required for various agents and specimens	
b. Specimen collection	Hospital and laboratory policies regarding specimen collection	
c. Specimen processing	Requirements for and use of protective clothing and gloves	
	Use and limitations of biological safety cabinets	
	Handling of infectious material	
d. Decontamination	Decontamination of surfaces	
	Autoclave use	
e. Disposal	Disposal of infectious waste	
f. Spills	Containment and decontamination of spills, including spilled clinical specimens and culture materials, contamination of personnel, large-volume spills, broken glass, and spills into laboratory equipment	
3. Chemical and reagent safety		
a. General	Labeling and storage of reagents and chemicals	
	Material safety data sheets	
	Use of chemical fume hoods	
b. Spills	Types, locations, and use of spill kits	
c. Disposal	Handling and disposal of hazardous waste	
4. Fire safety		
a. Equipment	Types, uses, operation, and locations of fire extinguishers	
	Use and locations of fire blankets	
b. Fires	Reporting and handling of laboratory fires	
	Fire codes	
	Fire drill procedures	
5. Gas cylinders	Handling and use	
6. Accidents and incidents	Procedures for reporting incidents relating to:	
	Patients	
	Visitors	
	Work-related injury, accident, or illness	
7. Employee Health Service	Services provided by Employee Health Service	
	Availability of vaccines	
8. Radiation safety	Source, shipment, receipt, and storage of radionucleotides	
	Disposal	
	Spills	

Reportable Disease

Patient's name: Social security no.:

Address: Date of birth:

Telephone no.: Occupation:

Disease:

Test result:

Physician:

Comments:

Reported by: Date reported:

Personnel Record

Name:

Training:

College/university:

Specialized program:

Certification number:

Experience:

Tasks/procedures performed

Continuing education completed

Performance appraisal

14.4 Preparation and Quality Control of Laboratory Water

I. PRINCIPLE

The objectives of this procedure are to describe methods of preparation and QC testing of water used in microbiology laboratories and to compare water specifications published by the NCCLS (4), the American Society for Testing and Materials, the Environmental Protection Agency (2), the American Public Health Association-American Water Works Association-Water Pollution Control Federation (3), CAP (1), the American Chemical Society, and the U.S. Pharmacopeial Convention.

Classification of water (types I, II, III, and IV) is not based upon the method of preparation (e.g., distillation or deionization), which is typically how most individuals characterize water, but on a series of tests the water passes. The recommendations found in this chapter are largely based upon those published by the NCCLS (4).

II. SPECIMENS

A. Specifications

1. The specifications for various levels of water quality are presented in Table 14.4–1. A consensus is lacking among the various organizations regarding the specific parameters that should be tested and the criteria that should be met for defining the level of water quality.

2. The minimum testing criteria for defining a level of water quality acceptable for the procedures performed in a microbiology laboratory are defined by the NCCLS as follows.

- **a.** Resistivity
- **b.** pH
- **c.** Silicate content
- **d.** Bacterial count
- **e.** Particulate matter

  Particulate matter is a process specification achieved by filtration of type I water through a 0.22-μm-pore-size filter. It is not measured by the user and is not a requirement for other types of water.
- **f.** Organic content

  Organic content is a process specification for type I water achieved through pretreatment of raw water supplies with activated carbon or by posttreatment for applications requiring low levels of organic material.

3. The specifications published by the NCCLS (4) and the CAP (1) are most applicable to a clinical microbiology laboratory and will be the primary focus throughout the remainder of this procedure.

B. Types of water

1. Type I

- **a.** Must be utilized immediately after preparation.
- **b.** Must not contain particulate matter any larger than 0.2 μm in diameter. Many systems use a vinyl membrane filter with a pore size of 0.1 μm for removal of particulate contaminants.

Table 14.4–1 Laboratory pure-water specifications

Parameters[a]	Specification for water by[b]:																		
	NCCLS			ASTM				EPA				SM			CAP			ACS	USP
	I	II	III	I	II	III	IV	I	II	III	IV	I	II	III	I	II	III		
Conductivity at 25°C µS/cm [maximum]	0.1	0.5	1.0	0.6	1.0	1.0	5.0	0.06	1.0	1.0	5.0	0.1	1.0	10.0	0.1	0.5	1.0	2.0	0.15
Resistivity at 25°C (MΩ-cm [minimum])	10	1.0	0.1	16.67	1.0	1.0	0.2	16.67	1.0	1.0	0.2	10.0	1.0	0.1	10	2.0	0.1	0.5	6.66
pH at 25°C	NA	NA	5.0–8.0	NA	NA	6.2–7.5	5.0–8.0	NA	NA	6.2–7.5	5.0–8.0	NA	NA	5.0–8.0	NA	NA	5.0–8.0		5.0–7.0
Silicate (mg/liter [maximum])	0.05	0.1	1.0	ND	ND	0.01	No limit					0.05	0.1	1.0	0.05	0.1	1.0	0.01	
Total solids (mg/liter [maximum])				0.1	0.1	1.0	2.0	0.1	0.1	1.0	2.0	0.1	1.0	5.0					1.0
HPC (CFU/ml [maximum])	10	10^3	NA	Class A-1	Class B-10		Class C 100						1	10	100	10	10^3	NA	Compliance With EPA 40; CFR 141.14 and 141.21
$KMnO_4$ (minimum retention in min)				60	60	10	10	60	60	10	10							60	10
TOC (mg/liter [maximum])	NA	NA										0.05	0.2	1.0					
Ammonia (mg/liter [maximum])																			0.3
Total heavy metals (mg/liter [maximum])								0.1[c]										0.01	0.5

[a] HPC, heterotrophic plate count; TOC, total organic carbon.

[b] ASTM, American Society for Testing and Pharmacopeia; EPA, Environmental Protection Agency; SM, *Standard Methods for the Examination of Water and Wastewater* (3); ACS, American Chemical Society; USP, U.S. Pharmacopeia; NA, not applicable; ND, not done; CFR, Code of Federal Regulations.

[c] For microbiological use.

II. SPECIMENS *(continued)*

Include QC information on reagent container and in QC records.

c. The process of reverse osmosis, distillation, or passage through activated carbon removes most organic constituents.

2. Other types

Specifications for other levels of water quality are presented in Table 14.4–1.

3. Purchased or bottled water

a. Type I water cannot be bottled and sold because of degradation during storage.

b. Do not use sterile (pharmaceutical) water, as it does not meet the specifications for laboratory water.

c. Diluted water provided by a manufacturer can be used only for that specific system.

d. Purchase only water that is labeled as to bacterial count, resistivity, silicate content, and pH, as applicable.

e. At the time that the container is opened, determine the resistivity and bacterial count of the water.

f. Pour the water into a secondary container for use. Never dip pipettes into the original container. Discard water 7 days after a container is opened (3).

4. Special reagent water

Water quality greater than type I may be required for special procedures, such as high-performance liquid chromatography (HPLC), cell culture, or direct bacterial detection, that mandate the removal of specific contaminants.

C. Preparation methods

1. All methods producing water meeting the specifications listed in Table 14.4–1 are acceptable.

2. Water pressure, pH, temperature, pattern of usage (intermittent or continuous), and concentrations of contaminants in the feed water source influence each preparation method. Seek assistance from reputable manufacturers prior to purchasing a system.

3. Method of preparation (Table 14.4–2)

a. Distillation: a separation process that heats water to vapor phase and then condenses it back to a liquid, removing all nonvolatile contaminants

b. Deionization (demineralization or ion exchange)

(1) Process removes ionized molecules (anions and cations) as the feed water moves through a synthetic polymer resin.

Table 14.4–2 Comparison of water preparation methods[a]

Preparation method	Efficacy for classes of contaminants[b]					
	Dissolved ionized solids	Dissolved ionized gases	Dissolved organics	Particulates	Bacteria	Pyrogens and endotoxins
Distillation	E, G	P	G	E	E	E
Deionization	E	E	P	P	P	P
Reverse osmosis	G	P	G	E	E	E
Carbon adsorption	P	P	E, G	P	P	P
Microfiltration	P	P	P	E	E	P
Ultrafiltration	P	P	G	E	E	E
UV oxidation	P	P	E, G	P	P	P

[a] Adapted from reference 4 with permission. The current C3 edition may be obtained from NCCLS, 940 West Valley Rd. Suite 1400, Wayne, PA 19087.

[b] E, excellent (capable of complete or nearly complete removal); G, good (capable of removing large percentages); P, poor (little or no removal).

II. SPECIMENS *(continued)*

(2) Resin types
(a) Weak and strong acid cation resins remove cations.
(b) Strong basic anion resins eliminate CO_2 and silica.
(c) Weak basic anion resins remove anions but not CO_2 or silica.
(d) Mixed-bed resins are made up of a mixture of anion and cation resins.
(3) Feed water pretreatment may be essential to eliminate pyrogens, bacteria, heavy metals, and organic contaminants.

c. Reverse osmosis
(1) Process in which water is forced under pressure through a semipermeable membrane, effectively removing soluble ionic and organic contaminants, as well as insoluble contaminants.
(2) Removal efficiency depends upon the membrane type utilized (e.g., acetate, cellulose, polysulfone, or polyamide) and the feed water quality. Dissolved gases and low-molecular-weight organic compounds are not significantly reduced by reverse osmosis.
(3) Feed water pretreatment may be required if high levels of organic contaminants, bacteria, or insoluble materials are present.

d. Carbon adsorption
(1) Process utilizes a porous carbon material for adsorption of chlorine, organic contaminants, and gases.
(2) Adsorption efficiency depends upon the type of carbon, flow rate (contact time), filter depth, molecular size and solubility, and feed water quality.
(3) Process may be used as a pretreatment prior to reverse osmosis for removal of free chlorine or as a posttreatment of deionized water to eliminate trace organic materials.

e. Filtration
(1) Microfiltration utilizes membranes with pore sizes in the range of 0.1 to 10 μm for removal of impurities with molecular sizes of $>10^6$ Da. Microfiltration is useful for the removal of yeast cells, bacteria, fungi, and other large particles.
(2) Ultrafiltration is a process in which water is forced under pressure through membranes with pore sizes in the range of 0.001 to 0.1 μm for removal of impurities with molecular sizes of 10^4 to 10^6 Da. This process is utilized as a posttreatment after reverse osmosis, distillation, or deionization for removal of pyrogens and microorganisms.

f. UV oxidation
(1) UV oxidation converts trace organic materials to CO_2 and controls bacterial growth within the system.
(2) UV light (185-nm wavelength) is utilized as a posttreatment after deionization or reverse osmosis to oxidize trace organic materials.
(3) UV light (254-nm wavelength) is applied at various points in the water system (especially in holding tanks) to inhibit bacterial growth. The efficiency of this method depends upon the contact time, light intensity, and turbidity and the susceptibility of the microorganisms.

D. Storage and distribution of laboratory water

1. The construction and design of a water distribution system significantly influences the quality of the end product. Refer to NCCLS guidelines (4) for discussion of the important features of a water distribution system.

2. Storage containers should be constructed of polypropylene reinforced with stainless steel or fiberglass. Glass is inadequate.

a. Type I water cannot be stored and must be used immediately upon preparation.

II. SPECIMENS *(continued)*

b. Type II water may be stored for short periods.

3. In general, storage of laboratory grade water for long periods is unacceptable because of unpredictable deterioration rates.

E. Applications

1. The effect of reagent grade water on the various procedures performed in the clinical microbiology laboratory is, at best, poorly documented. Therefore, to obviate any unknown potential issues, the user is advised to use type I water whenever feasible. Realistically, most clinical microbiology laboratories will utilize deionized water meeting the criteria for type II water.

2. Suggested uses for various water types are presented below. This list is not intended to be exhaustive, but rather a presentation of common examples.

a. Type I

Use whenever feasible and particularly when a test method mandates minimal interference from water. Examples of situations in which Type I water might be used include enzyme immunoassays, radioimmunoassays, immunofluorescence assays, preparation of buffers, standard solutions, or reagents that will not contain preservatives, electrophoresis reagents, and virology and cell culture materials.

b. Type II

Use for most procedures unaffected by the presence of bacteria and not requiring type I water. Examples might include media and stains, and reagents that will be subsequently sterilized or to which preservatives will be subsequently added.

c. Type III, type IV

Utilize for washing and preliminary rinsing of glassware.

d. Special

In certain circumstances, water with other special properties may be required. Examples might include specific chromatographic procedures and the need for pyrogen-free water in certain tissue or cell culture media.

III. QUALITY CONTROL

A. Testing frequency (Table 14.4–3)

1. Perform QC procedures more frequently if any components of the production or distribution system are changed, if problems possibly related to the water occur during testing procedures, or if QC tests do not meet tolerance limits.

2. For some contaminants, the testing frequency may be seasonally dependent.

Table 14.4–3 Specifications and frequency of QC testing of laboratory water[a]

Test	Frequency	Tolerance limit[b]		
		Type I	Type II	Type III
Bacterial content (CFU/ml)	Weekly	≤10	≤1,000	NS
pH	With use	NS	NS	5.0–8.0
Resistivity (MΩ—cm at 25°C)	Type I, daily (in-line) Type II, daily (in- or off-line) Type III, with use (off-line)	10	1.0	0.1
Silicate (mg/liter)	Initially and annually thereafter	0.05	0.1	1.0

[a] Adapted from references 1 and 4 with permission. See Table 14.4–2, footnote *a.*

[b] NS, not specified.

III. QUALITY CONTROL *(continued)*

B. Tests

See Appendixes 14.4–1 to 14.4–4 for test procedures.

1. The number of tests performed may vary based on the specific accreditation body. As a minimum, pH, bacterial content, resistivity, and silica content should be determined (4).

2. Organic content

a. The purification system should include a process that significantly reduces concentrations of organic contaminants.

b. Water contamination with organic compounds can be determined by spectrophotometric evaluation of a sample in the far-UV range or by HPLC (4).

3. The total bacterial count may be determined using a standard plate count technique, a commercial filtration kit, a large-volume filtration methodology, or a commercial bacterial sampler method (4). Refer to Appendix 14.4–1 for the standard plate count methodology.

REFERENCES

1. **Commission on Laboratory Inspection and Accreditation.** 1985. *Reagent Water Specifications.* CAP, Chicago, IL.
2. **Environmental Protection Agency.** 1979. *Handbook for Analytical Quality Control in Water and Waste Water Laboratories.* Environmental Protection Agency, Cincinnati, OH.
3. **Greenberg, A. E., R. R. Trussell, and L. S. Clesceri.** 1989. *Standard Methods for the Examination of Waste and Wastewater,* 17th ed. American Public Health Association, Washington, DC.
4. **NCCLS.** 1997. *Preparation and Testing of Reagent Water in the Clinical Laboratory,* 3rd ed. Approved Guideline C3-A3. NCCLS, Wayne, PA.

SUPPLEMENTAL READING

American Society for Testing and Materials. 1990. *Annual Book of ASTM Standards,* vol. 11.1 and 11.2. American Society for Testing and Materials, Philadelphia, PA.

U.S. Pharmacopeial Convention. 1990. *The U.S. Pharmacopeia,* 22nd revision. The U.S. Pharmacopeial Convention, Inc., Rockville, MD.

APPENDIX 14.4–1

Determination of Total Bacterial Counts: Standard Plate Count

I. PRINCIPLE

The total bacterial count of a water sample measures the CFU of aerobic and facultatively anaerobic bacteria that will grow under incubation conditions.

II. SPECIMENS

Type I, II, and III water produced by a purification system

III. MATERIALS

A. Reagents and media: any clear medium capable of supporting growth of aerobic and facultatively anaerobic gram-negative bacilli (e.g., TSA, BHI agar, standard plate count agar)

B. Supplies

1. Petri plates (15 by 100 mm)
2. Micropipettors or pipettes
3. Sterile tubes

C. Equipment

1. Incubator, ambient air
2. Vortex mixer

IV. QUALITY CONTROL

See procedure 14.2.

APPENDIX 14.4–1 *(continued)*

V. PROCEDURE
- A. Flush the system by allowing water to flow for 1 min.
- B. Collect a minimum of 10 ml of water in a sterile container. Process the sample within 1 h or within 6 h if stored at 5°C.
- C. Melt 15 ml of the agar medium and cool it to 46 to 50°C.
- D. Vortex the 10-ml sample or invert it multiple times to mix.
- E. Transfer a 1-ml aliquot of the water sample to a petri plate. Pour the molten agar into the plate and mix by careful rotation.
- F. After the agar has solidified, invert the petri plate and incubate at 36 ± 1°C for 24 h and then at 23 ± 3°C for 24 h.

VI. RESULTS
- A. Count the colonies. Use a dissecting microscope with ×10 to ×15 magnification or a Quebec colony counter for optimal observation of growth.
- B. Acceptable results
  1. Type I, ≤10 CFU/ml
  2. Type II, ≤1,000 CFU/ml

VII. RESULT REPORTING

Report the CFU in 1 ml of sample

VIII. PROCEDURAL LIMITATIONS

Not all bacteria produce detectable colonies under these growth conditions.

Supplemental Reading

NCCLS. 1997. *Preparation and Testing of Reagent Water in the Clinical Laboratory,* 3rd ed. Approved Guideline C3-A3. NCCLS, Wayne, PA.

APPENDIX 14.4–2

Determination of pH of Laboratory Water

I. PRINCIPLE

Because type III water has so few ions present, potassium chloride (KCl) must be added to obtain a stable pH reading.

II. SPECIMEN

Type III laboratory water

III. MATERIALS
- A. Reagents
  1. Reference buffer solutions
     Use two commercial reference buffers at pH 4.0 and 7.0.
  2. KCl, saturated
     Slowly add KCl crystals to warm type II water. Stir constantly until the crystals no longer go into solution. Store indefinitely at room temperature (RT) in a stoppered glass or plastic container.
  3. Type I water in a squirt bottle for rinsing electrodes between measurements
- B. Supplies
  1. Large-mouth beaker for rinsing
  2. Small plastic tubes or beakers
- C. Equipment
  1. pH meter and electrode
  2. Stirring heating plate
  3. Thermometer

IV. QUALITY CONTROL

Refer to procedure 12.16 in CMPH for QC of pH meters.

V. PROCEDURE
- A. Allow all solutions to come to RT. Measure the temperature of the solution and adjust the temperature control on the pH meter to coincide with the reading.
- B. Standardize the pH meter (refer to procedure 12.16 in CMPH).
- C. Rinse the electrode a minimum of three times with a flowing stream of water.
- D. Add 1 drop of saturated KCl to a 50-ml sample of water to be tested.

APPENDIX 14.4–2 *(continued)*

VI. RESULTS
- A. Record the pH as quickly as possible.
- B. The pH of type III water should be 5.0 to 8.0.

VII. RESULT REPORTING

Report the pH to the nearest 0.1.

Supplemental Reading

NCCLS. 1997. *Preparation and Testing of Reagent Water in the Clinical Laboratory,* 3rd ed. Approved Guideline C3-A3. NCCLS, Wayne, PA.

APPENDIX 14.4–3

Determination of Laboratory Water Resistivity

I. PRINCIPLE

Resistivity is inversely proportional to the concentration of ionized compounds in the water.

II. SPECIMENS

Type I, II, and III water

III. MATERIALS
- A. Reagents
  1. Potassium chloride (0.01 mol/liter)
     Dissolve 0.7440 g of KCl in type I water. Dilute to 1 liter at 20°C.
  2. Type I water in a rinse bottle
- B. Supplies
  Water sample containers
- C. Equipment
  1. Resistivity meter
     - a. In-line meter (built into the purification system)
       - (1) Measures the resistivity of type I or type II water as it is produced.
       - (2) Follow manufacturer's instructions.
     - b. Off-line, dip-type meter
       - (1) Manually measures the resistivity of type II and II water.
       - (2) The accuracy of the meter must be $\pm 3\%$ of the full scale or $\pm 5\%$ of the actual reading.
  2. Thermometer: graduations of 0.1°C

IV. QUALITY CONTROL
- A. In-line meters
  Follow manufacturer's instructions.
- B. Off-line (dip-type) meters
  1. Follow manufacturer's instructions.
  2. Measure the resistivity of the 0.01-mol/liter KCl solution daily or with each meter use.
  3. The 0.01-mol/liter KCl has a resistivity of 708 Ω-cm at 25°C if the resistivity of the water used in the KCl is not taken into consideration.

V. PROCEDURE
- A. Type I water
  Resistivity can only be measured using an in-line meter. Measure daily.
- B. Type II and III water
  1. Follow manufacturer's instructions.
  2. Rinse the container and the meter cell a minimum of three times with separate aliquots of the water to be tested.
  3. Place the cell in the water. Move it up and down in a circular fashion to eliminate any trapped bubbles. Read immediately, because dissolving atmospheric CO_2 will result in a falsely low resistivity reading.
  4. If the resistivity meter does not automatically compensate for temperature, simultaneously measure the temperature to the nearest 0.1°C. Following the instructions, correct the resistance reading to 25°C.

APPENDIX 14.4–3 *(continued)*

VI. RESULTS
- A. Type I water, 10 MΩ-cm at 25°C
- B. Type II water, 1 MΩ-cm at 25°C
- C. Type III water, 0.1 MΩ-cm at 25°C

VII. RESULT REPORTING

Record the meter reading in megaohms per centimeter at 25°C.

VIII. PROCEDURE NOTES
- A. The resistance of pure water varies significantly, depending upon the temperature.
- B. Dissolving atmospheric CO_2 will result in lower resistivity readings.

Supplemental Reading

NCCLS. 1997. *Preparation and Testing of Reagent Water in the Clinical Laboratory,* 3rd ed. Approved Guideline C3-A3. NCCLS, Wayne, PA.

APPENDIX 14.4–4

Determination of Soluble Silica by the Molybdate Method

I. PRINCIPLE

Soluble silica in source water is a major problem in some geographical areas. Silicates react with the molybdate ions to form complexes that generate a blue color after reduction by 1-amino-2-naphthol-4-sulfonic acid.

II. SPECIMENS

Type I, II, or II water

III. MATERIALS
- A. Reagents
  1. 1-Amino-2-naphthol-4-sulfonic acid solution (14.45 mmol/liter)
     - a. Dissolve 1 g of sodium sulfite (Na_2SO_3) in 50 ml of type I water.
     - b. Add 0.5 g of 1-amino-2-naphthol-4-sulfonic acid. Mix.
     - c. Add this solution to 100 ml of a solution containing 30 g of hydrogen sulfite ($NaHSO_3$).
     - d. Dilute to 200 ml.
     - e. Store in a dark polyethylene bottle at room temperature (RT) for up to 2 weeks.
  2. Ammonium molybdate solution, 100 g/liter (85.9 mmol/liter)
     - a. Dissolve 10 g of ammonium molybdate in type II water and dilute to 100 ml.
     - b. Store in a polyethylene bottle at RT for up to 3 months.
  3. Hydrochloric acid
     - a. Slowly add 100 ml of concentrated HCl to 100 ml of type II water.
     - b. Store indefinitely in a polyethylene bottle at RT.
  4. Oxalic acid solution, 100 g/liter (1.11 mmol/liter)
     - a. Dissolve 10 g of oxalic acid in type II water and dilute to 100 ml.
     - b. Store in a polyethylene bottle at RT for up to 3 months.
  5. Silica standard solution (1 ml = 1 mg of SiO_2; 16.64 μmol)
     - a. Dissolve 4.732 g of sodium metasilicate ($Na_2SiO_3 \cdot 9H_2O$) in type II water and quantity sufficient to 1 liter
     - b. Store in a polyethylene bottle at RT for up to 3 months.
- B. Equipment
  1. Spectrophotometer with capability to read at 700-to 850-nm wavelength
  2. Balance with capability to measure to the nearest milligram

IV. QUALITY CONTROL
- A. Purchase or prepare QC standards containing silica over the range 0.01 to 1.0 mg/liter.
- B. Assay QC standards with each use.

V. STANDARD-CURVE PREPARATION
- A. Prepare a series of 0.01-, 0.05-, 0.1-, and 1.0-mg/liter SiO_3 standards (100 ml each) by diluting the stock SiO_3 solution in type I water.
- B. Treat 50-ml samples of each standard as described in item VI below.
- C. Use 50 ml of assayed type I water as a blank. This water must not generate any visible blue color in the assay procedure.

APPENDIX 14.4–4 *(continued)*

VI. PROCEDURE

A. Transfer 50-ml aliquots of the water samples, standards, and blank to polyethylene containers.

B. Add, in rapid succession, 1 ml of the HCl solution and 2 ml of ammonium molybdate. Mix well. Let stand for 5 min.

C. Add 1.5 ml of the oxalic acid solution. Mix. Let stand for 1 min.

D. Add 2 ml of amino-naphthol-sulfonic acid solution. Mix well. Let stand for 10 min.

E. Zero the spectrophotometer with the blank. Measure the A_{803} of the standards and the samples.

VII. RESULTS

A. Record the absorbance of each sample and standard.

B. Prepare a standard curve by plotting the absorbance values of the standards against the known silicate concentration in milligrams of SiO_3 per liter.

VIII. RESULT REPORTING

A. Determine the silica concentrations of the samples using the calibration curve.

B. Report the concentrations in milligrams per liter.

C. Acceptable values for laboratory grade water

1. Type I, 0.05 mg/liter
2. Type II, 0.1 mg/liter
3. Type III, 1.0 mg/liter

IX. PROCEDURE NOTES

A. A commercially prepared kit methodology may be used as a substitute.

B. Ideally, the silica content of the source water should be determined and a water purification system should be designed to yield low levels of silicates, which would obviate the need to test routinely for silicates.

Supplemental Reading

NCCLS. 1997. *Preparation and Testing of Reagent Water in the Clinical Laboratory,* 3rd ed. Approved Guideline C3-A3. NCCLS, Wayne, PA.

SECTION 15 Biohazards and Safety

SECTION EDITORS: *Gerald A. Denys and Larry D. Gray*

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15.1 Introduction

Laboratory workers are at high risk for occupational exposure to infectious agents. Infections can be acquired from exposure to contaminated blood, tissue, and other material. The greatest risks for clinical microbiologists are associated with the processing of specimens and the manipulating of pathogens isolated from these materials. The actual incidence of laboratory-acquired infections is probably higher than recognized due to subclinical symptoms and poor compliance in reporting.

Laboratory practices that can reduce the risk of infection include standard precautions, PPE, safety devices, and the proper decontamination and disposal of biohazardous material. The concept of standard precautions evolved by incorporating the 1991 OSHA standard for occupational exposure to blood-borne pathogens, universal precautions, and the 1996 Hospital Infection Control Practices Advisory Committee guidelines for isolation precautions in hospitals, body substance isolation. The intent of standard precautions is to include a broader range of pathogens than universal precautions implied. Standard precautions recognize that all patient specimens should be handled as if they are infectious and capable of transmitting disease. This applies to all body fluids, secretions, excretions, and tissue specimens, regardless of whether they contain visible blood, as well as QC strains and proficiency test samples.

A renewed interest in improving safety in the laboratory has occurred because of the recognition of new infectious agents, emergence of antimicrobial resistance, new diagnostic and treatment methods, and the potential threat of agents of bioterrorism. Numerous guidelines for the prevention of laboratory-associated infections have appeared in the literature. Adherence to these guidelines can reduce, but not eliminate, the risks of occupational exposure to infectious agents.

This section deals with basic strategies and procedures for the prevention, containment, and management of laboratory-acquired infections in the microbiology laboratory. The items in Supplemental Reading below include examples of laboratory-associated infections and published guidelines and regulations for improving laboratory safety. Additional biosafety information can be found on the following website: http://www.cdc.gov/OD/ohs/biosfty/biosfty.htm.

SUPPLEMENTAL READING

Centers for Disease Control and Prevention. 1994. Guidelines for preventing the transmission of *Mycobacterium tuberculosis* in health-care facilities. *MMWR Morb. Mortal. Wkly. Rep.* **43:**1–128.

Centers for Disease Control and Prevention and National Institutes of Health. February 2007, posting date. *BioSafety in Microbiological and Biomedical Laboratories*, 5th ed. U.S. Government Printing Office, Washington, DC. http://www.cdc.gov/OD/ohs/biosfty/bmbl5/bmbl5toc.htm.

CLSI. 2005. *Protection of Laboratory Workers from Occupationally Acquired Infections.* Document M29-A3. CLSI, Wayne, PA.

Garner, J. S. 1996. Hospital Infection Control Practices Advisory Committee. Guideline for isolation precautions in hospitals. *Infect. Control Hosp. Epidemiol.* **17:**53–80.

Noble, M. A. 2007. Prevention and control of laboratory-acquired infections, p. 97–106. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.

Occupational Safety and Health Administration. 1991. Occupational exposure to bloodborne pathogens: final rule. *Fed. Regist.* **56:**64004–64182.

Occupational Safety and Health Administration. 1997. Occupational exposure to tuberculosis: proposed rule. *Fed. Regist.* **62:**54160–54308.

Sewell, D. L. 2006. Laboratory-acquired infections: are microbiologists at risk? *Clin. Microbiol. Newsl.* **28:**1–7.

Sewell, D. L. 2004. Nosocomial infections in diagnostic laboratories, p. 1431–1441. *In* C. G. Mayall (ed.), *Hospital Epidemiology and Infection Control*, 3rd. ed. Lippincott Williams & Wilkins, Philadelphia, PA.

Sewell, D. L. 1996. Laboratories at risk: the threat of exposure to infectious agents, and the role of the biosafety program. *Lab. Med.* **27:**673–678.

Sewell, D. L. 1995. Laboratory-associated infections and biosafety. *Clin. Microbiol. Rev.* **8:**389–405.

15.2.1 Routes of Infection and Laboratory Activities

Many different categories of microbiological hazards are encountered from the time a specimen is collected until it is disposed of permanently. The management of risks engendered by working with pathogens is accomplished by the development and implementation of standard procedures and practices for handling infectious material and will prevent microbial transmission. "Infection" as used in this procedure implies overt clinical manifestations of disease. Exposure to infectious agents can occur by several routes. The actual occurrence of an infection depends on the concentration and virulence of the infecting agent, the route of exposure, and the susceptibility of the host.

I. INHALATION: ACTIVITIES THAT GENERATE AEROSOLS

Selected agents may include *Bacillus anthracis*, *Brucella* species, *Coxiella burnetii*, *Burkholderia pseudomallei*, *Francisella tularensis*, and *Mycobacterium tuberculosis.*

A. Manipulating needles and syringe
- **1.** Expelling air from tubes or bottles
- **2.** Withdrawing needles from stoppers
- **3.** Separating needles from syringes

B. Manipulating inoculation needles or loops
- **1.** Flaming loops
- **2.** Cooling loops in culture media
- **3.** Subculturing and streaking culture media

C. Manipulating pipettes
- **1.** Mixing microbial suspensions
- **2.** Spilling microbial suspensions on hard surfaces

D. Manipulating specimens and cultures
- **1.** Centrifugation
- **2.** Mixing, blending, grinding, shaking, sonication, and vortexing of specimens or cultures
- **3.** Pouring or decanting fluids
- **4.** Removing caps or swabs from culture containers
- **5.** Spilling infectious material
- **6.** Filtering specimens under vacuum

II. INGESTION: ACTIVITIES RELATED TO ORAL TRANSMISSION

Selected agents may include *Salmonella* species, *Shigella dysenteriae*, *Campylobacter jejuni*, *Escherichia coli*, *Vibrio cholerae*, *Yersinia pestis*, and *Cryptosporidium* spp.

A. Pipetting by mouth
B. Splashing contaminated material into the mouth
C. Placing contaminated material or fingers in the mouth
D. Eating, drinking, using lipstick, and smoking in the workplace

III. INOCULATION: ACTIVITIES RELATED TO DIRECT INTRAVENOUS AND SUBCUTANEOUS TRANSMISSION

Selected agents may include human immunodeficiency virus, hepatitis B and C viruses, *Plasmodium vivax,* and Venezuelan equine encephalitis virus.

A. Manipulating needles and syringes
B. Handling broken glass, scalpels, and other sharp objects

IV. INOCULATION: ACTIVITIES RELATED TO CONTAMINATED SKIN AND MUCOUS MEMBRANES

Selected agents may include *Streptococcus pyogenes, Staphylococcus aureus, Neisseria meningitidis, Cryptococcus neoformans,* and free-living amoebae.

A. Splashing or spilling material into eyes, mouth, and nose and onto skin
B. Exposing nonintact skin to contaminated material
C. Working on contaminated surfaces
D. Handling contaminated equipment
E. Inappropriate handling of loops, inoculating needles, or swabs containing specimen or culture material

15.2.2 Safe Work Practices

All laboratory personnel must follow standard precautions guidelines. Specimens received in the laboratory cannot be distinguished between containing potentially infectious and noninfectious material. The implementation of safe work practices is essential for the prevention of laboratory-associated infections.

I. HANDLING OF SPECIMENS

Observe standard precautions.

A. Gloves and gowns

1. Wear gloves and gowns (impervious to liquids) at all times when handling and processing patient specimens, decontaminating instruments and countertops, and cleaning spills. Coats with snug-fitted sleeves and aprons are optional.
2. Bandage open cuts and scratches on hands and then wear gloves.
3. Wear gloves (e.g., latex free or vinyl) when performing phlebotomy and when handling actual blood specimens.
4. Wash hands immediately after gloves are removed, after a task that involves heavily contaminated matter, and before leaving the laboratory.

B. Specimen transport

1. Place specimens in plastic bags and transport in leakproof containers with the biohazard symbol affixed (*see* Fig. 15.6–4).
2. *Do not* accept grossly soiled or contaminated specimens. Notify an individual responsible for submitting such a specimen immediately, and follow the laboratory's specimen rejection policy.

C. Needles and syringes

1. Use needle-locking syringes or plastic disposable syringe-needle units.
2. Never recap or bend needles or remove them from syringes.
3. *Do not* accept specimens received in syringes with needles attached. Notify the individual responsible for submitting such a specimen immediately and follow the laboratory's specimen rejection policy.
4. Discard in a puncture-resistant container that has the biohazard symbol affixed.
5. Use needleless systems for blood samples if available.
6. Use mechanical devices or one-handed techniques when handling sharp objects.
7. Secure blood culture bottles before inserting needles into the bottles (e.g., place bottles in support racks).

I. HANDLING OF SPECIMENS *(continued)*

It is imperative that these cultures be handled in a biosafety cabinet.

D. Tubes
1. Carry tubes in racks.
2. Use plastic tubes when possible.
3. Uncap tubes behind a clear plastic shield to contain splashes or sprays (e.g., when removing tops from vacuum tubes).

E. Centrifuges
1. Centrifuge tubes must be intact and properly balanced when centrifuged.
2. Centrifuge tubes used in mycobacteriologic areas must be enclosed in sealed safety cups.
3. Use aerosol-free centrifuges when possible (e.g., in the mycobacteriology laboratory).
4. Centrifuge safety cups must be opened in a biological safety cabinet (BSC) after centrifugation.
5. *Do not* place tabletop centrifuges in a BSC because air turbulence within the cabinet can allow aerosols to escape.

F. Hand washing
1. Perform frequent hand washing after removing gloves, before leaving the laboratory, and before eating, drinking, or applying cosmetics.
2. Use nonirritating soap for routine washing.
3. Use antiseptic soap or an alcohol planchet followed by thorough hand washing for accidental skin contamination.

II. PROCESSING OF SPECIMENS

A. Process all specimens in a BSC (*see* procedure 15.3.4 for BSC operating procedures).

B. Sterilize bacteriological wire needles and loops to avoid spattering of material on heating.
1. Use of electric incinerators is preferred over Bunsen burners, and they should be used if at all possible.
2. Use Bunsen burners equipped with a safety sleeve.
3. Cool needle and loop tips enough to avoid searing the surface of the medium.
4. Alternatives to wire needles and loops which require no heating include plastic disposable loops and spreaders for streaking plates and spreading material onto slides.

C. Mix or transfer liquids by using disposable plastic pipettes and a rubber bulb. Alternatively, use mechanical pipetting devices.

D. Cover tubes when mixing, blending, vortexing, etc. (e.g., cap tubes or cover them with Parafilm).

E. Work over an absorbent covering or disinfectant-moistened mat (e.g., phenolic-compound-soaked pad for *Mycobacterium tuberculosis*).

F. Plan tasks to minimize exposure to known hazards.

G. Follow standard precautions when performing nonculture techniques (e.g., antibody, antigen, and molecular detection methods).

III. HOUSEKEEPING AND MISCELLANEOUS SAFE PRACTICES

Observe standard precautions.

A. General
1. Avoid or minimize activities associated with the transmission of infectious agents.
2. Designate clean and contaminated work areas.
   a. Wear gloves in contaminated areas.
   b. Clean and disinfect all surfaces after spills and at the end of each work shift.
3. Keep all work areas neat and uncluttered.
   a. *Do not* store personal items in the work areas.

III. HOUSEKEEPING AND MISCELLANEOUS SAFE PRACTICES *(continued)*

   b. *Do not* store large quantities of disposable items in the work areas.
4. Remove coats and gowns before leaving the laboratory. Place contaminated laundry (e.g., reusable lab coats and gowns) in designated bag for cleaning by the institution.
5. Dispose of all contaminated material in containers for treatment (e.g., by autoclaving).

B. Compressed gases

1. Secure cylinders in an upright position with wall mounts.
2. Store cylinders away from open flames and sources of heat.
3. Use the correct pressure regulators.
4. Verify the contents of the cylinder before gas is used.
5. Transport cylinders in secured hand trucks or carts.

C. Chemicals

1. Wear appropriate PPE when handling hazardous chemicals.
2. Label all reagents with their chemical names and appropriate hazard warnings provided from the MSDS information.
3. Keep MSDSs for all chemicals, either in the laboratory or in a nearby office.
4. Store flammable and combustible liquids in fire-rated storage cabinets and explosion-proof refrigerators.
5. Store all hazardous chemicals, including reagents and dyes, below eye level.
   a. Use plastic bottles when appropriate.
   b. Store on the bench volumes necessary for daily work.
6. Place chemical waste in a flume hood until final disposal.

SUPPLEMENTAL READING

CLSI. 2005. *Protection of Laboratory Workers from Occupationally Acquired Infections*. Document M29-A3. CLSI, Wayne, PA.

Collins, C., and D. Kennedy. 1999. *Laboratory-Acquired Infections: History, Incidence, Causes, and Prevention*, 4th ed., p. 65–110. Butterworth-Heinemann, Oxford, United Kingdom.

NCCLS/CLSI. 2004. *Clinical Laboratory Safety*. Document GP17-A2, p. 17–21. NCCLS/CLSI, Wayne, PA.

Sewell, D. L. 2004. Nosocomial infections in diagnostic laboratories, p. 1431–1441. *In* C. G. Mayall, (ed.), *Hospital Epidemiology and Infection Control*, 3rd ed. Lippincott Williams & Wilkins, Philadelphia, PA.

Sewell, D. L. 1995. Laboratory-associated infections and biosafety. *Clin. Microbiol. Rev.* **8:**389–405.

15.2.3 Decontamination

Routine decontamination and cleaning of the work environment are the responsibility of all laboratory workers at both the basic and highly sophisticated laboratory levels. To accomplish this effectively, the work areas should be uncluttered, with clean and dirty materials separated and clearly identified. Table 15.2.3–1 lists procedures and products commonly used for sterilization and disinfection (1). The manufacturer's instructions for preparation and use should be carefully followed. No single product and procedure, however, are adequate for all decontamination purposes. When selecting a procedure, the degree of microbial killing, nature and composition of the surface or device to be treated, cost, safety, and ease of use of available agents should be considered. More information on Environmental Protection Agency (EPA)-approved general-purpose disinfectants and Food and Drug Administration (FDA)-regulated sterilants and high-level disinfectants can be found on the websites http://www.epa.gov and http://www.fda.gov/cdrh/index.html. A new website, http://www.sustainablehospitals.org, is also available that provides information on safer alternatives to products containing latex, polyvinyl chloride, mercury, and disinfectants. This procedure presents common decontamination practices used in the microbiology laboratory.

Table 15.2.3–1 Activity levels of selected liquid germicides[a]

Procedure and product	Aqueous concn	Activity level
Sterilization		
Glutaraldehyde	Variable	
Hydrogen peroxide	6–30%	
Formaldehyde	6–8%	
Chlorine dioxide	Variable	
Peracetic acid	Variable	
Disinfection		
Glutaraldehyde	Variable	High to intermediate
ortho-Phthalaldehyde	0.5%	High
Hydrogen peroxide	3–6%	High to intermediate
Formaldehyde	1–8%	High to low
Chlorine dioxide	Variable	High
Peracetic acid	Variable	High
Chlorine compounds	500–5,000 mg of free, available chlorine/liter	Intermediate
Alcohols	Ethyl, isopropyl: 70%	Intermediate
Phenolic compounds	0.5–3%	Intermediate to low
	30–50 mg of free iodine/liter; up to 10,000 mg of available iodine/liter	Intermediate to low
Quaternary ammonium compounds	0.1–0.2%	Low

[a] Reproduced with permission from reference 2. A large number of commercial products based on the generic compounds listed can be considered for use. Users should ensure that commercial formulations are registered with the EPA or by the FDA.

I. MATERIALS

A. Alcohol (e.g., 70% ethanol, isopropanol)
Use to decontaminate work surfaces only.

B. Glutaraldehyde or suitable alternative
1. Dirty glassware
2. Equipment decontamination

C. Chlorine, iodophors, phenolic compounds, quaternary ammonium compounds (e.g., T.B.Q., germicidal detergent; Calgon Vestal Labs, St. Louis, MO)
1. Work surfaces
2. Dirty glassware
3. Equipment decontamination

II. PROCEDURES

Observe standard precautions.

A. Preparation of 10% household bleach (0.5% sodium hypochlorite), working solution
1. Prepare fresh daily.
2. Add one part household bleach to nine parts tap water (5,000 mg of available chlorine per liter).
3. Dispense in wash bottles.
4. Record the date prepared on the bottles.
5. Leave wash bottles in the work areas.
6. After 24 h, pour unused bleach solution down the drain and flush the drain with running water to prevent corrosion of pipes.
7. Allow bottles to air dry.

B. Decontamination of work surfaces
1. Follow manufacturer's instructions for cleaning and appropriate disinfecting solutions. Allow bleach solutions to air dry (minimum contact time should be 10 min).
2. Use a paper towel or soft cloth soaked with the recommended disinfectant solution.
3. Decontaminate
a. Before and at the end of the work shift
b. Upon completion of a procedure
c. When surfaces become overtly contaminated. *See* procedure 15.2.4.

C. Decontamination of equipment (including vortex, centrifuge, and telephone)
1. Follow manufacturer's instructions for cleaning and suitable disinfecting solutions.
2. Use a soft cloth moistened with recommended disinfecting solution.
3. Decontaminate
a. Upon completion of a procedure
b. When surfaces become overtly contaminated. *See* procedure 15.2.4.
c. Rinse equipment with tap water (except when alcohol is used).
4. If the equipment needs to be replaced or serviced, it must be decontaminated before removal.

D. Decontamination of reusable biohazard pail
1. Remove biohazard lining bags and tape shut with white tape.
a. If a bag is ripped or torn, place it inside a second bag.
b. Place red biohazard tape on each bag.
c. Place each bag on a biohazard cart or in a waste dumpster for disposal.
2. Wipe the entire inside surface of the pails with soft cloths soaked with a fresh solution of 10% bleach.
3. Allow a pail to air dry before placing a new biohazard lining bag into it.
4. Inspect each pail for holes or leakage.
5. Decontaminate
a. Whenever the biohazard lining bag is changed or at least once per week
b. Whenever a biohazard lining bag is torn

II. PROCEDURES *(continued)*

E. Decontamination of computer keyboards

1. Clean and disinfect keyboards at the end of every shift and when the keyboards are visibly contaminated.
2. Unplug the keyboard from the computer.
3. Clean the keyboard surface with a soft cloth moistened with 70% ethanol, or use an alcohol prep pad.
   - **a.** If the keyboard has a fitted covered, inspect the cover for holes or tears.
   - **b.** If the keyboard is uncovered, avoid spilling alcohol underneath the keys.
4. Plug the keyboard back into the computer.
5. If the computer is overtly contaminated with a specimen, clean with a 10% bleach solution and rinse with water.

III. LIMITATIONS OF THE PROCEDURES

Observe standard precautions.

A. Disposable gloves, impervious gowns, eye protection, and masks should be worn when preparing disinfectants and when cleaning and decontaminating soiled equipment.

B. Overtly contaminated reusable equipment and surfaces should be cleaned with an aqueous detergent solution before application of the disinfectant for optimum effectiveness.

C. *Do not* use alcohol solutions for equipment decontamination in poorly ventilated areas or near open flames.

D. *Do not* use sodium hypochlorite solutions on metal parts (aluminum and stainless steel) because they may cause rusting and pitting.

E. *Do not* use glutaraldehyde solutions as general surface disinfectants because of their irritating vapors and prolonged contact time.

F. Inactivation of the causative agent of transmissible spongiform encephalopathies, or prion disease (e.g., Creutzfeldt-Jakob disease), is not achieved by conventional inactivation procedures, heat, irradiation, and chemical germicides. Specimens containing prion proteins include tissues of the central nervous system (high risk); CSF, lymph nodes, spleen, pituitary gland, and tonsils (medium risk); and bone marrow, liver, lungs, thymus, and kidneys (low risk). Blood samples carry no risk of transmitting prion disease.

1. Avoid the generation of aerosols or droplets during the manipulation of tissues or fluids and during necropsy of experimental animals.
2. Wear gloves for all activities that skin contact with infectious tissues and fluids may occur.
3. Use disposable plasticware whenever possible.
4. Formaldehyde-fixed and paraffin-embedded tissues, especially of the brain, remain infectious (they may be immersed for 30 min in 96% formic acid or phenol before processing, but this may distort microscopic neuropathology or alter immunohistochemical reactions).
5. Contaminated surfaces should be cleaned and then decontaminated with a 1:10 dilution of sodium hypochlorite.
6. Contaminated items and medical devices should be decontaminated by either of the following conditions (2).
   - **a.** 134°C for 18 min in a prevacuum sterilizer (liquids must be removed before sterilization)
   - **b.** 121 to 132°C for 1 h in a gravity displacement sterilizer
   - **c.** Soak in 1 N NaOH or 2.5% NaOCl for 1 h at room temperature.
7. For reusable instruments, the World Health Organization (3) recommends more stringent guidelines for disinfection (e.g., combined use of 1 N NaOH and autoclaving at 134°C).

III. LIMITATIONS OF THE PROCEDURES *(continued)*

8. Instruments too delicate for harsh treatments or difficult to clean devices should be discarded.
9. Decontaminate paraformaldehyde-vaporized biosafety hoods with 1 N NaOH, followed by 1 N HCl, and rinse with water. HEPA filters should be autoclaved or incinerated.

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

Sign initials in the appropriate box on the decontamination worksheet after completion of each task. See Appendix 15.2.3–1 for a sample QC daily recording worksheet.

REFERENCES

1. **Favero, M. S., and W. W. Bond.** 2001. Chemical disinfection of medical and surgical materials, p. 881–917. *In* S. S. Block (ed.), *Disinfection, Sterilization, and Preservation,* 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
2. **Rutala, W. A., and D. J. Weber.** 2001. Creutzfeldt-Jakob disease: recommendations for disinfection and sterilization. *Clin. Infect. Dis.* **32:**1348–1356.
3. **World Health Organization.** 1999. *WHO Infection Control Guidelines for Transmissible Spongiform Encephalopathies: Report of a WHO Consultation.* WHO/CDS.CSR/APH/2000.3. World Health Organization, Geneva, Switzerland.

SUPPLEMENTAL READING

Baron, H., J. Safar, D. Groth, S. J. DeArmond, and S. B. Prusiner. 2001. Prions, p. 659–674. *In* S. S. Block (ed.), *Disinfection, Sterilization, and Preservation,* 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.

CLSI. 2005. *Protection of Laboratory Workers from Occupationally Acquired Infections.* Document M29-A3. CLSI, Wayne, PA.

Favero, M. S., and M. J. Arduino. 2006. Decontamination and disinfection, p. 373–381. *In* D. O. Fleming and D. L. Hunt (ed.), *Biological Safety: Principles and Practices,* 4th ed. ASM Press, Washington, DC.

Widmer, A. F., and R. Frei. 2007. Decontamination, disinfection, and sterilization, p. 65–96. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology,* 9th ed. ASM Press, Washington, DC.

APPENDIX 15.2.3–1 Decontamination Worksheet

Month _____ Year _____ Location _____

Date	Equipment	Biohazard pail	Work surface
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			
30			
31			
Corrective action		Date:	Reviewed by:

Note: Sign initials in appropriate box after completion of decontamination tasks.

15.2.4 Biohazardous Spills

Management of accidental biohazardous spills depends on the infectious agent, the quantity of the material spilled, and whether an aerosol was generated. A number of effective disinfectants have been described for decontamination; however, a tuberculocidal (e.g., T.B.Q.) disinfectant is appropriate for most spills. This procedure is for the management of accidental biohazardous spills of infectious material or release of infectious microorganisms into the laboratory. Factors that can influence the effectiveness of decontamination procedure include the volume of the spill, type of body fluid, protein content, infectious agent present and concentration, and spill surface (porous or water resistant).

I. MATERIALS

(Keep readily available.)

A. Disinfectants
- **1.** 10% Household bleach
- **2.** See Table 15.2.3–1.

B. Equipment and supplies
- **1.** Paper towels
- **2.** PPE
- **3.** Autoclavable squeegee and dust pan
- **4.** Tongs, forceps, or hemostats
- **5.** Autoclavable plastic bags
- **6.** Biohazardous-waste container
- **7.** BioZorb (biological absorbent and disinfectant; Ulster Scientific, Inc., Highland, NY)

II. PROCEDURE

Observe standard precautions.

A. Cleanup of major spills (possible aerosol formation)
- **1.** Evacuate the area or room, taking care not to breathe in aerosolized material.
- **2.** Alert personnel in the laboratory to evacuate the area.
- **3.** Close the doors to the affected area.
- **4.** After 30 min, when aerosols have settled, enter the area to clean.
  - **a.** Cleanup should be performed by the individual who committed the spill or by assigned personnel.
  - **b.** PPE should include gloves (e.g., heavyweight utility), disposable booties or water-impermeable shoe covers, long-sleeved gowns, and masks.
  - **c.** For high-risk agents, a full-faced respirator or HEPA-filtered mask should also be used.
- **5.** Remove and discard any broken glass or other objects.
  - **a.** *Do not* allow contact with hands.
  - **b.** Use rigid cardboard or squeegee and dustpan, tongs, forceps, or hemostats.
  - **c.** Discard these items into a plastic biohazardous-waste container.
- **6.** Cover the spill with disposable absorbent material (e.g., paper towels).
- **7.** After absorption of liquid, discard all contaminated material in a biohazardous-waste container.
- **8.** Carefully clean the spill site of any visible material, from the edges of the spill into the center, with an aqueous detergent solution.

II. PROCEDURE *(continued)*

9. Pour fresh disinfectant on the spill site or wipe down the site with disinfectant-soaked disposable towels.
10. Allow disinfectant to remain on the site for 20 min.
11. Absorb disinfectant solution with disposable material or allow the disinfectant to dry.
12. Rinse the spill site with water, and air dry to prevent slipping.
13. Discard all paper towels, gloves, and other disposable items into an autoclavable plastic bag or biohazardous-waste container.
14. Place gowns in a container for autoclaving after the cleanup process is completed.
15. Wash hands with soap and water.

B. Cleanup of minor spills

1. PPE should include gloves, gowns, and facial protection.
2. Wipe up contaminated material or spilled material with disinfectant-soaked paper towels.
3. Rinse materials with water if necessary.
4. Discard all materials in a biohazardous-waste container.
5. Wash hands with soap and water.

C. Cleanup of spills in biological safety cabinet (BSC)

1. See procedure 15.3.4 for operation of the BSC.
2. *Do not* turn off the cabinet.
3. Pour disinfectant (e.g., T.B.Q.) over the spill area and then apply paper towels to absorb liquids. *Do not* use alcohol.
4. Allow 20 min of contact with the disinfectant.
5. Discard paper towels in a biohazardous-waste container.
6. Using a soft cloth soaked in disinfectant, wipe down all cabinet surfaces and equipment as needed.
7. If spills leak through the vent cover, remove and clean the gutter area with disinfectant.
8. Place gowns in a container for autoclaving after the cleanup process is completed.
9. Allow the cabinet blower to run for 10 min before resuming activity.
10. For major spills of potentially infectious materials, contact a technical service consultant for decontamination.
11. *Do not* turn off the cabinet.

D. Commercial products (BioZorb)

1. Apply powder liberally over small spills, and wait until the spill has been completely absorbed.
2. Remove the absorbed material with the disposable scoop and scraper provided.
3. Rinse spill site with water, and air dry to prevent slipping.
4. Discard all materials in a biohazardous-waste container.
5. Wash hands with soap and water.

III. LIMITATIONS OF THE PROCEDURE

A. *Do not* pour hypochlorite solutions into pools of urine, blood, or feces. Highly irritating gases may be produced.

B. *Do not* use low-level disinfectants, such as quaternary ammonium compounds, for disinfecting spills.

IV. QUALITY ASSURANCE

Include QC information on reagent container and in QC records.

Complete Biohazardous-Spill Report and submit it to a laboratory safety committee representative. See Appendix 15.2.4–1 for a sample report form.

SUPPLEMENTAL READING

CLSI. 2005. *Protection of Laboratory Workers from Occupationally Acquired Infections.* Document M29-A3. CLSI, Wayne, PA.

Favero, M. S., and M. J. Arduino. 2006. Decontamination and disinfection, p. 373–381. *In* D. O. Fleming and D. L. Hunt (ed.), *Biological Safety: Principles and Practices*, 4th ed. ASM Press, Washington, DC.

APPENDIX 15.2.4–1

Biohazardous-Spill Report

Department _____

Biohazard name:
Time and date of spill:
Location of spill:
Cleanup procedure:
Names of staff in affected area:
Apparent injuries:
Reported by:
Cleaned by:
Signature: Date:
Note: Return form to department safety officer.

15.3.1 Introduction

In addition to implementing standard microbiological procedures and practices, management of the biohazards associated with working with pathogens includes physical barriers and administrative controls. Physical barriers include primary safety equipment and secondary facility design. Procedures 15.3.2 through 15.3.5 describe general physical and administrative controls for microbial containment.

SUPPLEMENTAL READING

Kimman, T. G., E. Smit, and M. R. Klein. 2008. Evidence-based biosafety: a review of the principles and effectiveness of microbiological containment measures. *Clin. Microbiol. Rev.* **21:**403–425.

15.3.2 Risk Assessment

Table 15.3.2–1 outlines a sample risk assessment and exposure control plan. Individual laboratories should modify this table to conform with the requirements in their own institution. The risk assessment should focus on the appropriate safety practices for handling infectious materials and cultures rather than on specific infectious agents. More information on risk assessment can be found at http://www.cdc.gov/OD/ohs/biosfty/bmbl5/sections/SectionII-BiologicalRiskAssessment.pdf.

SUPPLEMENTAL READING

Gilchrist, M. J. R., J. Hindler, and D. O. Fleming. 1992. Laboratory safety management, p. xxix–xxxvii. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook*, vol. 1. American Society for Microbiology, Washington, DC.

Table 15.3.2–1 Risk assessment and exposure control plan for the clinical microbiology laboratory[a]

Laboratory section and task	Exposure risk		PPE[b]				Engineering controls[d]		
	Blood and body fluid contact	Cultured biological agent exposure	Gloves	Lab coat/gown[c]	Face shield	Splash shield	BSC	Sharps containers readily accessible[e]	Safety centrifuge[f]
General									
Inventory: media and supplies	Low			Coat					
Clerical: computer entry, telephones, record reports, calculations, writing	Low		P	Coat					
Instrument maintenance									
Parts contaminated with blood or body fluids	High	Variable	R	Gown					
Parts not contaminated with blood or body fluids	Low	Variable	D	Coat					
Surface decontamination	Low	Variable	R	Coat					
Infectious-waste disposal	High	Variable	R	Gown	A (D)			Sharps	
Bacteriology									
Primary specimen processing	High	BSL 2	R	Coat			R	Sharps	
Subculture blood culture bottles	High	BSL 2	R	Coat	A[g]	A[g]	A[g]	Needles, vents	
Subculture colonies or broth tubes	Low	BSL 2[h]		Coat				Sharps	
Identification tests and AST	Low	BSL 2		Coat				Sharps	
Prepare smears and fix slides	Low	BSL 2		Coat				Sharps	
Stain fixed slides and read	Low			Coat				Slides	
Mycobacteriology and mycology									
Primary specimen processing	High	BSL 2/3	R	Gown			R[i]	Sharps	R[i]
Prepare smears, wet mounts, India ink; fix slides	High	BSL 2/3	R	Gown			R	Sharps	
Read wet mounts; India ink preps from specimens	High	BSL 2	R	Coat				Slides/covers	
Read wet mounts, India ink preps from cultures	Low	BSL 2/3	R	Coat				Slides/covers	
Examine sealed cultures	Low	BSL 2/3[j]		Coat					
Stain fixed slides and read	Low			Coat				Slides	
Handling yeast cultures, smears, and fixed slides	Low	BSL 2		Coat			D	Slides	
Handling molds and mycobacterial cultures	Low	BSL 3[k]	R	Gown			R[l]	Sharps	R[l]
Virology[m]									
Primary specimen processing	High	BSL 2	R	Coat			R	Sharps	
Feed and manipulate uninoculated cells	Low			Coat				Pipettes	
Read inoculated cells, tubes, or vials for CPE	Low	BSL 2		Coat					

(continued)

Table 15.3.2–1 Risk assessment and exposure control plan for the clinical microbiology laboratory[a] *(continued)*

Laboratory section and task	Exposure risk		PPE[b]				Engineering controls[d]		
	Blood and body fluid contact	Cultured biological agent exposure	Gloves	Lab coat/gown[c]	Face shield	Splash shield	BSC	Sharps containers readily accessible[e]	Safety centrifuge[f]
Feed and manipulate inoculated cells	High	BSL 2	R	Coat			R	Pipettes	
Perform tests to identify viruses	High	BSL 2	R	Coat			D	Sharps	
Stain fixed slides and read	Low			Coat				Slides	
Parasitology									
Concentrate fecal specimens, smears, wet mounts	Low	BSL 2	R	Coat				Pipettes/sticks	
Read fecal wet mounts	Low	BSL 2	R	Coat				Slides/covers	
Prepare thick and thin blood smears; fix slides	High	BSL 2	R	Coat	A	A		Slides/covers	
Stain and read slides (fecal and fixed blood)	Low			Coat				Slides/covers	
Antigen detection/PCR/DNA probes									
Primary specimen processing	High	BSL 2/3	R	Coat	A[n]	A[n]	A[n]	Pipettes	*M. tuberculosis*
Cultured microorganisms	Low	BSL 2	D	Coat			R	Sharps	
		BSL 3	R	Gown			R[o]	Sharps	
Antibiotic levels (Schlichter test)									
Manipulate organisms (without serum)	Low	BSL 2		Coat				Pipettes/loops	
Manipulate serum	High	BSL 2	R	Coat	A[n]	A[n]	A[n]	Pipettes	
Serology									
Manipulate serum	High	BSL 2	R	Gown	A	A		Pipettes	
Arrange tubes; prepare and dispense reagents	Low			Gown					
Mix serum and reagents; read and discard tests	High	BSL 2	R	Gown	A[n]	A[n]	A[n]	Pipettes	

[a] P, prohibited; R, required; D, discretionary; A, one of the required alternatives; AST, antimicrobial susceptibility testing; BSC, biological safety cabinet; CPE, cytopathic effect.

[b] *Remove PPE when leaving the laboratory.*

[c] Gowns with solid front and impervious to liquid. Many employers provide and launder gowns, thus replacing the need for lab coats.

[d] Recapping of needles should be prohibited. Carry tubes in racks, or use plastic tubes. Plan each task to minimize known hazard. Wash hands when leaving the laboratory.

[e] Sharps include scalpel blades, pipettes, plastic loops, sticks, needles, syringes, slides, and coverslips.

[f] Open sealed cups only in BSCs.

[g] Use a BSC or acrylic splash shield.

[h] Requires surveillance and action plan for occasional isolation of BSL 3 organisms (e.g., *Brucella* species, *Francisella* species, *Mycobacterium* species, and systemic fungi), especially if plates are held for ≥3 days.

[i] *M. tuberculosis* requires a BSC and safety centrifuge.

[j] Requires a contingency plan for breakage of culture containers.

[k] Mycobacteria other than those causing tuberculosis (MOTT group) may be handled at BSL 2; however, use BSL 3 practices since most manipulations precede organism identification.

[l] Use HEPA-filtered mask or respirator in addition to BSC for culture of *M. tuberculosis* group.

[m] Special precaution for BSL 4 agents (e.g., hemorrhagic fever virus) should be arranged (e.g., call CDC).

[n] Vortexing or other splatter-generating steps require use of a BSC or safety shield.

[o] Requires BSL 3 practices if there is a potential for aerosols.

15.3.3 Biosafety Levels

BSLs are guidelines that describe appropriate containment equipment, facilities, and procedures for use by laboratory workers. The BSLs range from BSL 1 to BSL 4. Each BSL is based on the increased risk associated with the pathogenicity of the microorganisms encountered. Most clinical microbiology laboratories follow BSL 2 practices. When working with highly infectious agents for which the risk of aerosol transmission is greater (e.g., *Brucella* species, *Francisella* species, *Mycobacterium tuberculosis*, and systemic fungi), clinical microbiology laboratories should follow BSL 3 practices. Because of the threat of bioterrorism, new facility designs for public health laboratories are conforming to BSL 3 requirements. Information on BSL recommendations for specific microorganisms can be found on the website http://www.cdc.gov/OD/ohs/biosfty/bmbl5/sections/SectionIV-LaboratoryBiosafetyLevelCriteria.pdf.

I. BSL 1

BSL 1 is recommended for work with microorganisms not known to cause disease in healthy adults (e.g., *Bacillus subtilis*).

It is imperative that these cultures be handled in a biological safety cabinet.

A. Restrict access to authorized personnel.
B. Make sinks for hand washing readily accessible.
C. Make eyewash stations readily accessible.
D. Make appropriate PPE available and ensure use.
E. Ensure that laboratory bench tops are impervious to liquids and resistant to chemicals.
F. Ensure that laboratory surfaces and equipment are easily cleaned and disinfected and that these procedures are done on a regular basis or whenever the surfaces or equipment is contaminated.
G. Decontaminate solid waste within the laboratory (e.g., by autoclaving), or package the waste to be transported off-site.

II. BSL 2

BSL 2 is recommended for microorganisms associated with human disease but not transmitted by aerosols (e.g., *Salmonella* species).

It is imperative that these cultures be handled in a biological safety cabinet.

A. Follow BSL 1 practices plus the following.
B. Display universal biohazard signs outside of the laboratory (*see* Fig. 15.6–4).
C. Perform specimen processing in a biological safety cabinet (BSC) (*see* procedure 15.3.4).
D. Perform centrifugation of mycobacteriologic specimens by using centrifuge safety cups (*see* procedure 15.4.3).
E. Ensure that an autoclave or other decontamination equipment is available and used for treatment of infectious waste (*see* procedures 15.4.2 and 15.8).
F. Use the appropriate PPE (e.g., gowns, gloves, and facial barriers).
G. Place all sharps carefully in conveniently located, puncture-resistant containers.
H. Trained personnel must observe good microbiological practices and techniques.

III. BSL 3

BSL 3 is recommended for hazardous microorganisms primarily transmitted by aerosols (e.g., *M. tuberculosis*).

It is imperative that these cultures be handled in a biological safety cabinet.

A. Follow BSL 1 and BSL 2 practices plus the following.
B. Control access to the laboratory.
C. Perform all manipulations of cultures and clinical material in a BSC (class II).
D. Maintain a negative-pressure airflow in the laboratory.
E. Include double doors and an anteroom in the laboratory design.
F. Discharge HEPA-filtered exhaust air from BSCs outside the facility.
G. Use all appropriate PPE and containment devices.
H. Use HEPA-filtered respirators or masks when aerosols may be generated.
I. Collect baseline serum samples from all personnel for serological determination of immune status.

IV. BSL 4

BSL 4 is recommended for agents causing life-threatening or untreatable diseases by aerosols or unknown transmission (e.g., Ebola virus).

It is imperative that these cultures be handled in a biological safety cabinet.

A. Follow BSL 3 practices plus the following.
B. Change to protective clothing before entering, and shower on exit.
C. Decontaminate all waste on exit.
D. Use a BSC (class III) and full-body, air-supplied, positive-pressure suit for all procedures.
E. Locate facility with specialized ventilation and waste management systems separate from other laboratories.

SUPPLEMENTAL READING

Centers for Disease Control and Prevention and National Institutes of Health. February 2007, posting date. *BioSafety in Microbiological and Biomedical Laboratories*, 5th ed. U.S. Government Printing Office, Washington, DC. http://www.cdc.gov/OD/ohs/biosfty/bmbl5/bmbl5toc.htm

Fleming, D. O., and D. L. Hunt (ed.). 2006. *Biological Safety: Principles and Practices*, 4th ed. ASM Press, Washington, DC.

Noble, M. A. 2007. Prevention and control of laboratory-acquired infections, p. 97–106. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.

15.3.4 Biological Safety Cabinet

The biological safety cabinet (BSC) is the most important primary containment equipment in the clinical microbiology laboratory. Class IIA laminar-flow cabinets are the most commonly used for BSL 2 and 3 practices. Air is drawn into the cabinet by negative air pressure and passes through a HEPA filter. The air flows in a vertical sheet that serves as a barrier between the outside and the inside of the cabinet. The cabinet exhaust air is also passed through HEPA filters. Aerosols are contained within the BSC, and the work area is protected from outside contamination when the cabinet is operating under the manufacturer's recommended conditions. The class III BSC is a completely enclosed, ventilated cabinet of airtight construction and provides the highest level of protection to laboratory workers and the environment. Class III cabinets are used for working with agents that require BSL 3 or 4 containment. More detailed information on the selection, installation, and use of BSCs can be found on the website http://www.cdc.gov/OD/ohs/biosfty/bmbl5/sections/AppendixA.pdf.

I. MATERIALS

A. Disinfectants
1. 10% Household bleach solution (0.5% sodium hypochlorite). Metal surfaces must be rinsed to avoid corrosion.
2. 70% Ethanol. Store outside of the cabinet.
3. *See* Table 15.2.3–1. Follow the manufacturer's directions for preparation.

B. Common supplies
1. Sterile disposable loops and spreaders
2. Bacteriological wire needles and loops
3. Sterile swabs
4. Sterile disposable pipettes
5. Sterile forceps
6. Glass slides
7. Absorbent mats or towels

C. Common equipment
1. Incinerator
2. Heat block
3. Vortex
4. Sonicator
5. Tissue grinders
6. Filtering apparatus
7. Pipetting devices
8. Test tube rack
9. Discard container

II. PROCEDURES

It is imperative that these cultures be handled in a biological safety cabinet.

A. Start-up
1. Turn on blowers for at least 10 min before QC checks and specimen processing.
2. Open the viewscreen to operating height (8 in.).
3. Turn off UV lights (if present).
4. Turn on a fluorescent light.
5. Perform daily QC as outlined in item III.A below.
6. Clean the cabinet working surfaces with appropriate disinfectant.

B. Operation
1. Avoid outside sources of air currents (e.g., personnel walking by, doors being opened or closed, etc.).
2. Wear long-sleeved coats or cuffed gowns and gloves during specimen processing.

II. PROCEDURES *(continued)*

3. Place all items that will be used in a planned activity inside the cabinet prior to starting work.
   a. Segregate clean from contaminated materials.
   b. Place the minimum number of large devices and supplies inside the cabinet.
      (1) *Do not* block intake or rear grills.
      (2) *Do not* place sterile material or specimens near the sides, front, or back of the cabinet.
      (3) *Do not* place or tape paper notes or procedures on the window.
      (4) *Do not* use a flame in the cabinet.
      (5) *Do not* operate centrifuges in the cabinet.
4. Plan work flow to minimize movements.
   a. Work at least 6 in. inside the front grill intake.
   b. Avoid rapid arm movement in and out of the cabinet while a procedure is in progress.
   c. If necessary, slowly move arms in and out of the cabinet.

C. Shutdown

1. Allow the cabinet to continue running for 15 to 20 min after work is completed and before removing materials.
2. Allow the cabinet to continue running for at least 3 h after processing acid-fast or fungal specimens.
3. Place contaminated materials in covered containers or closed bags, or immerse them in disinfectant, before removal from the cabinet.
4. Disinfect the surfaces of any contaminated materials before removal from the cabinet.
5. Clean up spills by following the steps outlined in procedure 15.2.4, item II.C.
6. Clean interior surfaces with disinfectant (e.g., 70% ethanol)
7. Turn off the blower and lights.
8. Turn on UV light (if present).
9. Close viewscreen (if present).
10. *Do not* shut down cabinets that function 24 h a day, unless a malfunction occurs or routine maintenance is required.

III. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Daily

1. Disinfect all cabinet surfaces while cabinet is running.
2. Check air velocity (recommended for BSCs not equipped with velocity gauges and airflow indicators in the work area).
   a. Place a Vaneometer (Lab Safety Supply, Janesville, WI) at the opening of the cabinet toward the work space area but not on the airflow grill.
   b. Level the Vaneometer and record face airflow velocity.
      (1) The air velocity should be greater than 100 ft/min.
      (2) *Do not* use the BSC if velocity remains less than 100 ft/min. Contact a technical service consultant.
3. Check the blower function.
   a. Record the manometer gauge reading (if provided).
   b. Perform a visible-smoke test.
      (1) Pass a smoke source (e.g., smoke bottle or smoke stick; Lab Safety Supply) from one end of the cabinet opening to the other.
      (2) The smoke should show a smooth downward flow with no dead spots or reflux.
      (3) Record results.

III. QUALITY CONTROL *(continued)*

c. Observe airflow check strips (optional).
(1) Strips should be drawn toward the work space area.
(2) Record results.

B. Weekly
Clean UV lights (if present) with 70% ethanol.

C. Monthly
Clean the gutter area with disinfectant.

D. Semiannually
1. Have mycobacteriology and mycology BSCs recertified by certified personnel.
2. Have certified personnel measure UV light output (if present).

E. Annually (or every 1,000 h)
Have routine and virology BSCs recertified by certified personnel.

F. Complete and initial the Biological Safety Cabinet Quality Control and Maintenance Record worksheet after each task is completed (*see* Appendix 15.3.4–1).

IV. LIMITATIONS OF THE PROCEDURES

A. BSCs should not be used as fume hoods.
1. Toxic, radioactive, or flammable vapors or gases are not removed by HEPA filters.
2. Potentially hazardous amounts of volatile material may build up in class II-type cabinets.
3. Exhausted vapors can be vented into the laboratory air.

B. The use of gloves when performing routine manipulations should not substitute for proper hand washing practices. Wash hands with soap and water between tasks and when heavily soiled with clinical material.

SUPPLEMENTAL READING

CLSI. 2005. *Protection of Laboratory Workers from Occupationally Acquired Infections*, Appendix B. Document M29-A3. CLSI, Wayne, PA.

Kruse, R. H., W. H. Puckett, and J. H. Richardson. 1991. Biological safety cabinet. *Clin. Microbiol. Rev.* **4:**207–241.

National Sanitation Foundation. 1988. *Standard 49 for Class II (Laminar Flow) Biohazard Cabinetry*. National Sanitation Foundation, Ann Arbor, MI.

U.S. Department of Health and Human Services. 1995. *Primary Containment of Biohazards: Selection, Installation and Use of Biological Safety Cabinets.* U.S. Department of Health and Human Services, Washington, DC.

APPENDIX 15.3.4–1 Biological Safety Cabinet Quality Control and Maintenance Record

Month _____ Year _____ Cabinet no./Class _____ Location _____

Date	Visual airflow reading	Blower/gauge reading (manometer)	Vaneometer reading (optional) ≥100 ft/min	Cleaning	Other
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					
21					
22					
23					
24					
25					
26					
27					
28					
29					
30					
31					
Corrective action				Date:	Reviewed by:
Note: Sign initials in appropriate box after each task is completed.					

15.3.5 PPE and Engineering Controls

I. PPE AND OSHA STANDARDS

OSHA standards for exposure to blood-borne pathogens require the laboratory to provide PPE for its employees.

Observe standard precautions.

A. Protective clothing is worn while in the laboratory. When leaving the laboratory, either dispose of protective clothing in the laboratory or have it laundered by the institution.
  1. Coat, gowns, smocks, or aprons impervious to liquids
  2. Garments fitted snugly around the wrists

B. Gloves are worn when handling infectious materials, contaminated surfaces, or equipment. Dispose of gloves when contaminated, after handling infectious materials is completed, and before leaving the laboratory. Carefully remove gloves by folding cuff back over exposed portion (or inside out) without creating an aerosol. Hands should be washed before and after each use.
  1. Disposable and powderless gloves (should not be washed or reused)
  2. Glove liners
  3. Hypoallergenic or latex-free gloves

C. Face and eye protection is used when splashes or sprays of infectious materials may occur outside the biological safety cabinet (BSC). Laboratory workers who wear contact lenses should also wear protective eyewear.
  1. Masks (e.g., N-95)
  2. Goggles or safety glasses
  3. Face shields
  4. Acrylic splash shields

II. ENGINEERING CONTROLS

Engineering controls should be in place within the laboratory. The controls outlined below are designed to help prevent or minimize infectious aerosols, splashes, or sprays and sharps injury. The OSHA standard for occupational exposure to blood-borne pathogens (1) requires employers to select safer needle devices as they become available. Information on safe needle devices can be found on the website http://www.sustainablehospitals.org.

Observe standard precautions.

A. Automatic or mechanical pipetting devices
B. Bench tops impervious to water and resistant to acids
C. Biohazard bags
D. Biohazard labels are required on the following.
  1. Refrigerators and freezers containing blood or infectious materials
  2. Containers used to store, transport, or ship regulated waste, blood, or infectious materials

II. ENGINEERING CONTROLS *(continued)*

   3. Sharps containers
   4. Contaminated equipment
E. BSC
F. Centrifuge safety cups
G. Containers
   1. Secondary containers (transport of specimens)
   2. Sharps (puncture resistant)
   3. Waste (including stainless steel buckets)
H. Eyewash stations
I. Hand washing sinks
J. HEPA-filtered respirators or masks
K. Laboratory furniture that is sturdy and accessible for cleaning
L. Plasticware (substitute for glassware)
M. Syringes
   1. Needle-locking syringes or disposable syringe-needle units
   2. Syringes that resheath needles
   3. Needleless systems or other safe devices
N. Waste collection carts

III. COMMERCIAL SAFETY PRODUCTS

Observe standard precautions.

Numerous commercial safety products are available for laboratory use. A partial list of common devices or instruments used in the microbiology laboratory is given below. Manufacturers of instruments (e.g., autoanalyzers and centrifuges) are encouraged to incorporate new safety design features and decontamination procedures during regular use and before servicing.

A. Needlestick prevention devices (e.g., those from Becton Dickinson Microbiology Systems, Cockeysville, MD)
B. Ergonomic pipettes (Rainin Instrument Company, Inc., Emeryville, CA)
C. Biogel Revel glove (Regent Hospital Products, Greenville, SC)
D. Automated petri dish streaking instrument (e.g., Isoplater 180; Vista Technologies, Inc., Edmonton, Alberta, Canada)
E. Automated tissue processor (e.g., Stomacher; Seward Medical Ltd., London, United Kingdom)

REFERENCE

1. **Occupational Safety and Health Administration.** 2001. Occupational exposure to bloodborne pathogens; needlestick and other sharps injuries: final rule. *Fed. Regist.* **66:**5318–5325.

SUPPLEMENTAL READING

Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health. 1999. Preventing needlestick injuries in health care settings. U.S. Department of Health and Human Services (NIOSH) publication no. 2000-108. U.S. Department of Health and Human Services, Washington, DC.

Centers for Disease Control and Prevention and National Institutes of Health. February 2007, posting date. *BioSafety in Microbiological and Biomedical Laboratories*, 5th ed. U.S. Government Printing Office, Washington, DC. http://www.cdc.gov/OD/ohs/biosfty/bmbl5/bmbl5toc.htm.

Occupational Safety and Health Administration. 1991. Occupational exposure to bloodborne pathogens: final rule. *Fed. Regist.* **56:**64004–64182.

15.4.1 Introduction

Procedures 15.4.2 through 15.4.6 describe common operational procedures performed in the clinical microbiology laboratory. They are intended to present proper safety practices when working with equipment and handling infectious material.

15.4.2 Autoclave

An autoclave or steam sterilizer is an insulated pressure chamber in which saturated steam is used to elevate temperature above the normal boiling point of water. A gravity displacement autoclave, in which lighter steam is fed into the chamber to displace heavier air, is the most common type used in the laboratory. The higher the temperature and pressure, the shorter the time required to kill microorganisms. In addition to time, temperature, and direct steam contact, other factors which can effect kill include density, physical state and size, and organic content of the material treated. Autoclaves are used for sterilization of both culture media and heat-stable supplies and the treatment of infectious waste. Many operating rooms and laboratories today use a rapid enzymatic indicator to monitor the sterilization process (http://www.3m.com/healthcare). The activity of a *Bacillus stearothermophilus* enzyme is measured by reading a fluorescent product produced by the enzymatic breakdown of a nonfluorescent substrate. Detection of the enzyme after 1 to 3 h of incubation indicates a sterilization process failure.

I. MATERIALS

A. Physical monitors (recorder chart)
- **1.** Exposure time
- **2.** Exposure temperature
- **3.** Chamber pressure

B. Biological indicators (*B. stearothermophilus*)
- **1.** Ampoule
- **2.** Spore strip
- **3.** Enzymatic (Attest Rapid Readout Biological Indicators and Monitoring System; 3M, St. Paul, Minn.)

C. Chemical indicators
- **1.** Chemical-impregnated strip
- **2.** Autoclave tape

D. Containers and packaging
- **1.** Autoclave bags, trays, or pans
- **2.** Metal pails
- **3.** Linen packs

E. PPE
- **1.** Splash goggles
- **2.** Heat-resistant gloves

II. PROCEDURES

Observe standard precautions.

A. Media, supplies, and linen packs

1. Initial preparation
- **a.** Loosen all caps or lids.
- **b.** *Do not* fill vessels more than two-thirds full.
- **c.** Place autoclave tape on items to be sterilized.

2. Load the autoclave with material to be sterilized.
- **a.** Allow adequate space between objects (at least 2 in. apart).
- **b.** Use test tube or other wire racks to allow the free flow of steam.

3. Close the autoclave and set the sterilization time and temperature (*see* manufacturer's instructions).
- **a.** Autoclave culture media for 15 min at 121°C, with a 15-min exhaust time. Suggested autoclaving times are 18 min for 500 ml, 21 min for 1,000 ml, and 24 min for 1,500 ml. A general rule is to add 3 min more for each 500 ml of medium.
- **b.** Autoclave dry-wrapped material for 25 min at 121°C with fast exhaust and a 30-min dry cycle.

II. PROCEDURES *(continued)*

4. Start the cycle and confirm that temperature and pressure monitors are functioning.
5. After the cycle has ended and the chamber pressure has reached 0 lb/in^2, open the door slowly.
   - **a.** Stand behind the door to avoid steam.
   - **b.** Wear eye protection and heat-resistant gloves.
   - **c.** Allow 20 min for contents to cool.
   - **d.** Handle all liquid material gently to prevent splattering of hot fluid.
6. Carefully remove material from the autoclave and place on designated area (e.g., bench top or cart) labeled "autoclaved."
7. Tighten caps.

B. Infectious waste

1. For initial preparation, infectious waste should be clearly marked and separated from microbiological media and supplies to be sterilized.
2. Autoclave bags
   - **a.** Place infectious waste in double autoclave bags with biohazard labels.
   - **b.** Fill bags no more than three-fourths full.
   - **c.** Tie bags loosely or add 1 cup of water to bags before sealing (optional).
   - **d.** Place autoclave tape on the outside of bag.
   - **e.** Place the bags on autoclave pans or trays.
   - **f.** Place liquid waste in vessels (not to exceed two-thirds full) in pails.
3. Load autoclave bags on pans or trays or in pails to be sterilized.
4. For routine waste, set the time and temperature for 15 min at 121°C, with a 15-min exhaust time.
5. For mycobacteriology waste (e.g., stock cultures), set the time and temperature for 30 to 45 min and 121°C, with a 15-min exhaust time.
6. Proceed as outlined in item II.A above.
7. Drain excess fluid from the pans and clean them with soap and water. Allow molten agar to solidify and then discard it in normal trash.

III. LIMITATIONS OF THE PROCEDURES

A. Steam under pressure and at high temperature presents the risks of scalding and explosion to the operator.

B. Antineoplastic agents, toxic chemicals, or radioisotopes in waste may not be destroyed. In the microbiology laboratory, the most likely types of radiation are ^{14}C and ^{3}H, which can be autoclaved without any radiation hazard.

C. Highly volatile chemicals could become vaporized and disseminated by heat and ignited by a spark from operating equipment.

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Each run

1. Check the data recorder to verify correct time and temperature.
2. Check the autoclave tape, chemical indicator strip, or rapid enzymatic indicator to verify operating conditions.

B. Daily

1. Remove the sediment screen from the chamber drain hole and clean thoroughly.
2. Periodically clean accessories such as shelves, racks, and trays with soap and water.

C. Weekly

Include a biological indicator ampoule or spore strip in a routine run based on the manufacturer's instructions.

D. Troubleshooting and maintenance

Contact a technical service representative.

SUPPLEMENTAL READING

Joslyn, L. J. 2001. Sterilization by heat, p. 695–728. *In* S. S. Block (ed.), *Disinfection, Sterilization, and Preservation*, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.

15.4.3 Centrifuge

A centrifuge produces centrifugal force that is used to separate particles of various densities. In the microbiology laboratory, centrifugation is used for concentrating microorganisms and cells, separating components of body fluids or mixed suspensions, and removing particulate matter. Cytocentrifugation is also used in the laboratory as a rapid means of concentrating small volumes of body fluid specimens for staining. This procedure focuses on general safety issues related to centrifugation. The laboratory should follow the manufacturer's recommendations for the operation and maintenance of specific instruments.

I. MATERIALS

A. Supplies
- **1.** Disinfectants and cleaning solutions
- **2.** Paper towels
- **3.** Gloves, gowns, masks (for spills)

B. Equipment
- **1.** Centrifuge
  - **a.** Horizontal rotor, fixed-angle rotor, microtube, etc.
  - **b.** Cytocentrifuge (Shandon, Inc., Pittsburgh, Pa.)
- **2.** Centrifuge tubes, bottles
- **3.** Blank containers
- **4.** Safety cups with O-rings
- **5.** Balance (two pan)
- **6.** Calibrated thermometer
- **7.** Tachometer (e.g., photoelectric)

II. PROCEDURE

Observe standard precautions.

A. Balancing of load
- **1.** Weigh similar items and balance to within 0.5 g.
- **2.** Use blank tubes or containers filled with water for balancing.
- **3.** Weigh buckets with tubes.
  - **a.** Add balance tubes until both buckets are equal in weight.
  - **b.** Distribute tubes and adapters in rotor or bucket holder in a symmetrical arrangement.
  - **c.** Place all buckets (empty or full) in place.

B. Containment of material
- **1.** All specimens must be capped or covered.
- **2.** Use nonbreakable plastic screw-cap tubes.
- **3.** Place tubes in tightly covered safety cups or rotors (e.g., tubes containing specimens of mycobacteria and systemic fungi)
- **4.** Use aerosol-free centrifuges if available.
- **5.** Cytocentrifuge (double-enclosed system)
  - **a.** *Do not* overfill specimen containers.
  - **b.** All cytospin funnels must be capped.
  - **c.** Open sealed heads in a biological safety cabinet (BSC).
- **6.** Open sealed safety cups in a BSC after centrifugation.

II. PROCEDURE *(continued)*

C. Operation

1. Set appropriate time, rotation speed, temperature, and brake speed (if necessary).
2. Close and lock the top.
3. Turn the centrifuge on. For manual speed adjustment, slowly increase the speed until the desired number of revolutions per minute is reached.
4. Once the desired speed is reached, check for undue vibration.
a. Turn the centrifuge off if excess vibration occurs.
b. *Do not* apply the brake.
5. *Do not* open the top of the centrifuge until it has come to a complete stop.

III. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Each run

1. Check tubes for cracks.
2. Check inside of carrier cups for rough walls or glass and other debris, and remove any of these from the base of the cushion.
3. Check that each load is properly balanced.
4. Check the operating temperature.
5. Clean up any spills immediately (*see* procedure 15.2.4).
a. Use a 10% bleach solution, and rinse surfaces thoroughly after cleaning.
b. Use alternative disinfectants (*see* Table 15.2.3–1).

B. Daily

1. Clean inside of the bowl with disinfectant and rinse thoroughly.
2. Refrigerated centrifuges
a. Allow the bowl to dry if the centrifuge is turned off at night.
b. Leave the top closed when the unit is under refrigeration.

C. Weekly

1. Clean and open all vents.
2. Clean and disinfect rotors, carriers, and the inside of the bowl. Rinse and dry thoroughly.

D. Monthly

1. Vacuum clean the condenser coils, fan, screens, and filters (refrigerated centrifuges).
2. Check brushes.
3. Check electrical connections.

E. Semiannually

1. Check the internal temperature of refrigerated units with calibrated thermometer.
2. Check brushes.
3. Calibrate speed with a photoelectric tachometer.

F. Annually

Have speed and temperature control recertified by qualified personnel.

IV. TROUBLESHOOTING

A. Vibration

1. Check for proper balance.
2. Check all tube holders and buckets.
3. Check that the centrifuge is on a level surface.

B. Breakage

1. Check balance.
2. Check tubes for proper size or use.
3. Check tube holders for proper cushion.

IV. TROUBLESHOOTING *(continued)*

C. Corks or stoppers pop off.
- **1.** Use screw-cap top.
- **2.** Use Parafilm over the stopper.
- **3.** Use clean tube holders, free of glass.
- **4.** Use lower temperature in (refrigerated) centrifuge.

D. Fine gray dust in rotor chamber
- **1.** Thoroughly vacuum clean the chamber, and run the centrifuge empty several times.
- **2.** Clean between operations until dust is gone.

V. LIMITATIONS OF THE PROCEDURE

A. *Do not* centrifuge large volumes of flammable liquids which could become vaporized and then be ignited by a spark from operating equipment.

B. Concentration of infectious material for culture, such as bronchoalveolar lavage specimens, by cytocentrifugation may be performed in the routine culture area. Preparations should not produce spatters or aerosols if specimen containers are properly filled.

C. Samples of infectious material prepared for smears by cytocentrifugation for detection of acid-fast bacilli should be pretreated with equal amounts of fresh 5% sodium hypochlorite (household bleach).

SUPPLEMENTAL READING

Saceanu, C. A., N. C. Pfeiffer, and T. McLean. 1993. Evaluation of sputum smears concentrated by cytocentrifugation for detection of acid-fast bacilli. *J. Clin. Microbiol.* **31:**2371–2374.

APPENDIX 15.4.3–1 Centrifuge Quality Control and Maintenance Record

Year _____ Location: _____ Date in use: _____

Centrifuge: Model no. _____ Serial no. _____ Identification no. _____

Date	Temperature check	Speed calibration check	Cleaning	Other

15.4.4 Gas Cylinders

Laboratory personnel should be cognizant of hazards from compressed gases. A falling cylinder can cause physical injury to personnel. A gas cylinder with ruptured pressure-reducing fittings or broken valve heads can become an "unguided missile," destroying everything in its path. Some gases may be toxic and/or flammable. If heated, cylinders may explode.

I. PROCEDURE

Observe standard precautions.

A. Transportation

1. *Do not* move a cylinder with a regulator attached. Valve safety covers must be tightened securely during transport.
2. *Do not* drag or roll cylinders.
   - **a.** Use hand trucks, dollies, carts, etc., to move cylinders.
   - **b.** Properly secure cylinders to hand trucks, etc.
3. *Do not* place cylinders in a horizontal position.

B. Storage

1. *Do not* accept cylinders that are marked with wired-on tags or are color coded. All cylinders must be labeled with the name of the contents and appropriate warning labels (e.g., U.S. Department of Transportation labels).
2. Secure all gas cylinders in an upright position in secure racks or to wall mounts.
3. *Do not* remove valve safety covers until pressure regulators are attached.
4. *Do not* store cylinders with or near flammable material.
5. *Do not* store cylinders with highly combustible material in the laboratory. A separate room or enclosure should be reserved for stored cylinders.
6. *Do not* store cylinders at temperatures of >125°F (55°C).
7. *Do not* discard empty cylinders.
   - **a.** Mark cylinders as empty and keep them secured in an upright position with safety cover in place.
   - **b.** Return empty or unused cylinders to the manufacturer.

C. Pressure regulators and needle valves

1. Use only designated fittings for gases indicated.
2. *Do not* use oil, grease, or lubricants on valves, regulators, or fittings. Threads and surfaces should be clean and tightly fitted.
3. *Do not* attempt to repair damaged cylinders or to force open frozen cylinder valves.
4. Use a proper-size wrench to tighten regulators.

I. PROCEDURE *(continued)*

5. Open the diaphragm control knob completely before the control valve is opened.
6. Open all valves slowly.
   a. Stand to the side of gauges.
   b. *Do not* force valves that stick.
7. Check all connections for leaks.
   a. Test before and after regulators or gauges are attached.
   b. Test piped lines before use and periodically thereafter.
   c. Use a "snoop" or soap solution.
8. Leave valve handles attached to cylinders.
9. Regulation of gas flow rate
   a. Use high-pressure valve on the cylinder to set the maximum rate of gas flow.
   b. Use needle valve for fine tuning of gas flow.
10. Shut off valves on gas cylinders when not in use.

II. QUALITY CONTROL

Include QC information on reagent container and in QC records.

Complete and initial the Gas Cylinder Quality Control Record worksheet after each task is completed (*see* Appendix 15.4.4–1).

SUPPLEMENTAL READING

NCCLS/CLSI. 2004. *Clinical Laboratory Safety.* Document GP17-A2, p. 45–46. NCCLS/CLSI, Wayne, PA.

APPENDIX 15.4.4–1 Gas Cylinder Quality Control Record

Year _____ Location _____

Date cylinder received in lab	Cylinder identification code	Date placed into use	Pressure when placed into use	Date empty and replaced
Corrective action			Date:	Reviewed by:
Note: Complete information and sign initials in appropriate box after each task is completed.				

15.4.5 Pneumatic Tube System

A safe and efficient way to transport specimens and materials from one point to another is through an automated and computerized pneumatic tube system. Such a system typically consists of carriers, sending/receiving stations, blowers, transfer units, and a control center. The primary concern in the transportation of clinical specimens in a pneumatic tube system is leakage of the specimen into the carrier and potentially the system tubing, thus exposing workers to hazardous materials. The system should be operated according to the manufacturer's instructions. All specimens must be handled in accordance with standard precautions.

I. MATERIALS

A. Primary containers (e.g., clear plastic biohazard Ziploc bags)
B. Secondary containers (e.g., conical, hard plastic devices which close when latches are engaged)
C. Foam liner/packing material
D. Laboratory requisition slips
E. PPE

II. PROCEDURE FOR SENDING SPECIMENS

Observe standard precautions.

A. Ensure that container tops are tightly secured.
B. Place specimen in biohazard Ziploc bag and secure it.
C. Place requisition slip in the outer pouch of the biohazard Ziploc bag.
D. Select the appropriate foam liner/packaging material for the specimens.
  1. Use slotted foam for shipping Vacutainer tubes.
  2. Select material to provide immobility to the specimen.
E. Place the foam liner/packaging material in the carrier.
F. Place the biohazard Ziploc bag with specimens inside the packing material in the carrier.
G. Secure the carrier lid tightly, making sure that no packing material is visible outside the carrier.
H. Holding the carrier in both hands, flip it over and back in a smooth but quick fashion, and observe for movement in the carrier. If movement is detected, repeat the packing procedure until the specimen is immobile.
I. Place the closed carrier in the dispatcher unit, select the destination address, and press send.

III. PROCEDURE FOR RECEIVING CARRIERS

Observe standard precautions.

A. An incoming carrier message should be displayed on the control panel indicating that the station has been selected by a user to receive a carrier.
B. The carrier is then dropped from the dispatcher into a bin at the station.
C. Remove the carrier from the bin.
D. Examine all specimens for visible contamination or breakage before removing from the secondary container.

III. PROCEDURE FOR RECEIVING CARRIERS *(continued)*

E. If contamination is detected, the following should be carried out.
  1. Primary containers should be decontaminated before being sent to the work areas for testing or contents should be transferred to a clean container.
  2. Laboratory requisition slips should be discarded and replaced.
  3. Padding material should be decontaminated or discarded.
  4. Remove the carrier from the system for cleaning if contaminated.
  5. Clean contaminated material with bleach, wearing appropriate PPE.

F. If a spill has occurred or if the leak has escaped into the system, notify maintenance personnel so that appropriate decontamination procedures may be done (e.g., use of bleach canister).

G. Sign off the pneumatic tube system at your location so the carriers do not accumulate in the bin.

IV. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Complete a Biohazard Spill Report (*see* Appendix 15.2.4–1) and submit it to a laboratory safety committee representative.

B. Report improperly packaged specimens to a manager or regulatory specialist for follow-up with the sending unit.

V. LIMITATIONS OF THE PROCEDURES

A. Approximately 95% of all system problems are a result of user error in packaging and sending specimens. Cloth towels are not acceptable as padding.

B. Blood culture bottles are especially prone to breakage if not properly packaged.

C. Items with screw caps, such as urine and stool containers, are prone to leakage if caps are not fastened tightly.

D. Syringes with needles are not acceptable for transport.

15.4.6 Specimen/Microorganism Storage and Retention

Clinical specimens and microorganisms are routinely stored for extended periods of time for retrieval and testing or before shipment to other laboratories. Specimens should be stored in a secure, well-organized area separate from reagents. Microorganisms should be stored in an area with restricted access and clearly identified. All containers (e.g., tubes, vessels) should be placed in appropriate racks, baskets, freezer boxes, or other devices to maintain them upright. Standard precautions should be followed at all times when handling these specimens as well as QC and proficiency test microorganisms.

I. ROOM TEMPERATURE STORAGE

Observe standard precautions.

A. Store culture plates and capped tubes in suitable racks or baskets in the work area for 1 to 2 weeks.
B. Store samples away from high-traffic areas (e.g., in cabinets).

II. REFRIGERATOR STORAGE

Observe standard precautions.

A. Store specimens in a designated refrigerator designed for easy access and cleaning.
B. Maintain a reasonable cleaning schedule to prevent contamination.

III. FREEZER STORAGE

Observe standard precautions.

A. Place specimens or microorganisms to be frozen in containers or freezer vials designed for low-temperature storage (e.g., Protect tubes).
B. Use plastic containers whenever possible.
C. Fill containers to no more than two-thirds of their capacity.
D. Place culture tubes in a designated secondary freezer box.
E. Remove any broken tubes in the freezer using appropriate PPE, and decontaminate the area.
  1. Remove and decontaminate any intact tubes.
  2. Place frozen specimens into a secondary container, allow to thaw, and then transfer to a new container and refreeze (if needed).
  3. Disinfect the freezer before further use.

IV. LIQUID NITROGEN STORAGE

Observe standard precautions.

A. Handle cryogenic liquid nitrogen containers with care.
  1. Use insulated thermal gloves when handling any object that has been in contact with liquid nitrogen.
  2. Safety glasses are recommended during transfer of liquid nitrogen.
  3. *Do not* allow objects cooled by liquid nitrogen to touch bare skin.

IV. LIQUID NITROGEN STORAGE *(continued)*

B. Cryobiological storage container
- **1.** Storage
  - **a.** Always store in an upright position.
  - **b.** Store only in areas that are fully ventilated.
  - **c.** Store in clean dry areas.
- **2.** *Do not* roll, drag, or tip containers.
- **3.** *Do not* vibrate, jolt, or drop a container (which might damage its vacuum insulation system).
- **4.** *Do not* overfill containers.
- **5.** *Do not* seal containers tightly.
- **6.** *Do not* tamper with relief values.
- **7.** Use solid metal or wooden dipsticks.
- **8.** Avoid moisture, caustic cleaners, chemicals, or other substances that might cause corrosion.

SUPPLEMENTAL READING

CLSI. 2005. *Protection of Laboratory Workers from Occupationally Acquired Infections.* Document M29-A3. CLSI, Wayne, PA.

Petty, C. A., K. C. Carroll, and L. G. Reimer. 2007. Procedures for storage of microorganisms, p. 55–61. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.

15.4.7 Other Equipment and Devices

Preventative maintenance and decontamination procedures for all instruments and devices should be performed per the manufacturer's instructions. Copies of these instructions must be available in the laboratory, including biological-hazard information. All activities performed on these devices should follow standard precautions. Prior to maintenance or repair, contaminated devices or the portion to be worked on should be decontaminated. All repairs should be performed by properly trained personnel. Service personnel need to wear the appropriate PPE and follow the laboratory safety policies.

15.5.1 Introduction

The purpose of the information in this subsection is to help reduce the chances of clinical microbiology laboratory employees' being exposed to microorganisms associated with bioterrorism and with laboratory-acquired infections, including Creutzfeldt-Jakob disease.

15.5.2

Early (Bench Level) Recognition of Agents of Bioterrorism and Laboratory-Acquired Infections

Clinical microbiologists, especially those who work at the bench level in sentinel laboratories, must be aware that they can work with, manipulate, culture, and be exposed (even for days) to microorganisms which cause the more common laboratory-acquired infections (LAI) *before* these microorganisms are recognized as such. These microorganisms include agents of bioterrorism. The result can be an LAI. One way to reduce potential LAI in clinical microbiology laboratories is to make clinical microbiologists aware of the appearances of these microorganisms early in the diagnostic process, i.e., in Gram stain preparations and as early as possible in culture. Such early recognition can alert workers to potential dangers and prompt them to seal plates, work subsequently within biological safety cabinets, and use other appropriate safety precautions while working with suspicious cultures.

Table 15.5.2–1 shows selected agents of LAI and some characteristics of these agents early in the bench level laboratory diagnostic process. Except for *Neisseria meningitidis*, these agents usually are not readily recognizable early in growth as are routine isolates such as *Staphylococcus* spp., *Streptococcus* spp., and members of the *Enterobacteriaceae*. Table 15.5.2–1 shows the Gram stain morphology, colonial morphology, and biochemical reactions which can be helpful to sentinel laboratory workers to rule in and rule out a particular agent. If a particular agent has sufficient characteristics within its indicative column, the agent *cannot* be ruled out, and the agent should be examined by a reference laboratory in the Laboratory Response Network. Table 15.5.2–1 can be modified to suit the needs or opinions of individual laboratories.

Table 15.5.2–1 Early (bench level) recognition of microorganisms associated with bioterrorism and with LAI[a,b]

Characteristic	*Bacillus anthracis* (BT)	*Yersinia pestis* (BT)	*Burkholderia mallei* and *Burkholderia pseudomallei* (BT)	*Francisella tularensis* (BT)	*Brucella* spp. (BT)	Smallpox virus (BT)	*N. meningitidis*
Colony morphology (BAP)	Nonhemolytic Ground-glass appearance Irregular or wavy edges Tenacious "Beaten egg white" appearance when touched with loop	Pinpoint at 24–48 h "Fried egg" or "hammered copper" or shiny appearance at 48–72 h Nonhemolytic	*B. mallei:* smooth, gray, translucent, not pigmented *B. pseudomallei:* days 1 to 2, small, smooth, creamy; days 2+, dry, wrinkled	Does not pass well on BA Usually slightly alpha-hemolytic Colonies *do not* have a central dark dot which looks like a star or "crossed cigars"	Small colonies Punctate after 48 h Nonhemolytic		Poor growth to NG
			***Stop!* If a MAC was not inoculated in the original setup, subculture (within a BSC) growth to a MAC.**				
Colony morphology (MAC)		Growth Lactose$^-$	*B. mallei:* lactose$^-$ or NG *B. pseudomallei:* lactose$^-$	NG	Poor growth to NG		NG
Growth	Standard conditions Extremely rapid	28°C, optimal 35–37°C, slower	35–37°C Ambient air or increased CO_2	Standard conditions; very slow growth (2, 4, or more days)	Grows in blood culture media Can require blind subcultures	Grows in most cell lines Unusual or unrecognizable CPE	Grows best in CO_2 CHOC Beige to gray-brown, translucent, smooth, 0.5–1 mm
			***Stop!* Perform the following procedures *only* within a BSC!**				
Gram stain morphology	Large GPR From BA: no capsule; central to subterminal spores which do not swell cell From blood: capsule; usually no spores	Plump GNR Gram: +/− bipolar or "safety pin" Wright-Giemsa: bipolar	*B. mallei:* small GNCB *B. pseudomallei:* small GNR +/− bipolar staining	Minute GNCB Smaller than *Haemophilus influenzae* Pleomorphic Poorly staining	Tiny GNCB Faintly staining "Pink sand"		GNDC

(continued)

Table 15.5.2–1 Early (bench level) recognition of microorganisms associated with bioterrorism and with LAI[a,b] *(continued)*

Characteristic	*Bacillus anthracis* (BT)	*Yersinia pestis* (BT)	*Burkholderia mallei* and *Burkholderia pseudomallei* (BT)	*Francisella tularensis* (BT)	*Brucella* spp. (BT)	Smallpox virus (BT)	*N. meningitidis*
Tests	Catalase$^+$ Motility$^-$	Catalase$^+$ Motility$^-$ Urease$^-$ Oxidase$^-$ Indole$^-$	Catalase$^+$ Colistin (10 µg) and polymyxin B (300 U): R *B. mallei:* motility$^-$ *B. pseudomallei:* motility$^+$ Indole$^-$ *B. mallei:* oxidase$^{+/-}$ *B. pseudomallei:* oxidase$^+$ MAC lactose$^-$	Catalase$^+$ (weakly) Motility$^-$ Urease$^-$ Oxidase$^-$ Satellite$^-$	Catalase$^+$ Motility$^-$ Urease$^+$ Oxidase$^+$ (usually)	CPE can be passed	Oxidase$^+$ Catalase$^+$ DNase$^-$
***Stop!* Consider sending isolate to a higher-level laboratory in the Laboratory Response Network!**							

[a] Consult the director or supervisor *immediately* if any of these microorganisms are suspected!

[b] Abbreviations: BT, bioterrorism strain(s); BA, blood agar; NG, no growth; BSC, biological safety cabinet; CPE, cytopathic effect; GPR, gram-positive rods; GNR, gram-negative rods; GNCB, gram-negative coccobacilli; GNDC, gram-negative diplococci.

15.5.3 Select Agents

"Select agents" are microorganisms, biological agents, or biological toxins that have been deemed by the U.S. government to be major threats to public health and safety because they could be used as agents of bioterrorism (Table 15.5.3–1). The U.S. Department of Health and Human Services (HHS) regulates and the CDC oversees the possession, use, and transfer of select agents. The National Select Agent Registry Program (http://www.selectagents.gov) oversees all activities with select agents. All facilities and persons who possess, use, and/or transfer select agents *must* be registered with the U.S. government.

Table 15.5.3–1 List of select agents specifically designated by the HHS

Type of organisms	Select agent(s)
Bacteria	*Bacillus anthracis* *Yersinia pestis* *Brucella abortus, Brucella melitensis, Brucella suis* *Burkholderia mallei, Burkholderia pseudomallei* *Francisella tularensis* Neurotoxin-producing strains of *Clostridium botulinum*
Viruses	Smallpox virus Herpes B virus Hemorrhagic fever viruses (Crimean-Congo, Junin, Machupo, Sabia, Flexal, and Guanarito) Tick-borne encephalitis flaviviruses Monkeypox virus Lassa fever virus Marburg virus Hendra virus Ebola virus Nipah virus Rift Valley fever virus Eastern, western, and Venezuelan equine encephalitis viruses Others
Rickettsia	*Rickettsia rickettsii, Rickettsia prowazekii* *Coxiella burnetii*
Fungi	*Coccidioides immitis, Coccidioides posadasii*
Toxins	Ricin Shiga toxin Aflatoxins *Staphylococcus* enterotoxin *Clostridium botulinum* neurotoxin Others
Others	Reconstructed replication forms of the 1918 influenza H1N1 virus

15.5.4 Environmental, Nonanimal, and Suspicious Specimens and Substances

Clinical microbiology workers should consider implementing a policy of *not* (literally *not*) accepting nonanimal and suspicious environmental specimens (e.g., powders, envelopes, papers, building and maintenance materials, pouches, containers, books, and mail) into the laboratory for any type of examination or culture. This policy should include step-by-step instructions regarding what to do if the aforementioned substances are brought to the laboratory and are accompanied by some sort of request for examination or culture. The form and content of such a policy vary with the nature, physical structure, communication systems, and services of a particular health care facility. The more important persons who need to know the existence and physical location of the policy and instructions are those who work in the laboratory, the security department, and the emergency department. These persons need to know what actions laboratory employees will take if the aforementioned scenario occurs.

15.5.5 Prions, Prion Diseases (Including Creutzfeldt-Jakob), and Safety Issues Specific to Specimens Suspected of Containing Prions

I. PRIONS AND PRION DISEASES

Prions are abnormally folded, protease-resistant, autocatalytically replicating forms of a naturally occurring, noninfectious protein found on the membranes of the cells of healthy humans and animals. Prions cause several neurodegenerative diseases: kuru, four forms of Creutzfeldt-Jakob disease (CJD), and other neurological diseases in humans and bovine spongiform encephalopathy (mad cow disease), scrapie, chronic wasting disease, and other neurological diseases in animals. All prion diseases are universally fatal.

The form of CJD of which laboratory workers in general and workers in surgical and anatomic settings in particular should be aware is the iatrogenic form (iCJD). iCJD is caused by exposure to tissues (especially brain and other neural tissues) during autopsies, surgery, and preparation of tissues for histology. As of 2006, only 405 cases of iCJD have been reported; most cases were associated with implantation of cadaveric human growth hormone or dura mater grafts manufactured and distributed before the mid-1980s. The numbers of reported cases have been declining steadily.

All of the aforementioned prion diseases (collectively called transmissible spongiform encephalopathies) are degenerative neurological disorders. Although an extremely rare disease, CJD is the most common of these encephalopathies. The incubation period for CJD varies from months to decades, and the disorder is rapidly fatal once symptoms develop. This disease is manifested clinically as a rapidly progressive dementia that includes psychiatric and behavioral abnormalities, coordination deficits, myoclonus, and a distinct triphasic and polyphasic electroencephalogram reading. Definitive diagnosis of CJD requires a histological examination of the affected brain tissue. *CJD is not transmitted by direct contact, droplet, or airborne spread. Iatrogenic transmission of CJD has resulted from the direct inoculation, implantation, or transplantation of infectious materials.* The variant form of CJD (vCJD) is most predominant in the United Kingdom. All patients with vCJD were potentially exposed to cattle with bovine spongiform encephalopathy. As of June 2008, the total number of vCJD cases identified in residents of the United States is three. All of these cases were epidemiologically linked to likely exposures to cattle products contaminated with the prion causing bovine spongiform encephalopathy while residing in the United Kingdom (two cases) or Saudi Arabia (one case).

Prions exhibit unusual resistance to conventional chemical and physical decontamination methods. Neither sterilization with ethylene oxide nor steam autoclaving at conventional exposure conditions (121°C for 15 min) inactivates prions. Additional harsh treatments are required to inactivate prions.

Prions cannot be cultured in the laboratory. Specimens (CSF, blood, and brain tissue) can be forwarded to the National Prion Disease Laboratory at Case Western Reserve University or commercial reference laboratories for analysis for the 14-3-3 protein, which is a marker for some prion diseases, such as CJD. This protein can also be present in other neurodegenerative diseases.

II. GENERAL SAFETY CONSIDERATIONS

Observe standard precautions.

A. Specimen collection and handling

Potential CJD specimens can be classified by level of risk to laboratory workers.

1. High-risk specimens: brain (includes dura mater and spinal cord) and eyes
2. Low-risk specimens: CSF, kidney, liver, spleen, lung, and lymph nodes
3. No-risk specimens: peripheral nerve, intestine, bone marrow, blood, leukocytes, serum, thyroid gland, adrenal gland, heart, skeletal muscle, adipose tissue, gingival tissue, prostate, testis, placenta, tears, nasal mucus, saliva, sputum, urine, feces, semen, vaginal secretions, and milk

II. GENERAL SAFETY CONSIDERATIONS *(continued)*

B. Notification of laboratory staff

Notification of laboratory personnel that potential CJD patient specimens are present can be given to or received from several and various sections of the laboratory and hospital: anatomic pathology, hospital epidemiology, infection control, referral (sendout) testing, surgery, central processing, etc. Anyone receiving this notification should notify the laboratory administration to send out a CJD notification to all appropriate section directors and supervisors. Notification should include the patient's name, medical record number, and other pertinent information. Persons receiving this notification should check to see if specimens were received from this patient, the type of specimen, and if the specimens were handled appropriately.

Observe standard precautions.

C. PPE

The BSL classification for prions from human tissue is BSL 2. Human prions propagated in animals should also be handled under BSL 2 conditions. Certain circumstances might require the use of BSL 3 facilities. All laboratory staff should follow standard precautions when working with clinical specimens, including those from patients with suspected cases of CJD, and should use appropriate PPE and engineering controls.

1. Gloves *Must* be worn when handling blood, body fluids, or tissue samples.
2. Masks and protective eyewear *Must* be worn if exposure of mucous membranes to blood or body fluids might occur.
3. All clinical specimens *Must* be handled in a laminar-flow class II (or higher) biological safety cabinet or behind a protective splash shield when specimen containers are opened.
4. Only disposable instruments or those amenable to harsh high-level sterilization should be used.
5. Shipping materials can be obtained from the National Prion Disease Pathology Surveillance Center, 216-368-0587 (http://www.cjdsurveillance.com/contact.html).

III. HANDLING AND PROCESSING TISSUES FROM SUSPECTED CJD PATIENTS

Observe standard precautions.

A. Handling and processing tissues

1. Histology technicians must wear gloves, an apron, a laboratory coat, and face protection. To minimize surface contamination, disposable-use cover sheets can be used on work surfaces.
2. Adequate fixation of small tissue samples (biopsy specimens) from a patient with suspected CJD-like disease can be followed by postfixation in 96% absolute formic acid for 30 min followed by 48 h in fresh 10% formalin.
3. Liquid waste should be collected in a 4-liter waste bottle initially containing 600 ml of 6 N NaOH.
4. Gloves, embedding molds, and all handling materials should be disposed of as regulated medical waste.
5. Tissue cassettes should be processed manually to prevent contamination of tissue processors.
6. Tissues should be embedded in a disposable embedding mold. If used, forceps are decontaminated as in item IV below.
7. In preparing sections, employees should collect section waste and discard it into a regulated medical waste receptacle. The knife stage should be wiped with 2 N NaOH, and the knife used should be discarded immediately in a regulated medical waste sharps receptacle. Slides should be labeled with "CJD Precautions." Sectioned block should be sealed with paraffin.
8. Routine staining
   - **a.** Slides should be processed by hand.
   - **b.** Reagents should be prepared in 100-ml disposable specimen cups.

III. HANDLING AND PROCESSING TISSUES FROM SUSPECTED CJD PATIENTS *(continued)*

c. Slides should decontaminated by soaking them for 1 h in 2 N NaOH.
d. Slides should be labeled as "Infectious—CJD."

9. Additional suggestions
a. Disposable specimen cups or slide mailers can be used for reagents.
b. Slides for immunocytochemistry can be processed in disposable petri dishes.
c. Equipment should be decontaminated as described below or disposed of as regulated medical waste.

B. Storage and disposal of specimens

If suspected or proven CJD specimens are to be stored, appropriate employees should be notified that special handling of these specimens is necessary. High-risk specimens should be incinerated with a minimum secondary temperature of 1,000°C. Pathological incinerators should maintain a primary chamber temperature in compliance with state regulations. Medical waste incinerators should also be in compliance with state regulations. Low-risk and no-risk specimens may be discarded in the conventional manner.

IV. DECONTAMINATION OF INSTRUMENTS AND SURFACES AND INACTIVATION OF PRIONS

Observe standard precautions.

A. Contact with low- or no-risk specimens

1. Instruments should be cleaned and disinfected by conventional protocols, heat, or chemical sterilization.
2. Clean environmental surfaces with a hospital- or laboratory-approved disinfectant.

B. Contact with high-risk specimens

1. Instruments should be kept moist to prevent fluids and tissues from drying on them.
2. Decontaminate all nondisposable instruments before treating them to inactivate prions.
3. To minimize surface contamination, disposable-use cover sheets can be used on work surfaces.

C. Options for decontaminating heat-sensitive instruments and surfaces

1. Treat surfaces and heat-sensitive instruments with 2 N sodium hydroxide (NaOH) or 20,000 ppm of sodium hypochlorite (NaClO) for 1 h.
2. Ensure that surfaces remain wet for the entire period, and then rinse well with water.
3. It is strongly recommended that gross contamination of surfaces be reduced before chemical treatment because the presence of excess organic material will reduce the strength of either NaOH or NaClO solutions.

D. Options for inactivation of prions on reusable instruments (select one of the following methods)

1. Immerse in 1 N NaOH and heat in a gravity displacement autoclave at 121°C for 30 min. Clean and sterilize by conventional means.
2. Immerse in 1 N NaOH or 20,000 ppm of NaClO for 1 h. Transfer into water and autoclave (gravity displacement) at 121°C for 1 h. Clean and sterilize by conventional means.
3. Immerse in 1 N NaOH or 20,000 ppm of NaClO for 1 h. Rinse instruments with water, transfer to an open pan, and autoclave at 121°C (gravity displacement) or 134°C (porous load) for 1 h. Clean and sterilize by conventional means.

E. Preparation of NaOH and NaClO working solutions

1. NaOH working solutions
a. Prepare stock solution of 10 N NaOH (40 g of NaOH in 100 ml of water).

IV. DECONTAMINATION OF INSTRUMENTS AND SURFACES AND INACTIVATION OF PRIONS *(continued)*

b. Make a 2 N NaOH working solution of the stock solution by diluting the stock solution 1:5 with water (1 part 10 N NaOH plus 4 parts water). This working solution *must* be prepared daily.

c. Make a 1 N NaOH working solution of the stock solution by diluting the stock solution 1:10 with water (1 part 10 N NaOH plus 9 parts water). This working solution *must* be prepared daily.

2. NaClO working solution

a. A 2% solution of NaClO is a 20,000-ppm NaClO working solution. Most commercial household bleach contains 5.25% sodium hypochlorite.

b. Make a 1:2.5 dilution (1 part 5.25% NaClO plus 1.5 parts water) to produce a 20,000-ppm solution. This ratio can also be stated as 2 parts 5.25% NaClO to 3 parts water. This working solution *must* be prepared daily.

Caution: *The above-described solutions are corrosive and require suitable PPE and proper secondary containment. These strong corrosive solutions require careful disposal in accordance with local regulations.*

F. Precautions when using NaOH or NaClO solutions in autoclaves

1. NaOH spills or gas can damage an autoclave if appropriate containers are not used.

2. The use of containers with a rim and lid designed for condensation to collect and drip back into the pan is recommended.

3. Persons who use this procedure should be cautious when handling hot NaOH solutions (postautoclave) and in avoiding potential exposure to gaseous NaOH.

4. Exercise caution during all sterilization steps. Allow autoclave, instruments, and solutions to cool before removal.

Caution: *Immersion in NaClO bleach can cause severe damage to some instruments.*

SUPPLEMENTAL READING

Brown, P., M. Preece, J. P. Brandel, T. Sato, L. McShane, I. Zerr, A. Fletcher, R. G. Will, M. Pocchiari, N. R. Cashman, J. H. d'Aignaux, L. Cervenáková, J. Fradkin, L. B. Schoenberger, and S. J. Collins. 2000. Iatrogenic Creutzfeldt-Jakob disease at the millennium. *Neurology* **55:**1075–1081.

Brown, P., J. P. Brandel, M. Preece, and T. Sato. 2006. Iatrogenic Creutzfeldt-Jakob disease: the waning of an era. *Neurology* **67:**389–393.

Centers for Disease Control and Prevention. 2008. *Questions and Answers: Creutzfeldt-Jakob Disease Infection Control Practices.* http://www.cdc.gov/ncidod/dvrd/cjd/qa_cjd_infection_control.htm.

Centers for Disease Control and Prevention and National Institutes of Health. 2007. Section VIII-H: prion diseases, p. 284–290. *In Biosafety in Microbiological and Biomedical Laboratories*, 5th ed. U.S. Government Printing Office, Washington, DC.

CLSI. 2005. *Protection of Laboratory Workers from Occupationally Acquired Infections; Approved Guideline—Third Edition*. CLSI document M29-A3. CLSI, Wayne, PA.

Rutala, W. A., and D. J. Weber. 2001. Creutzfeldt-Jakob disease: recommendations for disinfection and sterilization. *Clin. Infect. Dis.* **32:**1348–1356.

15.5.6 Resources for Information on Specific Pathogens

In coordination with the CDC and the Association of Public Health Laboratories, the American Society for Microbiology (ASM) has developed protocols designed to assist sentinel level clinical microbiology laboratories with information and techniques to rule out microorganisms that might be suspected as agents of bioterrorism, or to refer specimens to public health laboratories for confirmation. The *Sentinel Level Clinical Microbiology Laboratory Guidelines* offer standardized, practical methods to aid microbiologists in ruling out critical agents and referring specimens to public health laboratories (Laboratory Response Network Reference Laboratories) for confirmation. The guidelines can be found on the ASM website (http://www.asm.org/Policy/index.asp?bid=6342) and include for the following.

A. Anthrax
B. Avian influenza A (H5N1) virus
C. *Brucella*
D. Botulinum toxin
E. Bioterrorism readiness plan (PDF file)
F. Bioterrorism readiness plan (Word file)
G. *Burkholderia mallei* and *Burkholderia pseudomallei*
H. *Coxiella burnetii*
I. Packing and shipping infectious substances
J. Plague (*Yersinia pestis*)
K. Staphylococcal enterotoxin B
L. Tularemia
M. Unknown viruses

15.6 Packing and Shipping Infectious Substances

The information in this procedure is not intended to be an all-inclusive guide to packing and shipping regulations. The information is a summary of my interpretations of the current (as of 1 January 2009) requirements and regulations issued by the following: the International Civil Aviation Organization (ICAO; a specialized United Nations [UN] agency which promotes the international standardization of essentially all technical aspects of aviation, including the transport of dangerous goods), the International Air Transport Association (IATA; a commercial airline trade association), and the U.S. Department of Transportation (DOT; an agency of the federal government).

The requirements and regulations governing the transport of infectious substances change frequently. Shippers are responsible for being aware of these changes, adhering to current regulations, and interpreting applicable regulations for themselves and their facilities.

A list of significant changes in IATA requirements and DOT regulations since 2006 can be found in Appendix 15.6–1. See Appendix 15.6–2 for definitions of IATA and DOT terms used in this procedure.

I. GOVERNING AUTHORITIES AND REGULATIONS

A. Origin of regulations

Shipping requirements and regulations are developed and published by many authorities, the most notable of which are shown in Table 15.6–1. Most regulations for the air transport of dangerous goods throughout the world originate as decisions (called Model Regulations) made by the UN Committee of Experts (15). The ICAO uses these regulations to develop formal and standardized *Technical Instructions for the Safe Transportation of Dangerous Goods by Air* for use in international aviation (8, 15). These *Technical Instructions* are the standards for the international shipment of dangerous goods by air. The IATA uses these IACO *Technical Instructions* to develop *Dangerous Goods Regulations* which are used by essentially all commercial airlines involved in the transport of dangerous goods (7). IATA requirements have become the most widely recognized, copied, and used packing and shipping guidelines in the world. Most

Table 15.6–1 Agencies governing transportation of dangerous goods

Governing authority	Agency	Regulations (reference)
United Nations	ICAO	*Technical Instructions for the Safe Transport of Dangerous Goods by Air*
Commercial airline industry	IATA	*Dangerous Goods Regulations*
United States	DOT	*United States Hazardous Materials Uniform Safety Act*
United States	USPS	*Domestic Mail Manual. Hazardous Materials.*
Canada	Transport Canada	*Transportation of Dangerous Goods Regulations*
Other nations		Individual national regulations

I. GOVERNING AUTHORITIES AND REGULATIONS *(continued)*

national and international regulations are based on or are at least in substantial agreement (harmonization) with IATA requirements (11, 13).

In the United States, the DOT regulates the commercial transportation of dangerous goods by both air and ground carriers. Just as the IATA derives its requirements from the ICAO, the DOT also derives its regulations from the ICAO (6, 11). On 2 June 2006, the DOT revised its regulations for the transportation of infectious substances to be in substantial agreement (harmonization) with ICAO requirements (11). For practical purposes, shippers of infectious substances can consider compliance with IATA requirements to be compliance with DOT regulations (6, 11, 13).

B. Importance of regulations

Laboratory workers who ship or transport dangerous goods, in general, and infectious substances, in particular, by a commercial land or air carrier are required to follow a complex and often confusing set of national and international requirements and regulations. The purpose of these requirements and regulations is to protect the public, emergency responders, laboratory workers, and personnel in the transportation industry from accidental exposure to the contents of the packages (6, 8). An important non-safety-related benefit of adherence to these regulations and requirements is to minimize the potential for damage to the contents of the package during transport and to reduce the exposure of the shipper to the risks of criminal and civil liability associated with the improper shipment of dangerous goods (6, 8).

C. Effectiveness of regulations

Statistical data show that these regulations are extremely effective in protecting both the contents of packages and the persons who handle the packages. To date, there are no reported cases of illness due to the release of an infectious substance during transport. Only 106 (0.002%) of the 4,920,000 primary containers shipped in 2003 to worldwide laboratories and other destinations were reported broken during transit. In each of the 106 reported breakages, absorbent material in appropriately prepared packages contained the leaking material and none of the secondary or outer containers were reported damaged (15).

D. Exceptions

The transportation of small quantities of non-Category A substances (usually specimens being transported for clinical, diagnostic, or other patient care purposes) is exempt from most DOT regulations if the specimens are transported by private or contract carrier in a motor vehicle used exclusively to transport such substances (6, 11). Such substances must be packed and secured inside the vehicle according to DOT regulations; however, these regulations are relatively lenient and state that the substances need only be in leakproof containers, sealed securely, and secured within the vehicle during transport. Readers should be aware that the usual strict OSHA regulations still apply during this type of transportation of infectious substances.

E. Specific regulations

IATA requirements and DOT regulations mandate the minimum standards for packing infectious substances that can pose a threat to humans, animals, or the environment. The safe and legal transport of these substances is based on the following mandated activities, each of which is presented in detail below.

1. Training of individuals on the requirements for appropriate packaging and shipping of infectious substances, documentation of the training, and subsequent certification (by the employer) of the trainee
2. Classification and naming of the material to be shipped
3. Selection of packaging that will contain the contents if the package is damaged and, thus, will protect carrier personnel if the package is damaged
4. Packing the shipment correctly

I. GOVERNING AUTHORITIES AND REGULATIONS *(continued)*

5. Placing appropriate information (markings and labels) onto the outer package to alert carrier personnel to the hazardous contents of the package and to identify contacts if an accident occurs

6. Documenting relevant aspects of each package and its contents

F. USPS

The U.S. Postal Service (USPS) publishes its own regulations in the USPS *Domestic Mail Manual* (14). The USPS regulations for mailing hazardous materials generally adhere to DOT regulations.

II. CLASSIFICATION OF SUBSTANCES

A. Classification

Shipping of all dangerous goods begins with classification of the substances. Classification is a mandatory three-step process to define dangerous goods that are shipped by commercial carriers (4, 6, 7, 9, 11). Classification serves two purposes: (i) it allows the shipper to select the proper IATA packing instructions (PI) and directions to use, and (ii) if the substance is a Category A infectious substance, it provides important information necessary to complete documentation (a Shipper's Declaration) which must accompany shipments of Category A substances.

B. Steps of classification

1. Step 1

The material must be classified into one of the nine IATA-specified classes (Class 1 through Class 9) of dangerous goods (Table 15.6–2). Infectious and toxic substances are Class 6 dangerous goods; dry ice is a Class 9 dangerous good. Class 6 and Class 9 substances usually are the only dangerous goods shipped by clinical microbiologists.

2. Step 2

Class 6 substances must be divided into either Division 6.1 (toxic substances) or Division 6.2 (infectious substances) (Table 15.6–2).

3. Step 3

Division 6.2 infectious substances must be classified into one of nine IATA-specified types of infectious substances (Table 15.6–3):

- **a.** Category A infectious substances
- **b.** Category B infectious substances
- **c.** Patient Specimens
- **d.** Exempt Human or Animal Specimens
- **e.** Genetically Modified Organisms
- **f.** Exempt Substances
- **g.** Biological Products
- **h.** Infected Animals
- **i.** Medical Waste

If the substance is determined to be a patient specimen or a genetically modified microorganism and is not obviously a Category A or Category B substance but it *meets the criteria of* or *has characteristics of* a Category A or Category B substance, the shipper must classify it as a Category A or Category B substance. Otherwise, the substance must be classified as an Exempt Human or Animal Specimen or a Genetically Modified Organism (Table 15.6–3) (6, 7, 11). Fortunately, most clinical microbiologists will find that essentially all of their substances are either Category A, Category B, or Exempt Human or Animal Substances.

Decisions made in the third step can be subjective and can be difficult; however, these decisions will determine exactly how a substance must be packed and shipped. Shippers must not arbitrarily classify all substances as Biological Substance, Category B, Exempt Human or Animal Specimen, or even Exempt

Table 15.6–2 IATA-defined classes of dangerous goods

Class	Substance
1	Explosives
2	Gases
3	Flammable liquids
4	Flammable solids
5	Oxidizing substances and organic peroxides
6	Toxic and infectious substances Division 6.1: toxic substances Division 6.2: infectious substances[a]
7	Radioactive materials
8	Corrosives
9	Miscellaneous dangerous goods (e.g., dry ice)[a]

[a] Addressed in detail in the text.

Table 15.6–3 Types and classifications of IATA Division 6.2 infectious substances

Type of infectious substance	IATA classification
Category A Substance	Category A
Category B Substance	Category B[a]
Patient Specimen	
Meets Category A criteria	Category A
Meets Category B criteria	Category B
Does not meet Category A or B criteria	Exempt Human or Animal Specimen
Exempt Human or Animal Specimen	Exempt Human or Animal Specimen
Genetically Modified Microorganism	
Meets Category A criteria	Category A
Meets Category B criteria	Category B
Does not meet Category A or B criteria	Genetically Modified Organism
Exempt Substance	None
Biological Product[b]	
Infected Animal[b]	
Medical Waste[b]	

[a] The only acceptable proper shipping name for Category B substances is Biological Substance, Category B. The proper shipping names Diagnostic Specimen and Clinical Specimen are no longer allowed.

[b] Substance is not addressed in detail in this procedure.

II. CLASSIFICATION OF SUBSTANCES *(continued)*

Substances to avoid having to make important discriminatory shipping decisions or to make packing easier or less expensive. Such cavalier classification is illegal and can be overly expensive.

C. Category A infectious substances

A Category A substance (pathogen or agent) is "an infectious substance which is transported *in a form* that, when exposure to it occurs, is capable of causing permanent disability, or life-threatening or fatal disease to otherwise healthy humans or animals" (6, 7, 11).

1. List of Category A substances

Deciding whether an infectious substance is a Category A substance is relatively easy because Category A substances are specifically designated and listed by IATA and DOT (Table 15.6–4). The list of Category A substances is not all-inclusive, and a thorough risk assessment must be performed before assigning a substance to Category A. Category A pathogens are essentially the same as those previously classified as "forbidden substances" and risk group 4 substances.

2. Decisions to classify substances as Category A

IATA requirements allow shippers to use their discretion and professional judgment when deciding if a substance meets Category A criteria. IATA *Dangerous Goods Regulations* state the following:

a. Regarding judgment—"Assignment to UN2814 or UN2900 [i.e., Category A] must be based on the known medical history and symptoms of the source human or animal, endemic local conditions, or professional judgment concerning individual circumstances of the source human or animal."

b. Regarding assigning infectious agents which, in the shipper's opinion, meet Category A criteria, but which are not specifically listed as a Category A agent—"Infectious substances . . . which do not appear in the table but which meet the same criteria must be assigned to Category A."

c. Regarding uncertainty of Category A criteria—". . . if there is doubt as to whether or not a substance meets the criteria [of Category A] it must be included in Category A" (7).

Table 15.6–4 Examples of infectious substances (agents) included in Category A in any form unless otherwise indicated

UN number and proper shipping name	Organism
UN2814 (Infectious Substance, Affecting Humans)	*Bacillus anthracis* (cultures only) *Brucella abortus* (cultures only) *Brucella melitensis* (cultures only) *Brucella suis* (cultures only) *Burkholderia mallei* (cultures only) *Burkholderia pseudomallei* (cultures only) *Chlamydia psittaci* (avian) (cultures only) *Clostridium botulinum* (cultures only) *Coccidioides immitis* (cultures only) *Coxiella burnetii* (cultures only) Crimean-Congo hemorrhagic fever virus Dengue virus (cultures only) Eastern equine encephalitis virus (culture only) *Escherichia coli*, verotoxigenic (cultures only) Ebola virus *Francisella tularensis* (cultures only) Hantavirus (causing hemorrhagic fever with renal syndrome) Hantaan virus Hepatitis B virus (cultures only) Herpes B virus (cultures only) Human immunodeficiency virus (cultures only) Lassa virus Marburg virus Monkeypox virus *Mycobacterium tuberculosis* (cultures only) Poliovirus (cultures only) Rabies virus (cultures only) *Rickettsia rickettsii* (cultures only) Rift Valley fever virus (cultures only) *Shigella dysenteriae* type 1 (cultures only) Variola virus Venezuelan equine encephalitis virus (cultures only) West Nile virus (cultures only) Yellow fever virus (cultures only) *Yersinia pestis* (cultures only)
UN2900 (Infectious Substance, Affecting Animals)	Classical swine fever virus (cultures only) Foot-and-mouth disease virus (cultures only) Goatpox virus (cultures only) Lumpy skin disease virus (cultures only) Newcastle disease virus (cultures only) Sheeppox virus (cultures only) Swine vesicular disease virus (cultures only) Vesicular stomatitis virus (cultures only)

II. CLASSIFICATION OF SUBSTANCES *(continued)*

3. UN numbers of Category A pathogens
Category A pathogens and substances likely to contain Category A pathogens must be assigned the UN number UN2814 (proper shipping name: Infectious Substance, Affecting Humans) or UN2900 (proper shipping name: Infectious Substance, Affecting Animals) (Fig. 15.6–1) (6, 7, 11).

4. Agents of bioterrorism
Some Category A pathogens have been designated as agents of bioterrorism and are known as select agents (Appendix 15.6–3). U.S. federal regulations require shippers to have special permits to possess, use, transfer, and receive these agents (1, 2a, 2b, 3, 5).

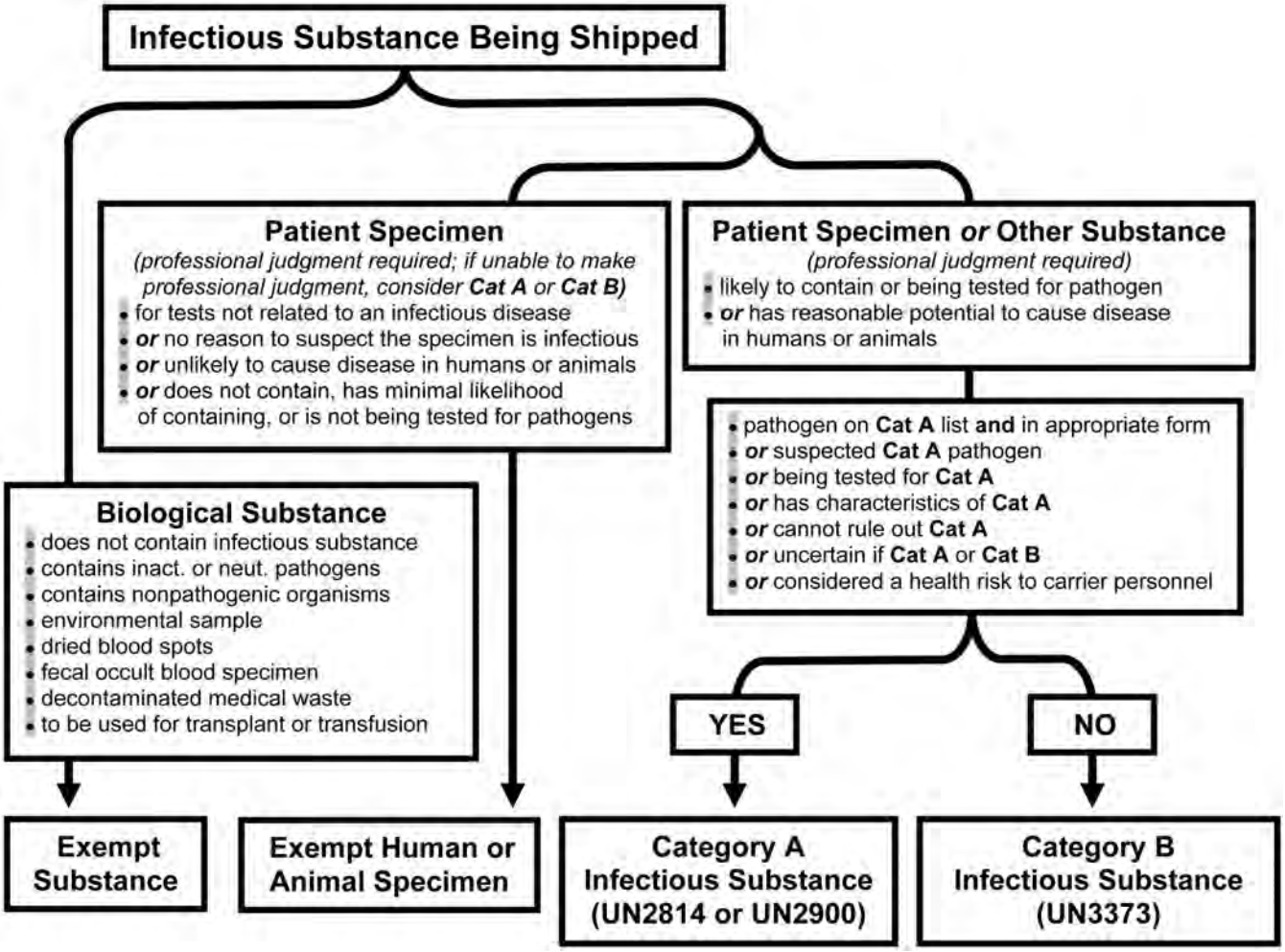

Infectious Substance Being Shipped
Patient Specimen
(professional judgment required; if unable to make professional judgment, consider Cat A or Cat B)
• for tests not related to an infectious disease
• or no reason to suspect the specimen is infectious
• or unlikely to cause disease in humans or animals
• or does not contain, has minimal likelihood of containing, or is not being tested for pathogens
Patient Specimen or Other Substance
(professional judgment required)
• likely to contain or being tested for pathogen
• or has reasonable potential to cause disease in humans or animals
• pathogen on Cat A list and in appropriate form
• or suspected Cat A pathogen
• or being tested for Cat A
• or has characteristics of Cat A
• or cannot rule out Cat A
• or uncertain if Cat A or Cat B
• or considered a health risk to carrier personnel
Biological Substance
• does not contain infectious substance
• contains inact. or neut. pathogens
• contains nonpathogenic organisms
• environmental sample
• dried blood spots
• fecal occult blood specimen
• decontaminated medical waste
• to be used for transplant or transfusion
YES
NO
Exempt Substance
Exempt Human or Animal Specimen
Category A Infectious Substance (UN2814 or UN2900)
Category B Infectious Substance (UN3373)

Figure 15.6–1 Algorithm for classifying infectious substances. Abbreviations: inact., inactivated; neut., neutralized.

II. CLASSIFICATION OF SUBSTANCES *(continued)*

D. Category B infectious substances

A Category B substance is defined by the IATA as "an infectious substance which does not meet the criteria for inclusion in Category A" (Fig. 15.6–1; Table 15.6–3) (7). Category B substances are *not* in a form generally capable of causing disability, life-threatening illness, or fatal disease. Examples of Category B substances could be the following.

1. Typical clinical, diagnostic, or patient specimens, e.g., blood, biopsy samples, swab specimens, excreta, secreta, body fluids, tissues, etc., (i) being shipped for routine culturing or other testing for a non-Category A infectious microorganism(s) or (ii) suspected of containing a non-Category A microorganism(s), and
2. Typical clinical laboratory cultures (usually on solid or in liquid media) of routinely encountered non-Category A microorganisms grown and used in clinical microbiology laboratories.

In my opinion, a clinical, diagnostic, or patient specimen suspected of containing or being tested for a "culture only" Category A substance may be shipped as a Biological Substance, Category B, because the suspected Category A sub-

II. CLASSIFICATION OF SUBSTANCES *(continued)*

stance is not in culture form, e.g., sputa being tested for *Mycobacterium tuberculosis* and serum to be cultured for human immunodeficiency virus.

Category B substances must be assigned UN number UN3373 (proper shipping name: Biological Substance, Category B) (6, 7, 11).

E. Exempt Human (or Animal) Specimens

Exempt Human or Animal Specimens are those for which there is "minimal likelihood there are pathogens present" (Fig. 15.6–1; Table 15.6–3) (6, 7, 11). Examples of such specimens include urine or serum to be tested for glucose, cholesterol, hormone levels, prostate-specific antigen, and analytes used to evaluate heart and kidney function. Professional judgment and knowledge of patient medical history may be used to determine if the specimen is an infectious risk or contains pathogens. Historically, such specimens were packed and shipped as Clinical Specimens or Diagnostic Specimens (such designations are no longer allowed). Exempt Human or Animal Specimens have less stringent packaging requirements than do Category A and Category B substances. The IATA requires outer packages which contain Exempt Human or Animal Specimens to be clearly labeled as "Exempt Human Specimen" or "Exempt Animal Specimen" (7, 10). The DOT does not require this label on outer packages (11).

F. Exempt substances

Many substances commonly encountered in clinical laboratories are exempt from the strict dangerous goods shipping requirements and regulations which apply to Category A and Category B substances and to Exempt Human or Animal Specimens (Fig. 15.6–1; Table 15.6–3). The following are examples of such exempt substances (7, 10).

1. Substances which do not contain infectious substances or are unlikely to cause disease in humans and animals
2. Substances which contain nonpathogenic microorganisms
3. Most environmental samples (food, soil, etc.) which do not pose a health risk to humans or animals
4. Substances which contain neutralized or inactivated microorganisms that no longer pose a health risk to humans or animals
5. Substances to be tested for therapeutic drug monitoring, insurance purposes, alcohol or drugs, pregnancy indicators, cancer, and antibodies
6. Dried blood spots and fecal occult blood screen specimens
7. Blood and blood components collected for the purpose of transfusion or for the preparation of blood products to be used for transfusion or transplantation and any tissue or organs intended for use in transplantation
8. FDA-approved and FDA-licensed biological products
9. $\leq$30 ml of 10% formalin per primary container when the formalin is used as a preservative of an infectious substance

G. Patient specimens

The IATA has defined a "patient specimen" as material collected directly from humans or animals for diagnostic, treatment, prevention, investigational, or research purposes (Fig. 15.6–1; Table 15.6–3) (7). Patient specimens which have Category A or Category B criteria should be classified, packed, and shipped as Category A or Category B substances (Fig. 15.6–1; Table 15.6–3). Patient specimens which have neither Category A nor Category B criteria should be packed and shipped as Exempt Human or Animal Specimens.

H. Genetically modified organisms

Genetically modified organisms usually meet either Category A or Category B criteria. If this is not the case, the organism must be classified as a Genetically Modified Microorganism (class 9; Miscellaneous Dangerous Goods) and packed and shipped as such (Table 15.6–3) (7).

II. CLASSIFICATION OF SUBSTANCES *(continued)*

I. Biological products

Virtually all commercially available biological products as defined by the IATA are exempt from the packing and shipping regulations presented in this procedure. However, if a biological product is determined to meet the criteria of one of the aforementioned infectious substances (Category A, Category B, Exempt Human or Animal Specimen, etc.) it must be packed and shipped as such (7). Examples of biological products include bacterial typing sera, vaccines, bacterial antigens, antimicrobial agents, reagents for identifying bacteria, and reagents used in antimicrobial susceptibility testing.

J. Medical waste

Medical waste which contains Category A or Category B infectious substances must be packed and shipped as such and assigned UN2814, UN2900, or UN3373 (Table 15.6–3) (7). Medical waste which is reasonably believed to have a low probability of containing infectious substances must be packed and shipped as Medical Waste, n.o.s. (UN3291) (7).

K. Infected animals

A live intentionally infected animal that is known to contain or reasonably expected to contain an infectious substance cannot be transported by air unless the substance cannot be transported by any other means (7). Consultation with individual commercial carriers is advised if either live or dead infected animals need to be shipped.

III. NAMING CATEGORY A AND CATEGORY B SUBSTANCES

After classifying the substance, the shipper must identify (officially name) a Category A and Category B substance by assigning the substance one of the over 3,000 IATA-specified and internationally recognized UN numbers and proper shipping names listed in the IATA requirements (7). Proper shipping names and their associated UN numbers are specifically listed and published internationally by the IATA so that most carriers around the world will recognize *the general group or kind* of infectious agent or dangerous good they are handling. This list provides 14 informational items (A through N) for each of the proper shipping names and UN numbers (Table 15.6–5). The 14 items correspond conveniently to the information

Table 15.6–5 Information provided for each proper shipping name in the IATA alphabetical *List of Dangerous Goods* and applicable to completing a Shipper's Declaration

Column[a]	Information
A	UN number of the proper shipping name/description
B	Proper shipping name/description
C	Class or division of dangerous good
D	NA[b]
E	The hazardous label required on the outer package
F	NA
G	NA
H	NA
I	PI to use for *passenger and cargo aircraft*
J	Maximum allowable amounts to be shipped in *passenger and cargo aircraft*
K	PI to use for *cargo aircraft only*
L	Maximum allowable amounts to be shipped in *cargo aircraft only*
M	Applicable special provisions and exceptions
N	Emergency response code

[a] Refers to the 14 columns in the IATA alphabetical *List of Dangerous Goods.*

[b] NA, not applicable to infectious substances.

Table 15.6–6 The seven types of infectious substances in the IATA alphabetical *List of Dangerous Goods*[a]

UN no. (A)	Proper shipping name/description (B)	Class (C)	Subsidiary risk (D)	Hazard label(s) (E)	Pk gp (F)	Passenger or cargo aircraft				Cargo aircraft only		Spec prov (M)	ERG code (N)
						Ltd qty		PI (I)	Max net qty/pkg (J)				
						PI (G)	Max net qty/pkg (H)			PI (K)	Max net qty/pkg (L)		
2814	Infectious Substance, Affecting Humans[b] (liquid)	6.2		Infectious substance				602	50 ml	602	4 liters	A81, A140	11Y
2814	Infectious Substance, Affecting Humans[b] (solid)	6.2		Infectious substance				602	50 g	602	4 kg	A81, A140	11Y
2900	Infectious Substance, Affecting Animals Only[b] (liquid)	6.2		Infectious substance				602	50 ml	602	4 liters	A81, A140	11Y
2900	Infectious Substance, Affecting Animals Only[b] (solid)	6.2		Infectious substance				602	50 g	602	4 kg	A81, A140	11Y
3373	Biological Substance, Category B	6.2		None				650	4 liters or 4 kg	650	4 liters or 4 kg		6L
3245	Genetically Modified Microorganisms	9		Miscellaneous				913	No limit	913	No limit	A47	9L
1845	Dry ice	9		Miscellaneous	III			904	200 kg	904	200 kg	A48	9L

[a] Pk gp, packing group; ltd, limited; qty, quantity; spec prov, special provisions; ERG, emergency response guide.

[b] On the Shipper's Declaration but not on the outer package, the proper shipping name of the substance must be followed by the technical name (in parentheses) of the substance, e.g., Infectious Substance, Affecting Humans (*Mycobacterium tuberculosis*).

III. NAMING CATEGORY A AND CATEGORY B SUBSTANCES *(continued)*

needed to complete the Shipper's Declaration. Fortunately, only 7 of the 3,000 proper shipping names are used by most clinical microbiology laboratories: 2 names for Category A infectious substances which affect humans (1 for liquids and 1 for solids), 2 names for Category A infectious substances which affect animals (1 for liquids and 1 for solids), 1 name for Category B substances, 1 name for genetically modified organisms, and 1 name for dry ice (Table 15.6–6). Table 15.6–6 shows the seven IATA- and DOT-designated infectious substances commonly shipped by clinical microbiologists. The table provides proper shipping names, UN numbers, PI, quantity limits, and other information related to packing and shipping these substances.

IV. PACKING INSTRUCTIONS AND PACKING SUBSTANCES

Observe standard precautions.

A. PI

DOT regulations, IATA requirements, and IATA PI describe the minimum standards for the safe transport of various biological materials. Shippers are legally responsible for complying with these regulations, for following prescribed PI, and for packing substances correctly to ensure the safety of all personnel who handle the package before, during, and even after shipment to the point of acceptance of the package by the consignee. After determining the exact nature and category of the substance to be shipped and the proper shipping name of the substance, the shipper must select the most appropriate PI and packing directions to use (Fig. 15.6–1; Table 15.6–6). Generally, the PI used by clinical laboratories are those that relate to shipping Category A infectious substances

Table 15.6–7 Comparison of IATA PI 650 and 602 and packing directions for exempt human specimens (7)

Requirement[a]	Exempt human specimens[b]	PI 650[c]	PI 602[d]
Inner containers			
Leakproof 1° and 2° containers	Yes	Yes	Yes
Pressure-resistant 1° or 2° container	—[e]	Yes	Yes
Absorbent between 1° and 2° containers[f]	Yes	Yes	Yes
List of contents between 2° and outer package	—	Yes	Yes
Positively sealed 1° container	—	No	Yes
Outer container			
Rigid outer packaging	—	Yes	Yes
Strict manufacturing specifications	None[g]	Few	Many
Name and number of responsible person	—	Yes[h]	Yes
Markings and labels	—	Less	More
Quantity limits for either passenger or cargo aircraft			
Maximum for each 1° container	—	1 liter (1 kg)	50 ml (50 g)
Total maximum for outer package	—	4 liters (4 kg)	50 ml (50 g)
Documentation: Shipper's Declaration for Dangerous Goods	—	No	Yes
Cost of labor and materials to pack substance	Least	More	Most

[a] 1° and 2°, primary and secondary, respectively.

[b] Includes substances with minimal likelihood of causing disease in humans and animals, and substances not likely to contain pathogens (see text).

[c] PI for Biological Substances, Category B.

[d] PI for Category A infectious substances and other biological substances shipper considers to be an infectious danger to carrier personnel.

[e] —, requirement not specified by the IATA or DOT.

[f] Not required for solid substances such as tissue and solid agar medium cultures or slants.

[g] Should be "of adequate strength for its intended capacity, mass, and intended use."

[h] May be placed either on the outer package or on the air waybill.

IV. PACKING INSTRUCTIONS AND PACKING SUBSTANCES *(continued)*

(PI 602), Category B infectious substances (PI 650), and dry ice (PI 904). There are no specifically numbered PI for specimens classified as Exempt Human or Animal Specimens; however, the IATA provides directions which must be followed (7). See Table 15.6–7 for a comparison of the details of packing instructions and directions.

B. Comparison of PI and directions

Details of the similarities of and differences between PI 602, PI 650, and the directions for packing Exempt Human or Animal Substances are shown in Table 15.6–7. The major similarity in these three instructions is commonly known as triple packaging. In its simplest form, triple packaging consists of a primary container, a secondary container, absorbent material between the containers, and an outer shipping container. The major differences between these instructions are those associated with documentation and with marking and labeling outer containers. The following are the main components of PI 602 and PI 650.

1. A leakproof primary container made of glass, metal, or plastic and, if it contains a Category A infectious substance, sealed by a positive method (e.g., heat seal, metal crimp, or taped screw-cap lid). For Category A and Category B substances to be shipped in either passenger or cargo aircraft, the maximum allowable volume per primary container is 50 ml (50 g) and 1 liter (4 kg) for Category A and Category B substances, respectively.

IV. PACKING INSTRUCTIONS AND PACKING SUBSTANCES *(continued)*

2. Absorbent material sufficient to absorb all liquid contained within the primary container(s) in case of breakage, placed between the primary and secondary containers. Absorbent material is not required if the material being shipped is a solid. Absorbent material should be used with liquids shipped in a frozen state.
3. A leakproof secondary container which contains the primary container(s)
4. Either the primary or secondary container must be able to withstand an internal pressure of at least 95 pKa (13.8 lb/in^2) because shipments are likely to be placed into unpressurized cargo sections of aircraft which fly at high altitudes.
5. A list of the contents and quantities of the primary container(s) must be attached to the outside of the secondary container.
6. A rigid and durable outer package of adequate strength for its intended use and constructed of cardboard, wood, or material of equivalent strength and which measures at least 4 by 4 in. on at least one surface. For shipping Category A infectious substances, these outer containers must meet strict UN manufacturing and testing specifications.

C. Packing directions for Exempt Human or Animal Specimens

Packaging used with Exempt Human or Animal Specimens is less strict than the aforementioned requirements in PI 650 and 602. However, such packaging must be composed of four important elements: (i) a leakproof primary container; (ii) a leakproof secondary container; (iii) for liquid substances, absorbent material of sufficient quantity to absorb the entire liquid, placed between the primary and secondary containers; and (iv) outer packaging "of adequate strength for its intended capacity, mass, and intended use" (Table 15.6–7) (7).

V. MARKING AND LABELING OUTER PACKAGES

Marking is the act of writing or typing information onto the outer surface of an outer package, and labeling is the act of placing informational labels or stickers onto the surface of an outer package. The two terms frequently are used interchangeably. The shipper is responsible for the proper marking and labeling of the outer shipping container. The markings and labels on the outer container communicate essential information regarding the shipper and consignee of the package, the nature and weight of the contents of the package, the potential hazard of the substance, how the substance is packed, and information to be used in case of an emergency. Some of these markings and labels can be seen in the IATA *Dangerous Goods Regulations* (6, 7, 11).

A. Specific markings and labels

1. Shipper and consignee—the shipper's and consignee's name and address
2. Responsible person—the name, address, and telephone number of a "person responsible" (IATA quote; IATA does not define "person responsible") must be on the outer container. If the substance being shipped is a Category B substance, this information may be provided either on the outer package or on the air waybill (7). My interpretation of a "person responsible" is someone who is familiar with the shipment and who can answer general (not necessarily emergency response) questions about the shipment.
3. Category A substances—(i) the Class 6 diamond-shaped "Infectious Substance. In Case of Leakage . . ." label and (ii) a label which shows the proper shipping name, UN number, and quantity of the substance (Fig. 15.6–2). The Class 6 infectious substance label is identical for all regulating agencies, except that the DOT version specifies notification of the CDC by use of a 1-800- (toll-free) number.

INFECTIOUS SUBSTANCE
IN CASE OF DAMAGE OR LEAKAGE IMMEDIATELY NOTIFY PUBLIC HEALTH AUTHORITY IN U.S.A. NOTIFY DIRECTOR - CDC ATLANTA, GA
6
Infectious Substance, Affecting Humans
UN 2814 4 mL

Figure 15.6–2 Labels which indicate an infectious substance (Class 6), proper shipping name, UN number, and quantity of substance.

Figure 15.6–3 Markings which indicate a Biological Substance, Category B, and appropriate UN number.

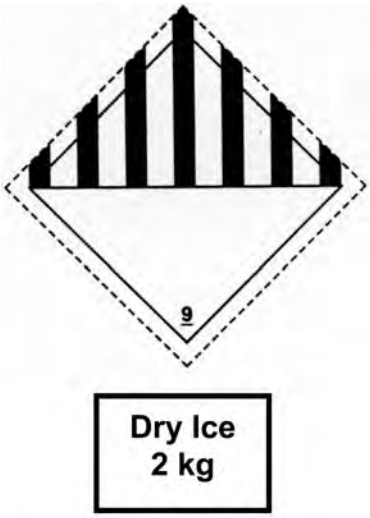


Figure 15.6–4 Labels which indicate a miscellaneous (Class 9) dangerous good (2 kg of dry ice).

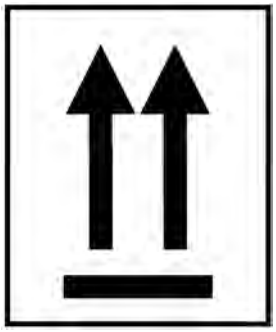


Figure 15.6–5 Label which indicates correct orientation of package during shipping.

Figure 15.6–6 Label which indicates that the substance must be transported only in cargo (not passenger) aircraft.

Figure 15.6–7 Label which indicates that an overpack is used and inner packages comply with regulations.

V. MARKING AND LABELING OUTER PACKAGES *(continued)*

4. Category B substances—(i) the label "Biological Substance, Category B" and (ii) the marking or label "UN3373" (Fig. 15.6–3)
5. Dry ice—Class 9 "Miscellaneous Dangerous Goods" label and the weight of dry ice (Fig. 15.6–4)
6. Package orientation—package orientation label (Fig. 15.6–5). Orientation labels (arrows) must be placed on opposite sides of all packages which contain >50 ml of a liquid or frozen liquid infectious substance to indicate the correct orientation of the package.
7. Cargo only—"Cargo Aircraft Only" label if the substance (because of its quantity) must be transported only by cargo aircraft (Fig. 15.6–6). This label is used if infectious substance amounts greater than 50 ml (5 g) but less than 4 liters (4 kg) per outer package are shipped.
8. Overpack—"Overpack" markings if overpacks are used (Fig. 15.6–7)
9. Exempt patient specimens—Patient specimens not classified as Category A or Category B must be labeled clearly as "Exempt Human Specimen" or

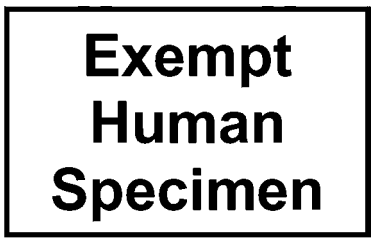


Figure 15.6–8 Label which indicates an Exempt Human Specimen.

Figure 15.6–9 Example of a label which indicates that the outer container has met UN-specified manufacturing standards.

V. MARKING AND LABELING OUTER PACKAGES *(continued)*

"Exempt Animal Specimen" (Fig. 15.6–8). This requirement is specified only by the IATA and not by the DOT (7, 10).

10. Outer package—all outer packaging used to ship Category A infectious substances and substances considered by the shipper to be an infectious risk to the health of carrier personnel must meet manufacturing and performance specifications established by the UN and must be marked as such by the manufacturer. Packaging that meets the UN specifications are marked by a "UN" inside of a circle, and a series of letters and numbers which indicate the type of package, class of goods the package is designed to carry, manufacturing date, authorizing agency, and manufacturer (Fig. 15.6–9). The designation "Class 6.2" in the marked code indicates that the container is approved for shipping infectious substances. These containers are commercially available and are preprinted with the appropriate UN marking. The strict UN specifications for outer packaging do not apply when shipping Category B substances. Outer boxes used to ship Category B substances need only to be rigid and strong enough for their intended purpose and be able to pass a 3.9-ft drop test (7).

B. Examples of labeled and marked outer packages

Figures 15.6–10, 15.6–11, and 15.6–12 show examples of completely labeled and marked outer shipping containers which contain an Exempt Human Specimen, a Category B infectious substance, and a Category A infectious substance, respectively. Packages in Fig. 15.6–11 and 15.6–12 also contain dry ice. For convenience and lower costs, one or more triple packages packed in full compliance with IATA regulations may be shipped within a single overpack which does not have to meet UN specifications. However, the overpack must be labeled "Overpack," and all inner packages must be completely labeled according to applicable IATA regulations (Fig. 15.6–7).

VI. DOCUMENTATION

A. Importance of Shipper's Declarations

A Shipper's Declaration is a legal contract between the shipper and carrier, is required to document the shipment of Category A infectious substances, must be accurate, and must be legible or the carrier may reject the package for transport. A Shipper's Declaration is required for dry ice (a dangerous good) if dry ice is used as a refrigerant for a Category A substance, but not for a Category B substance. Some carriers require the Shipper's Declaration to be typed; some require multiple copies. The original Shipper's Declaration given to the carrier must have vertical red candy stripes along the left and right edges of the document. Shippers must retain copies of Shipper's Declarations for 2 years (10). All corrections must be neatly "lined out," and all changes must be signed (not initialed) by the *same* person as who signed the document. A carrier may reject a shipment if each field on the Shipper's Declaration is not completed exactly

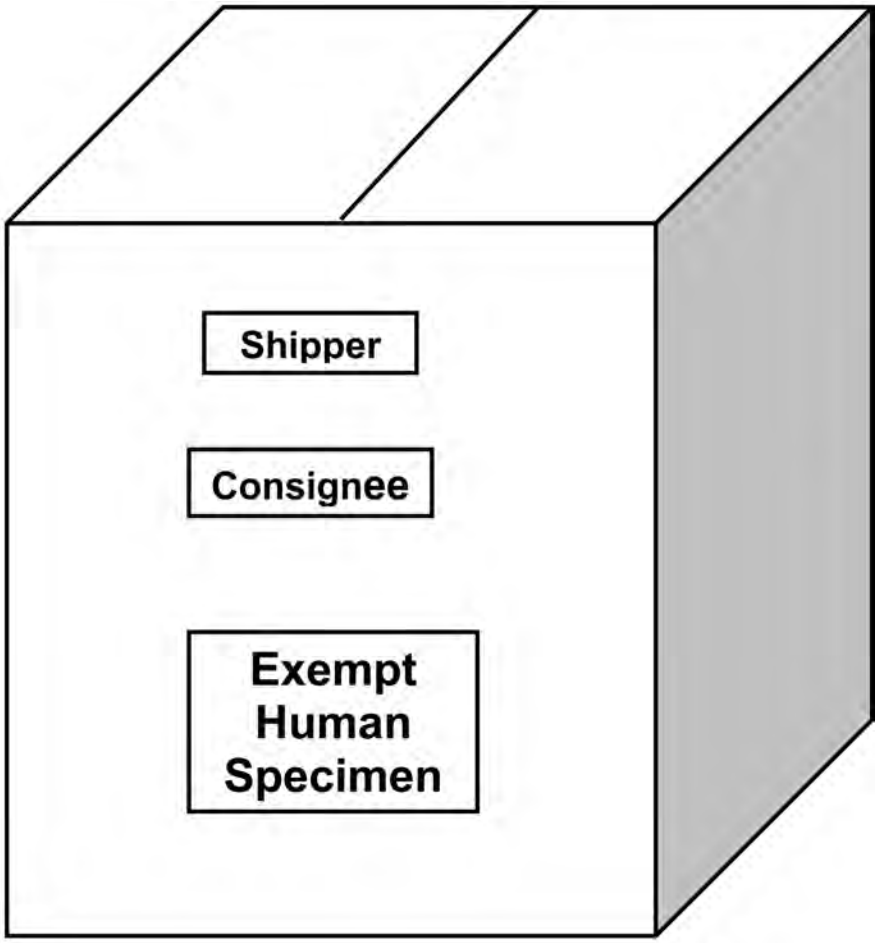


Figure 15.6–10 Example of an appropriately labeled outer package. The primary container inside the package contains an Exempt Human Specimen and is packed according to IATA directions.

VI. DOCUMENTATION
(continued)

to the carrier's satisfaction, and if the information and phrasing on the Shipper's Declaration do not match exactly the corresponding information on the outer package. Commercial carriers and the Federal Aviation Administration often exercise their authority at airports to examine Shipper's Declarations for compliance with applicable regulations and to open and inspect any package (whether or not the package is leaking) which contains or is suspected of containing an infectious substance. In addition, these agencies can and do examine documentation of perfectly packaged shipments, go to the facilities from which the packages originated, and request documentation of adequate training of employees. Figure 15.6–13 shows a blank Shipper's Declaration and the 13 sections which shippers must complete. Essentially all of the IATA-specified technical information required to complete the seven subsections of section nine (Nature and Quantity of Dangerous Goods) of the document can be found in Table 15.6–6 and reference 7. Figure 15.6–14 shows a completed and acceptable Shipper's Declaration.

B. Emergency response telephone number

DOT, but not IATA, regulations state that an "emergency response telephone number" must be provided on Shipper's Declarations which accompany shipments of Category A infectious substances (12). The number must be monitored

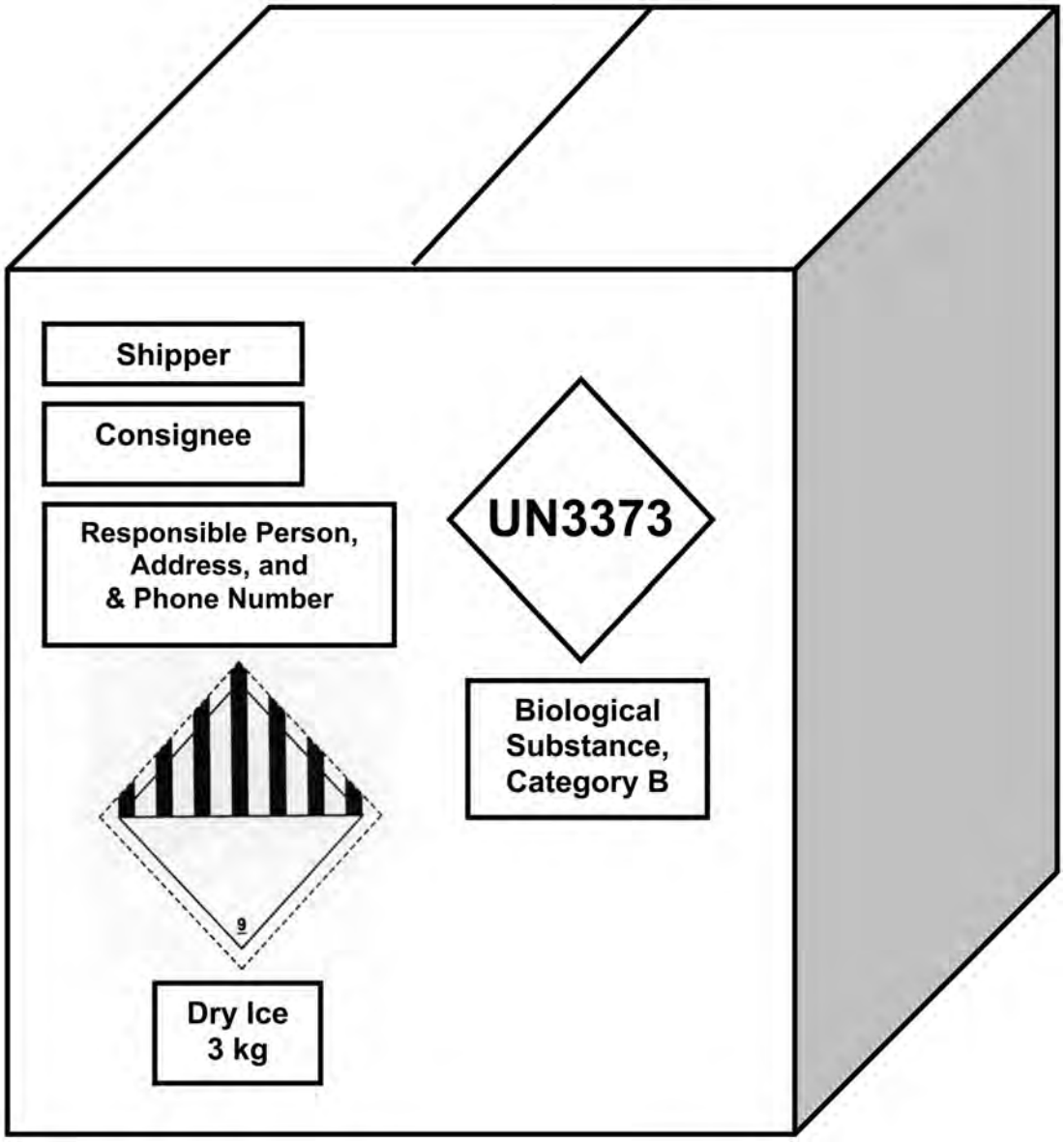


Figure 15.6–11 A completely labeled outer package. The primary container inside the package contains a Biological Substance, Category B (diagnostic or clinical specimen), and is packed according to PI 650.

VI. DOCUMENTATION *(continued)*

at all times by a person (*not* an answering machine, message service, pager, etc.) who has knowledge of (i) the hazards of the material being shipped and (ii) emergency response and accident mitigation information in case a handler contacts the released contents of the package. Alternatively, the number can be that of a person who has immediate access to a person who has such knowledge and information. The number of an agency, organization, or commercial company may be used instead of the aforementioned persons if the shipper can ensure that the agency, organization, or company can supply the required aforementioned emergency information in a timely manner.

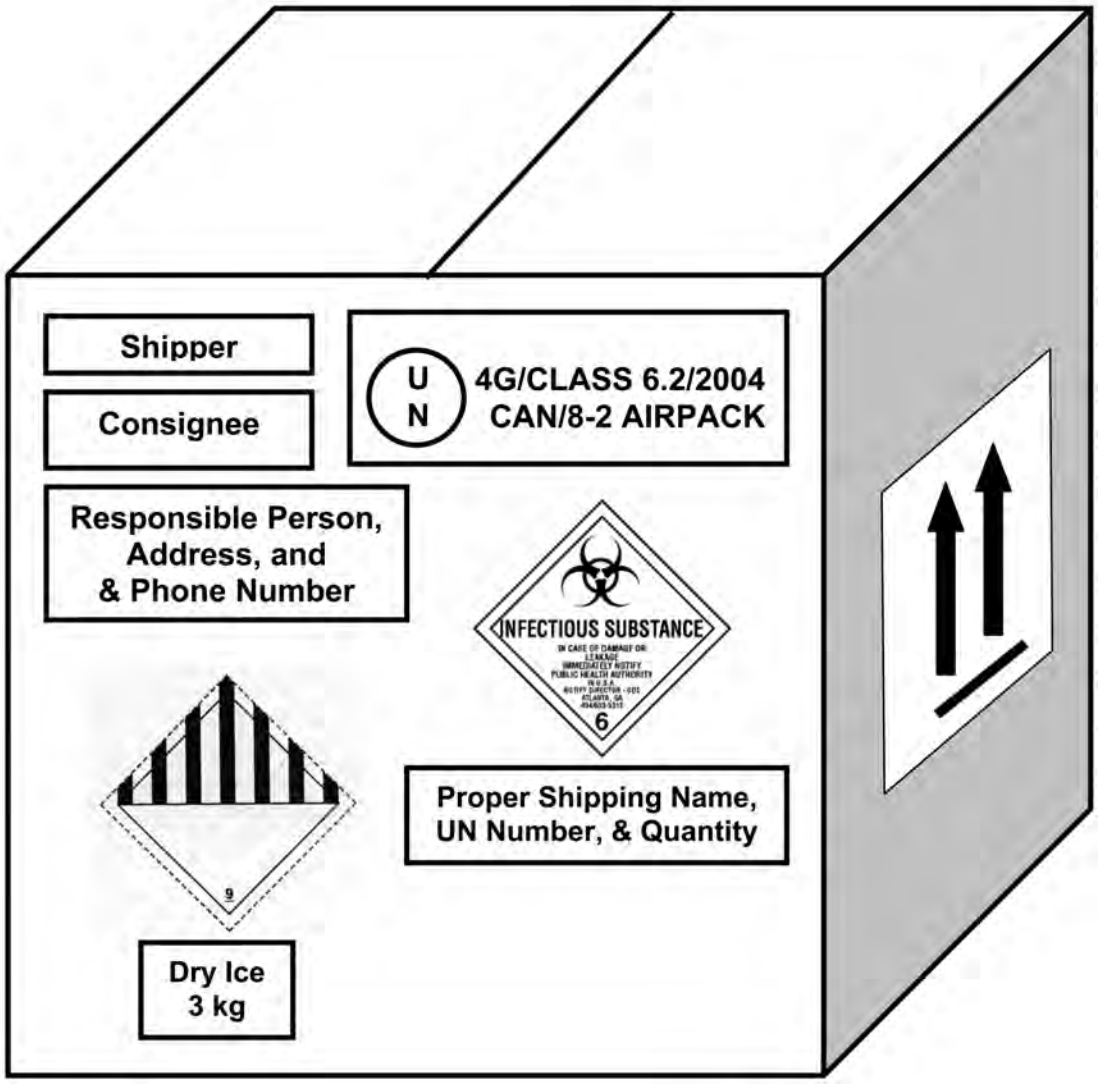
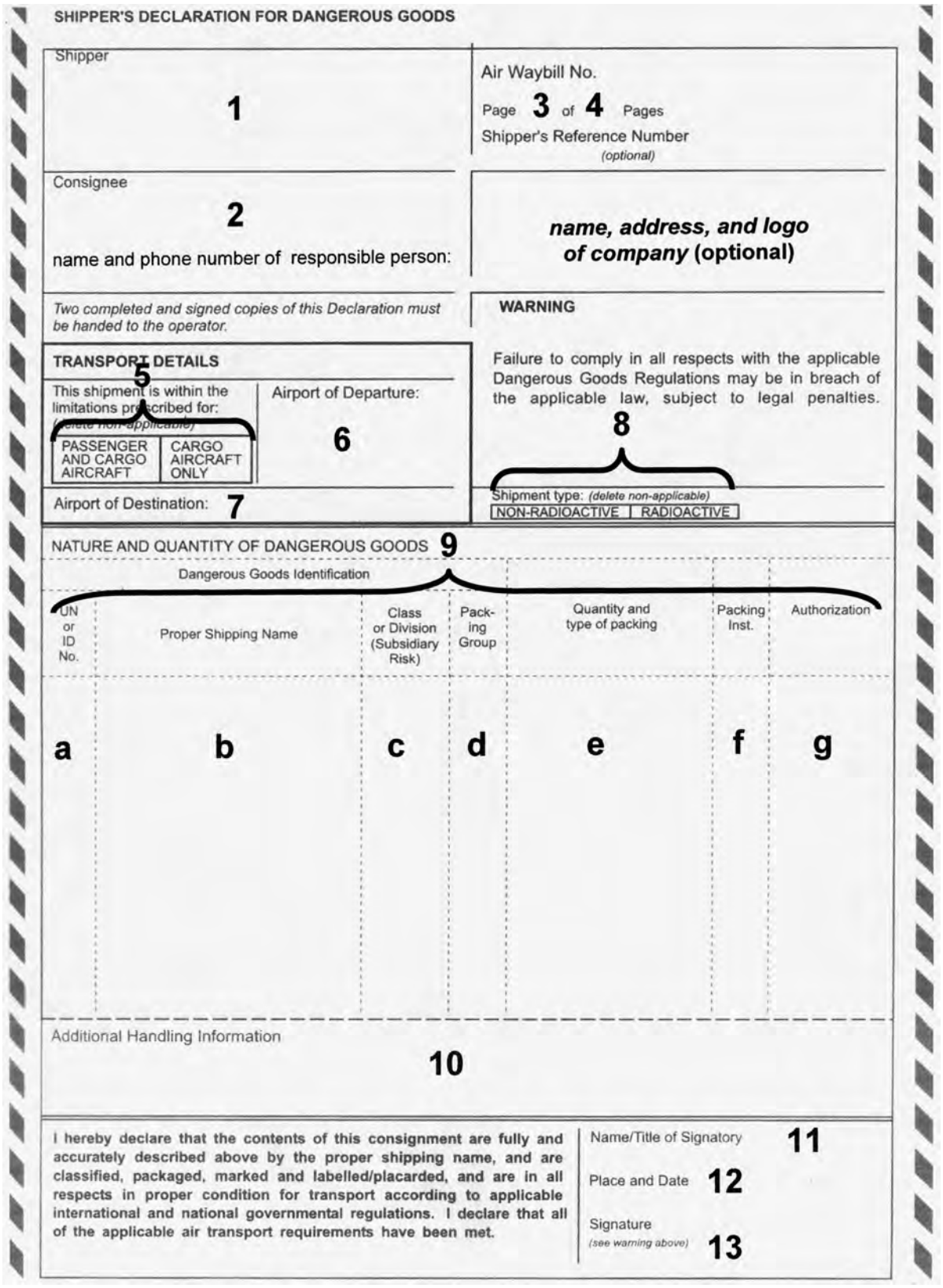


Figure 15.6–12 A completely labeled outer package. The primary container inside the package contains a Category A infectious substance and is packed according to PI 602.

VII. REFRIGERANTS

Wet and dry ice are two common refrigerants used to ship diagnostic specimens and infectious substances. Packaging must be leakproof when wet ice is used. Dry ice is a Class 9 dangerous good, it must be packaged according to PI 904, and its use requires completion of a Shipper's Declaration if it is used to ship a Category A substance. The secondary container must be secured so that it does not become loose as the dry ice sublimates. Outer packages must be labeled "Dry Ice," and the net weight of the dry ice must be indicated on the outside of the outer package and be recorded on the Shipper's Declaration (Fig. 15.6–4, 15.6–11, and 15.6–12). The maximum permitted net weight of dry ice per outer package is 200 kg.

◩ **NOTE:** Dry ice is an explosion hazard and must never be placed into a tightly sealed container! Dry ice must be placed *outside* the secondary container, and the outer packaging must permit the release of CO_2!



SHIPPER'S DECLARATION FOR DANGEROUS GOODS

Shipper **1**

Air Waybill No.

Page **3** of **4** Pages

Shipper's Reference Number *(optional)*

Consignee **2**

name and phone number of responsible person:

***name, address, and logo of company* (optional)**

Two completed and signed copies of this Declaration must be handed to the operator.

WARNING

Failure to comply in all respects with the applicable Dangerous Goods Regulations may be in breach of the applicable law, subject to legal penalties.

TRANSPORT DETAILS

5

This shipment is within the limitations prescribed for: *(delete non-applicable)*

PASSENGER AND CARGO AIRCRAFT | CARGO AIRCRAFT ONLY

Airport of Departure: **6**

Airport of Destination: **7**

8

Shipment type: *(delete non-applicable)*

NON-RADIOACTIVE | RADIOACTIVE

NATURE AND QUANTITY OF DANGEROUS GOODS **9**

Dangerous Goods Identification

UN or ID No.	Proper Shipping Name	Class or Division (Subsidiary Risk)	Packing Group	Quantity and type of packing	Packing Inst.	Authorization
a	**b**	**c**	**d**	**e**	**f**	**g**

Additional Handling Information **10**

I hereby declare that the contents of this consignment are fully and accurately described above by the proper shipping name, and are classified, packaged, marked and labelled/placarded, and are in all respects in proper condition for transport according to applicable international and national governmental regulations. I declare that all of the applicable air transport requirements have been met.

Name/Title of Signatory **11**

Place and Date **12**

Signature *(see warning above)* **13**

Figure 15.6–13 Shipper's Declaration for Dangerous Goods showing 13 sections which must be completed by the shipper.

SHIPPER'S DECLARATION FOR DANGEROUS GOODS

Shipper
Wilkins Laboratories
1970 Tarheel Avenue
Pacolet, SC 27105

Air Waybill No.

Page **1** of **1** Pages

Shipper's Reference Number *(optional)*

Consignee **Dr. William Truitt**
Elephants Foot Trail
Charlotte, NC 45227
Responsible Person and Number:
Janet Irwin (919) 271-5432

Wilkins Laboratories

Two completed and signed copies of this Declaration must be handed to the operator.

WARNING

Failure to comply in all respects with the applicable Dangerous Goods Regulations may be in breach of the applicable law, subject to legal penalties.

TRANSPORT DETAILS

This shipment is within the limitations prescribed for: *(delete non-applicable)*
PASSENGER AND CARGO AIRCRAFT / ~~CARGO AIRCRAFT ONLY~~

Airport of Departure:

Airport of Destination:

Shipment type: *(delete non-applicable)*
NON-RADIOACTIVE

NATURE AND QUANTITY OF DANGEROUS GOODS

Dangerous Goods Identification				Quantity and type of packing	Packing Inst.	Authorization
UN or ID No.	Proper Shipping Name	Class or Division (Subsidiary Risk)	Packing Group			
UN 2814	**Infectious substance, affecting humans (*Mycobacterium tuberculosis*)**	**6.2**		**2 mL**	**602**	
UN 1845	**Dry ice**	**9**	**III**	**4 kg** **packed in a single cardboard box**	**904**	

Additional Handling Information

Emergency Contact: (800) 745-1122

I hereby declare that the contents of this consignment are fully and accurately described above by the proper shipping name, and are classified, packaged, marked and labelled/placarded, and are in all respects in proper condition for transport according to applicable international and national governmental regulations. I declare that all of the applicable air transport requirements have been met.

Name/Title of Signatory
John E. Wilkins, Director

Place and Date
Pacolet, SC October 29, 2005

Signature *(see warning above)* ***John Wilkins***

Figure 15.6–14 Completed Shipper's Declaration for Dangerous Goods.

VIII. TRAINING AND CERTIFICATION

The DOT and IATA provide surprisingly little direction and details for training shippers. Neither organization provides much helpful information regarding who should or can be a trainer, how training should be performed, detailed contents of training, how testing is to be performed, the definition of a passing grade, and how to determine if a person is adequately trained.

A. Applicability

Anyone involved in the shipping or transportation of dangerous goods (including infectious substances) must be trained and certified in the shipment of dangerous goods (6, 7, 11). 2005 WHO guidelines state that only persons who pack and ship Category A infectious substances must receive the aforementioned formal training and certification (15). Persons who pack and ship Category B infectious substances and Exempt Human and Animal Specimens need to receive only general and practical training such as "clear instructions on the use of packaging" and "training and awareness" of the importance of packing substances appropriately (15). Such persons should receive clear instructions, guidance, and training appropriate for packing and shipping Category B infectious substances and diagnostic specimens, addressing spills, and protecting themselves (15).

◩ **NOTE:** As of this writing, the IATA and DOT have *not* adopted these recently relaxed and separate training requirements for shippers of Category B substances. Until such requirements are adopted by the DOT, shippers should be formally trained and certified as if they will pack and ship Category A and Category B substances. Readers are advised to review IATA and DOT regulations annually regarding this issue. New IATA regulations are published at the end of each year and are effective the following January 1.

B. Essential components

The essential components of a training program include the following:

1. General awareness and familiarity with the many aspects of shipping dangerous goods
2. Importance, nature, and contents of IATA and DOT regulations
3. Function-specific training (hands-on and/or demonstrations of packaging and packing techniques)
4. Marking and labeling
5. Documentation of shipments of dangerous goods
6. Safety training
7. Security training (if applicable to a trainee's job responsibilities)
8. Testing
9. Issuance of a certificate after successful completion of the training (6, 7)

C. Training materials

Acceptable training materials and methods include manuals, training courses, and workshops, all of which are commercially available from professional organizations and commercial suppliers of packaging materials for dangerous goods. Alternatively, a training program or workshop which includes hands-on training and demonstrations can be developed by any hospital, laboratory, school, institution, or other facility through the direction of a certified trainer. All training programs should be designed to provide initial and regular follow-up training to each employee responsible for shipping and packing infectious substances. Training and training material for the transportation of dangerous goods and infectious substances are available from the following sources.

1. American Society for Microbiology (http://www.asm.org)
2. IATA (training manuals) (http://www.iata.com)
3. Regional and national clinical microbiology meetings (workshops and presentations)

VIII. TRAINING AND CERTIFICATION *(continued)*

4. Many major universities and medical centers
5. Many national and state departments of health and public health
6. Many professional life science organizations and societies
7. SafTPak (http://www.saftpak.com)
8. CARGOpak (http://www.cargopak.com)
9. Dangerous Goods International (http://www.dgitraining.com)
10. ICC The Compliance Center (http://www.thecompliancecenter.com)
11. World Courier Training Course (http://www.worldcourier.com)
12. Casing Scientific (http://www.casingcorp.com)

D. Documentation of training

The IATA and DOT require all aspects of training to be documented. The most important document used to prove appropriate and timely training is a certificate which is issued after training is complete. Employers should keep a record for each employee who is trained. The record should include employee name, location and date of training, name of the trainer, course content, documentation of testing, and a copy of the certificate of training. IATA and DOT certification is valid for 2 and 3 years, respectively.

E. Enforcement of Compliance

The DOT and the Federal Aviation Administration have authority to perform unannounced inspections of facilities (e.g., clinical laboratories) that ship dangerous goods, and to inspect these facilities for compliance with the training regulations and to inspect training records at these facilities. Facilities which do not comply with prescribed regulations are subject to substantial fines.

REFERENCES

1. **Animal and Plant Inspection Service, U.S. Department of Agriculture.** 2005. Agricultural bioterrorism protection act of 2002: possession, use, and transfer of biological agents and toxins; final rule (7 CFR Part 331; 9 CFR Part 121). *Fed. Regist.* **70:**13242–13292.

2a. **Centers for Disease Control and Prevention and the Office of the Inspector General, U.S. Department of Health and Human Services.** 2005. Possession, use, and transfer of select agents and toxins; final rule (42 CFR Parts 72 and 73). *Fed. Regist.* **70:**13294–13325.

2b. **Centers for Disease Control and Prevention, U.S. Department of Health and Human Services.** 2008. Possession, use, and transfer of select agents and toxins; final rule (42 CFR Part 73). *Fed. Regist.* **73:**61363–61366.

3. **Centers for Disease Control and Prevention, U.S. Department of Health and Human Services.** 2005. Possession, use, and transfer of select agents and toxins—reconstructed replication competent forms of the 1918 pandemic influenza virus containing any portion of the coding regions of all eight gene segments; interim final rule (42 CFR Part 73). *Fed. Regist.* **70:**61047–61049.

4. **Denys, G. A., L. D. Gray, and J. W. Snyder.** 2004. *Cumitech 40, Packing and Shipping Diagnostic Specimens and Infectious Substances.* Coordinating ed., D. L. Sewell. American Society for Microbiology, Washington, DC.

5. **Gilchrist, M. J. R., W. P. McKinney, J. M. Miller, and A. S. Weissfeld.** 2000. *Cumitech 33, Laboratory Safety, Management, and Diagnosis of Biological Agents Associated with Bioterrorism.* Coordinating ed., J. W. Snyder. American Society for Microbiology, Washington, DC.

6. **Gray, L. D., and J. W. Snyder.** 2006. Packing and shipping biological materials, p. 383–401. *In* D. O. Fleming and D. L. Hunt (ed.), *Biological Safety: Principles and Practices*, 4th ed. ASM Press, Washington, DC.

7. **International Air Transport Association.** 2009. *Dangerous Goods Regulations*, 50th ed. International Air Transport Association, Montreal, Quebec, Canada.

8. **McKay, J., and D. O. Fleming.** 2000. Packaging and shipping biological materials, p. 411–425. *In* D. O. Fleming and D. L. Hunt (ed.), *Biological Safety: Principles and Practices*, 3rd ed. American Society for Microbiology, Washington, DC.

9. **Snyder, J. W.** 2002. Packaging and shipping of infectious substances. *Clin. Microbiol. Newsl.* **24:**89–93.

10. **U.S. Department of Transportation, Pipeline and Hazardous Materials Safety Administration.** 2005. Hazardous materials. Incorporation of statutory mandated revisions to the hazardous materials regulations. *Fed. Regist.* **70:**73164.

REFERENCES *(continued)*

11. **U.S. Department of Transportation, Pipeline and Hazardous Materials Safety Administration.** 2006. Hazardous materials: infectious substances; harmonization with the United Nations recommendations; final rule. *Fed. Regist.* **71:**32244–32263.
12. **U.S. Department of Transportation, Research and Special Programs Administration.** 2004. Hazardous materials. Miscellaneous changes to the hazardous communication requirements; final rule. *Fed. Regist.* **69:**64468.
13. **U.S. Department of Transportation, Research and Special Programs Administration.** 2004. Harmonization with the United Nations Recommendations, International Maritime Dangerous Goods Code, and International Civil Aviation Organization's Technical Instructions; final rule. *Fed. Regist.* **69:**76044–76187.
14. **U.S. Postal Service.** 2009. *Domestic Mail Manual*. http://pe.usps.com/text/dmm300/dmm300_landing.htm. Accessed 25 June 2009.
15. **World Health Organization.** 2005. *Guidance on Regulations for the Transport of Infectious Substances*. World Health Organization, Geneva, Switzerland. http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2005_22/en.

APPENDIX 15.6–1

Significant Changes in IATA Requirements and DOT Regulations Since 2006

Classification of infectious substances according to risk groups has been replaced by classification of substances into either Category A substance, Category B substance, Exempt Human or Animal Specimen, Exempt Substance, and Patient Specimen.

Culture and *patient specimen* have been defined, and the definitions are more user-friendly. The term *laboratory culture* is no longer used.

Packing directions for Exempt Human or Animal Specimen have been provided.

The technical name of a Category A substance packed according to PI 602 is no longer required on the outer package. For example, a package labeled "Infectious Substance, Affecting Humans (Hepatitis C Virus)" is now labeled "Infectious Substance, Affecting Humans." The technical name is still required on Shipper's Declarations.

PI 650: the only acceptable proper shipping name for a Category B substance is "Biological Substance, Category B." The terms "Clinical Specimen" and "Diagnostic Specimen" are not allowed.

PI 650: quantity limits have been revised to allow up to 1 liter of liquid per primary container.

PI 650 now mandates use of rigid outer containers.

PI 650: packages must be marked with a diamond symbol which contains "UN3373," and a "Biological Substance, Category B," marking adjacent to the diamond.

PI 650: If an air waybill is used, the "Nature and Quantity of Goods" box (or equivalent area on the air waybill) should indicate the appropriate proper shipping name, "Biological Substance, Category B (UN3373).

The "Prior arrangements as required . . ." statement in the Additional Handling Information section of the Shipper's Declaration is no longer required.

The requirement for an "air eligibility" label or marking (airplane symbol inside a circle) has been replaced by the following certification statement on the Shipper's Declaration: "I declare that all of the applicable air transport requirements have been met."

The sequence of information within the Nature and Quantity of Dangerous Goods section of the Shipper's Declaration of Dangerous Goods begins with the UN number in the first column.

Overpacks must now be labeled "Overpack" instead of "Inner Packages Comply. . . ."

Several exemptions to regulations have been defined.

Persons who pack and ship select agents and toxins and Category A agents must receive security training commensurate with their responsibilities.

Some hazardous materials, e.g., 10% formalin, used as a preservative are exempt from requirements and regulations if the quantity is $\leq$30 ml per primary container.

APPENDIX 15.6–2

Glossary

Biological product a substance which originated from living organisms (including humans and other mammals), and has been manufactured and distributed in accordance with compliance and licensing requirements set forth by the federal government; can be classified as an infectious substance if such is appropriate. Biological products can be finished or unfinished; are intended for use in the prevention, treatment, or diagnosis of disease in humans or animals; and are to be used for investigational, experimental, or development purposes. Biological products include such common items as clinical microbiology reagents and kits, serological reagents, diagnostic reagents, and vaccines. In certain parts of the world, some licensed biological products are regarded as biohazardous and either are subject to compliance criteria specified for infectious substances or must adhere to other restrictions imposed by the government of that country.

Biological substance, Category B any infectious substance which does not meet the criteria of a category A substance; formerly known as "Clinical Specimen" or "Diagnostic Specimen"; an infectious substance *not* in a form generally capable of causing disability, life-threatening illness, or fatal disease. Category B substances generally are (i) patient and clinical specimens reasonably expected to contain, or being cultured or otherwise tested for, a non-Category A pathogen and (ii) cultures of microorganisms not specifically listed in Category A.

Carrier (operator) individual or organization engaged in the commercial transportation of goods (e.g., Federal Express, United Parcel Service, Delta Airlines, and Northwest Airlines).

Category A substance an infectious substance or microorganism which is transported *in a form* that, when exposure to it occurs, is capable of causing permanent disability or life-threatening or fatal disease in an otherwise healthy human or animal. Category A substances are individually designated and specifically listed by the IATA.

Category B substance an infectious substance which does not meet Category A criteria. Category B substances generally are considered to be the following: (i) patient or clinical specimens reasonably expected to contain, or being cultured or otherwise tested for, a pathogen and (ii) microorganisms not specifically listed in Category A. The proper term for a Category B substance is Biological substance, Category B.

Code of Federal Regulation U.S. laws published in the *Federal Register* and available online at http://www.gpoaccess.gov/cfr/index.html

Consignee the receiver of the shipment (e.g., a reference laboratory)

Culture the result of a process by which pathogens are intentionally propagated. This definition refers to typical clinical laboratory microorganisms grown in broth or on solid media. Typical clinical cultures may be classified as either Category A or Category B, depending on the organism concerned and the professional judgment of the shipper.

Dangerous goods material which, when not properly handled and contained, can pose a risk to the health, safety, property, or environment and which are shown the list of dangerous goods in the IATA *Dangerous Goods Regulations*

Dangerous Goods Regulations a commercially available book of IATA requirements published by IATA; based on and incorporates ICAO regulations; provides packaging and shipping regulations for dangerous goods; generally recognized and accepted worldwide

Diagnostic (or clinical) specimen term no longer used or allowed; replaced by "Biological Substance, Category B"

Genetically modified microorganisms microorganisms that have had their genetic material purposely modified or altered through genetic engineering in a manner that does not occur naturally; must be classified in the same manner and to the same extent as any infectious substance

International Air Transport Association (IATA) a trade organization of the commercial airline industry; governs international aviation; publishes *Dangerous Goods Regulations* for use by anyone who packs, ships, transports, or handles dangerous goods

International Civil Aviation Organization (ICAO) a specialized agency of the UN; governs international aviation; regulates the transportation of dangerous goods for all international civil air carriers; the source of IATA requirements and DOT regulations

APPENDIX 15.6–2 *(continued)*

Infectious substance a substance which is known to contain or reasonably expected to contain pathogens (microorganisms which can cause disease in humans and animals); material known to contain or reasonably suspected of containing a Category A or B pathogen or substance; can be a class (Class 6), a division (Division 6.2), or a category (Category A or B) of dangerous goods as defined by the IATA

Overpack the outermost packaging used to enclose more than one complete package, each of which contains dangerous goods; usually used for convenience and to reduce shipping costs

Package end product of the packing process

Packaging all of the numerous materials used to contain a shipped substance and to prepare the substance for shipping; the container (receptacle) and its associated components (e.g., tubes, containers, absorbent material, boxes, and labels) used to contain and pack a substance and to ensure compliance with packing requirements

Packing the physical action and method by which packaging is used to secure articles or substances for shipment

Packing instructions IATA-defined directions shippers must follow to select, assemble, mark, label, and document the packing process for shipping dangerous goods, including infectious substances; includes manufacturing, testing, and performance specifications for packaging materials

Pathogen a microorganism (bacterium, mycobacterium, fungus, parasite, virus, plasmid, genetic element, proteinaceous infectious particle [prion], or genetically modified organism) that is known to cause or is reasonably expected to be able to cause disease in humans or animals

Patient specimen material collected from humans or animals, including, but not limited to, excreta, secreta, blood and its components, tissue, body fluids, body organs and parts, and swabs of human material being transported for purposes such as research, diagnosis, investigational activities, and disease treatment and prevention

Primary specimen container the innermost packaging containing a diagnostic specimen or infectious substance; composed of glass, metal, or plastic; must be leakproof; must be positively sealed if it contains an infectious substance

Proper shipping name any of over 3,000 IATA-listed and internationally recognized names of dangerous goods

Secondary specimen container the container that contains the primary specimen container

Shipper anyone who ships goods by a commercial carrier (usually an employee of a company or health care facility [e.g., laboratory staff member, contracted courier, and physician]); anyone who offers goods for transport to a member of IATA; anyone who completes and signs the Shipper's Declaration. The person who signs the Shipper's Declaration is the person who accepts responsibility for the accuracy of the information on the document.

Shipper's Declaration for Dangerous Goods (Shipper's Declaration) an IATA-defined and DOT-mandated form which must accompany each shipment of dangerous goods; contains information which describes the dangerous goods; is helpful to persons who handle the shipment; must be completed by the shipper

UN-certified container packaging material (usually a cardboard box) that has passed UN manufacturing standards and is labeled by the manufacturer as such for the transport of certain dangerous goods

U.S. Department of Transportation (DOT) the federal agency which regulates domestic transportation of all dangerous goods into and within the United States through regulations published in the *Federal Register*; publishes regulations which are based on and in substantial agreement with ICAO regulations

APPENDIX 15.6–3

Select Agents

Transfer of Select Agents

The U.S. Department of Health and Human Services (HHS) regulates and the CDC oversees the possession, use, and transfer of certain specifically listed biological agents and toxins (called "select agents") that have the potential to pose a severe threat to public health and safety. The Select Agent Program (http://www.cdc.gov/od/sap) oversees all activities with select agents and registers all U.S. laboratories, persons, and other entities that possess, use, and/or transfer select agents.

Select Agents

Select agents are microorganisms, biological agents, or biological toxins that have been deemed by the U.S. government to be major threats to public health and safety because they could be used as agents of bioterrorism. Select agents are listed in Table 15.5.3–1.

Packing and Shipping Select Agents

A select agent may be packaged and prepared for shipment as any other infectious substance is packed according to the guidelines in this procedure; i.e., packaging for select agents is the same as that for routine infectious substances: leakproof primary and secondary containers, absorbent, a sturdy and well-labeled outer container, shipper's declaration for Category A substances, etc. However, the requirements regarding *the possession, use, and transfer* of select agents are intricate, strict, and aggressively enforced.

If a select agent, specimen containing a select agent, or a substance suspected of containing a select agent is intended to be shipped, both the sender and the recipient must (read, it is imperative to) (i) contact the appropriate federal authorities for guidance, instructions, and permission to ship or receive the select agent *before* such transfer occurs and (ii) apply for and receive a site registration number from the CDC *before* the transfer occurs. In addition, the shipper must confirm that the recipient is approved for receiving select agents. The aforementioned CDC website provides complete information about select agents, the strict regulations related to transferring select agents, possession and transfer application forms, and additional resources.

15.7 Management of Laboratory Accidents

Despite improved control measures (engineering controls, safe work practices, and PPE), laboratory workers remain at risk for acquiring laboratory-associated infections. Reported cases of fatal meningococcemia in clinical laboratory workers underscore the potential risks of handling clinical samples (2). Every laboratory (e.g., anatomic pathology, clinical diagnostic, reference, and research laboratories) should implement a biosafety plan. The essential components of the plan should include written procedures to reduce risks of occupational exposure and mandatory training, health assessment of employees, and record keeping of all exposures and treatments. A risk assessment of the procedures carried out on samples, including the frequency of positive samples, should be determined. The potential risk of disease should also be evaluated along with the availability of postexposure prophylaxis (PEP) and preventive vaccines. The benefits and side effects of PEP and immunization must also be considered.

I. MANAGEMENT OF OCCUPATIONAL BLOOD-BORNE EXPOSURES

Observe standard precautions.

A. Provide immediate care to the exposure site.
- **1.** Wash wounds and skin with soap and water.
- **2.** Flush mucous membranes and conjunctiva sites with water.

B. Report the incident to a supervisor and complete an exposure report (Appendix 15.7–1).
- **1.** Record date and time of exposure.
- **2.** Record the procedure performed, including when, where, and how the exposure occurred and with what type of device, including brand name.
- **3.** Record details of the exposure, including route, body substance involved, volume, and duration of contact.
- **4.** Record information regarding source person, human immunodeficiency virus (HIV) status, stage of disease, history of antiretroviral therapy, and viral load status, if known.
- **5.** Record information about counseling, postexposure management, and follow-up.

C. Evaluate the risk of exposure.
- **1.** Appraise type of fluid (e.g., blood, visibly bloody fluid, concentrated virus).
- **2.** Appraise type of exposure (e.g., percutaneous, mucous membrane, or nonintact skin, bite resulting in blood exposure).

D. Evaluate the exposure source.
- **1.** Assess the risk of infection using available information. If the source of the exposure is unknown, further evaluation is required.
- **2.** Test known sources for HBsAg, anti-hepatitis C virus (anti-HCV), and HIV antibody (consider rapid testing if available).
- **3.** *Do not* test discarded needles, syringes, and other devices for virus contamination.

E. Evaluate the exposed person.

Assess the immune status for HBV infection using history of hepatitis B vaccination and tests for anti-HBs antibody.

I. MANAGEMENT OF OCCUPATIONAL BLOOD-BORNE EXPOSURES *(continued)*

F. Provide PEP based on the risk of infection transmission and institutional policy.
- **1.** HBV: see Appendix 15.7–2 (3)
- **2.** HCV: PEP is not recommended.
- **3.** HIV: see Appendixes 15.7–3 and 15.7–4 (3, 4)
  - **a.** Initiate PEP as soon as possible (within a few hours of exposure).
  - **b.** Offer pregnancy testing to all women of childbearing age not known to be pregnant.
  - **c.** Seek consultation if viral resistance is suspected.
  - **d.** Administer PEP for 4 weeks if tolerated.

G. Perform follow-up testing and provide counseling.
- **1.** Advise exposed workers to seek medical evaluation for any acute illness occurring during the follow-up periods.
- **2.** HBV exposures
  - **a.** Test for anti-HBs 1 to 2 months after the last dose of vaccine was administered if only a vaccine was given.
  - **b.** *Do not* follow up exposed workers if immune to hepatitis B or given hepatitis B immune globulin (HBIG) PEP.
- **3.** HCV exposures
  - **a.** Perform baseline and follow-up testing for anti-HCV and alanine aminotransferase activity 4 to 6 months after the exposure.
  - **b.** Perform HCV RNA testing at 4 to 6 weeks, if an earlier diagnosis for HCV infection is desired.
  - **c.** Confirm repeatedly reactive anti-HCV EIAs with supplemental tests.
- **4.** HIV exposures
  - **a.** Perform HIV antibody testing for at least 6 months postexposure.
  - **b.** Perform HIV antibody testing if an illness compatible with an acute retroviral syndrome occurs.
  - **c.** Advise exposed worker to use precautions to prevent secondary transmission during the follow-up period.
  - **d.** Evaluate exposed worker taking PEP within 72 h after exposure and monitor for drug toxicity for at least 2 weeks.

II. MANAGEMENT OF OTHER LABORATORY-ASSOCIATED EXPOSURES

Observe standard precautions.

A. Provide immediate care to the exposed sites.
- **1.** Wash wounds and skin with soap and water.
- **2.** Flush contaminated mucous membranes and conjunctiva sites with water.
- **3.** Rinse contaminated mouth with an antiseptic mouthwash.

B. Report the incident to a supervisor according to institutional policy (Appendix 15.7–1). Immediate reporting of an accident will help establish a time relationship if infection develops and allow preventative measures to be taken.

C. Evaluate the risk of exposure.
- **1.** Appraise type of infectious material (e.g., clinical specimens, stock cultures, and infectious waste).
- **2.** Appraise type of exposure (e.g., inhalation, ingestion, direct contact, or percutaneous inoculation).
- **3.** Appraise the volume of material or concentration of microorganisms tested.

D. Evaluate the exposed worker.
- **1.** Assess the immune status for infection using history of vaccination or tests (e.g., tuberculin skin test). Specific recommendations for the immunization of laboratory workers in high-risk situations are listed in Appendix 15.7–5.
- **2.** Collect baseline serum and follow-up serum for testing for specific agents when appropriate.

II. MANAGEMENT OF OTHER LABORATORY-ASSOCIATED EXPOSURES *(continued)*

E. Provide medical evaluation and follow-up.
- **1.** Seek medical evaluation by an occupational health or private physician.
- **2.** Exposed workers are advised to seek medical attention for any acute illness during the follow-up period.
- **3.** Receive appropriate postexposure treatment and prophylaxis. Specific recommendations for exposure treatment and prophylaxis depend on the infectious agent and the assessment of the risk of infection (1).

F. Analyze incident reports.
- **1.** Maintain all written records (e.g., accident reports, sharps exposure log) following any exposure to infectious agents.
- **2.** Review accident report data to identify any common patterns and eliminate risk factors.
- **3.** Develop a correct action plan to prevent or minimize future incidents.

REFERENCES

1. **Bolyard, E. A., O. C. Tablan, W. W. Williams, M. L. Pearson, C. N. Shapiro, and S. D. Deitchman, The Hospital Infection Control Practices Advisory Committee.** 1998. Guidelines for infection control in health care personnel. *Am. J. Infect. Control* **26:**289–354 and *Infect. Control Hosp. Epidemiol.* **19:**407–463.
2. **Centers for Disease Control and Prevention.** 2002. Laboratory-acquired meningococcal disease—United States, 2000. *MMWR Morb. Mortal. Wkly. Rep.* **51:**141–144.
3. **Centers for Disease Control and Prevention.** 2001. Updated U.S. Public Health Service guidelines for the management of occupational exposures to HBV, HCV, and HIV and recommendations for postexposure prophylaxis. *MMWR Morb. Mortal. Wkly. Rep.* **50**(RR-11)**:**1–42.
4. **Centers for Disease Control and Prevention.** 2005. Updated U.S. Public Health Service guidelines for the management of occupational exposures to HIV and recommendations for postexposure prophylaxis. *MMWR Morb. Mortal. Wkly. Rep.* **54:**1–17.

SUPPLEMENTAL READING

Beltrami, E. M., I. T. Williams, C. N. Shapiro, and M. E. Chamberland. 2000. Risk management of blood-borne infections in health care workers. *Clin. Microbiol. Rev.* **13:**385–407.

Centers for Disease Control and Prevention. 2008. Update: potential exposures to attenuated vaccine strain *Brucella abortus* RB51 during laboratory proficiency test—United States and Canada, 2007. *MMWR Morb. Mortal. Wkly. Rep.* **57:**36–39.

Centers for Disease Control and Prevention. 2007. Recommended adult immunization schedule—United States, October 2007–September 2008. *MMWR Morb. Mortal. Wkly. Rep.* **56:**Q1–Q4.

Centers for Disease Control and Prevention. 1997. Immunization of health-care workers: recommendations of the Advisory Committee on Immunization Practices (ACIP) and the Hospital Infections Control Practices Advisory Committee (HICPAC). *MMWR Morb. Mortal. Wkly. Rep.* **46**(RR-18)**:**1–42.

CLSI. 2005. *Protection of Laboratory Workers from Occupationally Acquired Infections.* Document M29-A3. CLSI, Wayne, PA.

Peacock, S. J., H. P. Schweizer, D. A. B. Dance, T. L. Smith, J. E. Gee, V. Wuthiekanun, D. DeShazer, I. Steinmetz, P. Tan, and B. J. Currie. 2008. Management of accidental laboratory exposure to *Burkholderia pseudomallei* and *B. mallei*. *Emerg. Infect. Dis.* **14:**e2.

Sejvar, J. J., D. Johnson, T. Popovic, J. M. Miller, F. Downes, P. Somsel, R. Weyant, D. S. Stephens, B. A. Perkins, and N. E. Rosenstein. 2005. Assessing the risk of laboratory-acquired meningococcal disease. *J. Clin. Microbiol.* **43:**4811–4814.

APPENDIX 15.7–1 Employee Accident and Exposure Report

Instructions for use:

Employee: 1) Fill out completely and promptly—within 24 hours. 2) Take report to supervisor for signature. 3) Take/mail form to your campus Employee Occupational Health Services (EOHS).

Supervisor: 1) Complete supervisor section and sign report. 2) Retain copy for your file. 3) Send employee for medical care, if requested.

Medical Care: 1) If treatment is required, employee to report to EOHS (or emergency room [ER] if EOHS closed) with complete report. 2) If no treatment is requested, mail form to your campus EOHS.

Blood and Body Fluid Exposures and Needle Sticks: Bring completed form to your campus EOHS for evaluation.

Employee Information ***(to be completed by the employee)***	Name (Print): _____ Signature: Date Signed:		
Employee ID (SS#):	Birth Date:	Sex (circle): M F	Prior Name:
Address (include city, state, and zip):	Home Phone:	Job Title:	
	Work Phone:	Cost Center Number and Name:	
Incident Information ***(to be completed by the employee)***	Date of Incident:	Time:	Exact Location:
List Names of Any Witnesses:	Date/Time Reported:	Name of Supervisor Reported to:	
What Part(s) of the Body Was Injured:		Type of Injury/Illness:	
Give a Complete Description of How the Incident Occurred:			
Blood & Body Fluid Exposures/Needle Sticks	*Reporting Instructions:* Report to your campus EOHS with completed report.		
Source Patient Name:	Source Patient Hospital ID #:	Hepatitis B Vaccine Completed?	
Treating MD:	Patient Room #:	☐ Yes ☐ No	

Supervisor Statement	Name and Title:	Work Phone #: _____ Pager #: _____
Identify any corrective or preventative actions to be taken, any safety violations, or any additional comments:		
Signature:		Date:

Medical Provider Statement	Diagnosis: Work Status/Restrictions/Comments:
Signature and Date:	

Claims Management	Type of Claim: Report Only 1st Aid Medical Only Lost Time
OSHA Case #:	Investigation Completed By: Date:

APPENDIX 15.7–2

Recommended PEP for exposure to HBV[a]

Vaccination and antibody response status of exposed workers[b]	Treatment		
	Source HBsAg positive	Source HBsAg negative	Source unknown or not available for testing
Unvaccinated	HBIG[c] × 1 and initiate HB vaccine series	Initiate HB vaccine series	Initiate HB vaccine series
Previously vaccinated			
Known responder[d]	No treatment	No treatment	No treatment
Known nonresponder[e]	HBIG × 1 and initiate revaccination or HBIG × 2[f]	No treatment	if known high-risk source, treat as if source were HBsAg positive
Antibody response unknown	Test exposed person for anti-HBs 1. If adequate,[d] no treatment is necessary 2. If inadequate,[e] administer HBIG × 1 and vaccine booster	No treatment	Test exposed person for anti-HBs 1. If adequate,[d] no treatment is necessary 2. If inadequate,[e] administer vaccine booster and recheck titer in 1–2 mo

[a] From **Centers for Disease Control and Prevention.** 2001. Updated U.S. Public Health Service guidelines for the management of occupational exposures to HBV, HCV, and HIV and recommendations for postexposure prophylaxis. *MMWR Morb. Mortal. Wkly. Rep.* **50**(RR-11)**:**1–42.

[b] Persons who have previously been infected with HBV are immune to reinfection and do not require PEP.

[c] Dose is 0.06 ml/kg intramuscularly.

[d] A responder is a person with adequate levels of serum antibody to HBsAg (i.e., anti-HBs ≥10 mIU/ml).

[e] A nonresponder is a person with inadequate response to vaccination (i.e., serum anti-HBs <10 mIU/ml).

[f] The option of giving one dose of HBIG and reinitiating the vaccine series is preferred for nonresponders who have not completed a second three-dose vaccine series. For persons who previously completed a second vaccine series but failed to respond, two doses of HBIG are preferred.

APPENDIX 15.7–3

Recommended HIV PEP for percutaneous injuries[a]

Exposure type	Recommendation with the following infection status of source				
	HIV positive, class 1[b]	HIV positive, class 2[b]	Source with unknown HIV status[c]	Unknown source[d]	HIV negative
Less severe[e]	Recommend basic 2-drug PEP	Recommend expanded 3-drug PEP	Generally, no PEP warranted; however, consider basic 2-drug PEP[f] for source with HIV risk factors[h]	Generally, no PEP warranted; however, consider basic 2-drug PEP[f] in settings where exposure to HIV-infected persons is likely	No PEP warranted
More severe[g]	Recommend expanded 3-drug PEP	Recommend expanded 3-drug PEP	Generally, no PEP warranted; however, consider basic 2-drug PEP[f] for source with HIV risk factors[h]	Generally, no PEP warranted; however, consider basic 2-drug PEP[f] in settings where exposure to HIV-infected persons is likely	No PEP warranted

[a] From **Centers for Disease Control and Prevention.** 2001. Updated U.S. Public Health Service guidelines for the management of occupational exposures to HBV, HCV, and HIV and recommendations for postexposure prophylaxis. *MMWR Morb. Mortal. Wkly. Rep.* **50**(RR-11)**:**1–42.

[b] HIV positive, class 1, asymptomatic HIV infection or known low viral load (e.g., <1,500 RNA copies/ml); HIV positive, class 2, symptomatic HIV infection, AIDS, acute seroconversion, or known high viral load. If drug resistance is a concern, obtain expert consultation. Initiation of PEP should not be delayed pending expert consultation, and because expert consultation alone cannot substitute for face-to-face counseling, resources should be available to provide immediate evaluation and follow-up care for all exposures.

[c] For example, deceased source person with no samples available for HIV testing.

[d] For example, a needle from a sharps disposal container.

[e] For example, solid needle and superficial injury.

[f] The designation "consider PEP" indicates that PEP is optional and should be based on an individualized decision between the exposed person and the treating clinician.

[g] For example, large-bore hollow needle, deep puncture, visible blood on device, or needle used in patient's artery or vein.

[h] If PEP is offered and taken and the source is later determined to be HIV negative, PEP should be discontinued.

APPENDIX 15.7–4

Recommended HIV PEP for mucous membrane exposures and nonintact skin[a] exposures[b]

Exposure type	Recommendation with the following infection status of source				
	HIV positive, class 1[c]	HIV positive, class 2[c]	Source with unknown HIV status[d]	Unknown source[e]	HIV negative
Small volume[f]	Consider basic 2-drug PEP[g]	Recommend basic 2-drug PEP	Generally, no PEP warranted; however, consider basic 2-drug PEP[g] for source with HIV risk factors[h]	Generally, no PEP warranted; however, consider basic 2-drug PEP[g] in settings where exposure to HIV-infected persons is likely	No PEP warranted
Large volume[i]	Recommend basic 2-drug PEP	Recommend expanded 3-drug PEP	Generally, no PEP warranted; however, consider basic 2-drug PEP[g] for source with HIV risk factors[h]	Generally, no PEP warranted; however, consider basic 2-drug PEP[g] in settings where exposure to HIV-infected persons is likely	No PEP warranted

[a] For skin exposures, follow-up is indicated only if there is evidence of compromised skin integrity (e.g., dermatitis, abrasion, or open wound).

[b] From **Centers for Disease Control and Prevention.** 2001. Updated U.S. Public Health Service guidelines for the management of occupational exposures to HBV, HCV, and HIV and recommendations for postexposure prophylaxis. *MMWR Morb. Mortal. Wkly. Rep.* **50**(RR-11)**:**1–42.

[c] HIV positive, class 1, asymptomatic HIV infection or known low viral load (e.g., <1,500 RNA copies/ml); HIV positive, class 2, symptomatic HIV infection, AIDS, acute seroconversion, or known high viral load. If drug resistance is a concern, obtain expert consultation. Initiation of PEP should not be delayed pending expert consultation, and because expert consultation alone cannot substitute for face-to-face counseling, resources should be available to provide immediate evaluation and follow-up care for all exposures.

[d] For example, deceased source person with no samples available for HIV testing.

[e] For example, splash from inappropriately disposed blood.

[f] In other words, a few drops.

[g] The designation "consider PEP" indicates that PEP is optional and should be based on an individualized decision between the exposed person and the treating clinician.

[h] If PEP is offered and taken and the source is later determined to be HIV negative, PEP should be discontinued.

[i] In other words, major blood splash.

APPENDIX 15.7–5

Immunization available for laboratory workers in special circumstances[a]

Generic name	Primary and booster dose schedule	Indications	Major precautions or contraindications
BCG vaccine (tuberculosis)	One percutaneous dose of 0.3 ml; no booster dose recommended	Not routinely indicated. Laboratory personnel who process large volumes of specimens from which *Mycobacterium tuberculosis* is isolated or high proportion of *M. tuberculosis* resistant to isoniazid and rifampin	Immunocompromised state and pregnancy
Hepatitis A vaccine	Two doses i.m., either (HAVRIX) 6–12 mo apart or (VAQTA) 6 mo apart	Not routinely indicated. Laboratory personnel who work with HAV-infected primates or with HAV in a research setting	History of anaphylactic reaction to alum or the preservative 2-phenoxyethanol. Vaccine safety in pregnant women has not been evaluated; risk to fetus is likely low and should be weighed against the risk of hepatitis A in women at high risk
Meningococcal polysaccharide vaccine (quadrivalent A, C, W 135, and Y)	One dose in volume and by route specified by manufacturer; need for boosters is unknown	Not routinely indicated. Research, industrial, and clinical laboratory personnel who are routinely exposed to *Neisseria meningitidis* in solutions that may be aerosolized	Vaccine safety in pregnant women has not been evaluated; vaccine should not be given during pregnancy unless risk of infection is high

APPENDIX 15.7–5 *(continued)*

Immunization available for laboratory workers in special circumstances[a] *(continued)*

Generic name	Primary and booster dose schedule	Indications	Major precautions or contraindications
Polio vaccine	IPV, two doses s.c. given 4–8 wk apart followed by 3rd dose 6–12 mo after 2nd dose; booster doses may be IPV or OPV	Laboratory personnel handling specimens that may contain wild poliovirus	History of anaphylactic reaction after receipt of streptomycin or neomycin; safety in pregnant women has not been evaluated.
Rabies vaccine	Primary, HDCV or RVA, i.m., 1.0 ml (deltoid area), one each on days 0, 7, 21, and 28, or HDCV, i.d., 1.0 ml, one each on days 0, 7, 21, and 28; booster, HDCV or RVA, i.m., 0.1 ml (deltoid area), day 0 only, or HDCV, i.d., 0.1 ml, day 0 only	Laboratory personnel who work with rabies or infected animals in diagnostic or research activities	The frequency of booster doses should be based on frequency of exposure.
Tetanus and diphtheria (Td) vaccine	Two doses i.m. 4 wk apart; 3rd dose 6–12 mo after 2nd dose; booster every 10 yr	All adults; tetanus prophylaxis in wound management	First trimester of pregnancy; history of a neurologic reaction or immediate hypersensitivity reaction. History of (Arthustype) reaction after previous dose of Td vaccine should not be given further routine or emergency dose of Td for 10 yr
Typhoid vaccine (i.m., s.c., and oral)	i.m. vaccine: one 0.5-ml dose, booster dose of 0.5 ml every 2 yr (Vi capsular polysaccharide). s.c. vaccine: Two 0.5-ml doses, ≥4 wk apart, booster 0.5 ml s.c. or 0.1 ml i.d. every 3 yr if exposure continues. Oral vaccine: four doses on alternate days; (Ty21a) vaccine manufacturer's recommendation is revaccination with the entire four-dose series every 5 yr	Personnel in microbiology laboratories who frequently work with *Salmonella enterica* serovar Typhi	Severe local or systemic reaction to a previous dose of typhoid vaccine; Ty21a vaccine should not be given to immunocompromised personnel or individuals receiving antimicrobial agents
Vaccinia vaccine (smallpox)	One dose administered with a bifurcated needle; boosters every 10 yr	Laboratory personnel who directly handle cultures of or animals contaminated with recombinant vaccinia viruses or orthopox viruses that infect humans	Pregnancy, presence or history of eczema, or immunocompromised status in potential vaccinees or in their household contacts

[a] Modified from **Centers for Disease Control and Prevention.** 1997. Immunization of health-care workers: recommendations of the Advisory Committee on Immunization Practices (ACIP) and the Hospital Infections Control Practices Advisory Committee (HICPAC). *MMWR Morb. Mortal. Wkly. Rep.* **46**(RR-18)**:**1–42. Abbreviations: HDCV, human diploid cell rabies vaccine; RVA, rabies vaccine absorbed; IPV, inactivated poliovirus vaccine; OPV, oral poliovirus vaccine; i.d., intradermally; i.m., intramuscularly; s.c., subcutaneously.

15.8 Management of Infectious Waste

I. PLAN DEVELOPMENT

For effective and efficient management of infectious wastes, a comprehensive waste management plan is essential in order to ensure the safety of the employees handling the waste, compliance with the various regulatory requirements (14, 17, 18, 20), meeting the standards of the JCAHO (11) and the guidelines of other professional groups such as the NCCLS (13), and implementation of cost-effective strategies for waste disposal. In developing a comprehensive plan for infectious-waste management, consider the following factors because of the constraints that they impose.

A. Regulatory requirements

1. Federal regulations

a. Comply with the requirements in OSHA's standard for exposure to bloodborne pathogens (14) that apply to infectious ("regulated") waste, especially those pertaining to contaminated sharps, sharps containers, other regulated waste, and hazard communication.

b. Comply with the requirements in the U.S. Department of Transportation's (USDOT's) regulations for transport of hazardous materials that apply to intrastate and interstate transport of "regulated medical waste," especially those pertaining to packaging and labeling (17, 18).

2. Federal guidelines

a. Follow CDC guidelines for the management of infectious wastes (1–4).

b. Follow CDC and NIH guidelines for the management of infectious wastes from microbiological and biomedical laboratories (5).

3. State regulations

Comply with state regulations that pertain to infectious-waste management and transport (e.g., definitions and requirements for packaging and labeling, transport, and treatment).

4. Local ordinances

Comply with local ordinances that pertain to infectious-waste management (e.g. restrictions on wastewater discharges and landfill disposal).

B. Management of other wastes

1. Microbiology laboratories generate various types of wastes, including infectious waste, chemical waste (some of which is probably hazardous per Resource Conservation and Recovery Act of 1976 regulations), radioactive waste, and wastes with mixed hazards, in addition to general trash and wastewater.

2. Institute an overall management system, with the infectious-waste management plan an integral part of the system (9, 13, 15).

I. PLAN DEVELOPMENT *(continued)*

C. Availability of treatment equipment and services

1. On-site treatment option. Review available treatment equipment (9).
- **a.** Will it meet regulatory requirements? (For example, does your state require destruction of the waste, and if so, does the available equipment both treat and destroy the waste?)
- **b.** How much of the infectious-waste stream can it treat? Is the capacity sufficient?
- **c.** What are the operating and maintenance costs?
- **d.** Is use of this equipment cost-effective?

2. Off-site treatment option. Consider the following.
- **a.** Is such a service available?
- **b.** Is this an appropriate option?
- **c.** What are the reliability and record of the vendor(s) offering such service?

3. Decision factors. Determine the following.
- **a.** What are the relative costs of on-site and off-site treatments?
- **b.** What are the liability considerations for your institution?

D. Waste minimization

1. Waste minimization decreases the quantities of infectious waste generated and/or the quantities of waste that must be managed as infectious, with a resultant reduction in the costs of infectious-waste management, handling, treatment, and disposal.

2. Institute waste minimization techniques (8), including the following.
- **a.** Define infectious wastes precisely.
- **b.** Separate waste streams at the source (i.e., point of discard).
- **c.** Segregate the infectious-waste stream throughout subsequent handling.
- **d.** Use new technology such as test miniaturization.
- **e.** Replace products using those that generate less waste.
- **f.** Reuse and recycle wastes.
- **g.** Treat wastes on-site.

II. MANAGEMENT PLAN

The elements of a comprehensive infectious-waste management plan are as follows.

A. Identification of infectious wastes

1. Institute a clear policy on waste identification. Such a policy will accomplish the following.
- **a.** It will help ensure that all infectious waste are directed into the infectious-waste stream.
- **b.** It will avoid risk and liability from infectious wastes that are not managed properly.
- **c.** It will keep noninfectious wastes out of the infectious-waste stream and thereby eliminate the cost of unnecessarily managing as infectious other wastes that do not need such management.

2. Consult various definitions of infectious waste in the OSHA blood-borne-pathogen regulations (14), the USDOT regulations (18), and the Environmental Protection Agency (EPA) regulations (20); the definitions in the guidelines issued by the CDC (1–4, 7), CDC and NIH (5), and EPA (17); and discussions in the literature (6, 9, 10, 15).

3. Define precisely the types of laboratory wastes that are managed as infectious.
- **a.** Human blood and blood products
  - (1) All human blood, serum, plasma, and blood products
  - (2) Blood-contaminated tubes, microscope slides, and coverslips
  - (3) Blood-soaked bandages

II. MANAGEMENT PLAN
(continued)

b. Contaminated waste from patient care
Suction canisters, tubing, and hemodialysis wastes
c. Cultures and stocks of infectious waste
d. Other contaminated laboratory waste
(1) Specimens and containers (cups, bottles, tubes, and flasks)
(2) Culture plates and devices used to transfer, inoculate, and mix cultures (swabs, pipettes, etc.)
(3) Diagnostic-kit components
(4) PPE grossly contaminated with blood, body secretions, or cultures (gloves, masks, gowns, or coats)
e. Contaminated sharps
(1) Hypodermic needles, syringes, and scalpel blades
(2) Disposable pipettes, capillary tubes, microscope slides, coverslips, and broken glass
f. Pathology waste
(1) Body tissue, organs, and body parts
(2) Body fluids removed during surgery, autopsy, or biopsy
g. Discarded live and attenuated vaccines
h. Contaminated animal waste, including carcasses, body parts, and bedding.

B. Waste discard

1. Use distinctive containers appropriate for the type of waste.
a. Place sharp objects directly into impervious, rigid, and puncture-resistant containers and plastic bags ("red bags") for most other types of infectious waste.
b. Place glass and liquids in disposable cardboard containers and leakproof boxes or containers with secure lids.
c. Comply with the OSHA requirements for container specifications and labeling and placement of sharps containers (14).
2. Institute source separation by a user knowledgeable about the nature of the waste directly into the specific container that is designated for that particular type of waste (e.g., general trash or infectious waste).
a. Eliminates the need for subsequent sorting of the waste.
b. Avoids hazards and expense of waste sorting.
3. Segregate mixed waste (infectious and radioactive or infectious and toxic chemical waste) and direct it to the appropriate treatment procedure based on the relative severity of the hazards.

C. Waste handling and collection

1. Seal waste containers before their removal from point of use.
2. Establish schedule for emptying, removal, and replacement of waste containers.
3. Use collection carts that are appropriate for various types of waste containers.
4. Determine best times and routes for movement of waste collection carts.
5. Disinfect waste collection carts routinely.

D. Waste storage

1. Treat all infectious waste as soon as possible.
2. When necessary, store the waste until it can be treated or shipped off-site for treatment.
3. Set aside a separate storage area for infectious wastes.
4. Post biohazard signs.
5. Limit access to authorized personnel.
6. Comply with state regulations that specify storage conditions (e.g., minimum temperature and maximum duration).

II. MANAGEMENT PLAN *(continued)*

E. Waste treatment (6, 9, 10, 12, 16)

1. Select treatment technology(ies) appropriate to types and quantities of infectious waste.

2. Use traditional treatment technologies.

a. Steam sterilization.

b. Incineration

3. Use alternative treatment technologies.

a. Chemical agents (sodium hypochlorite, chlorine dioxide, or peracetic acid).

b. Microwaves

c. Dry heat

d. Radio waves

e. Infrared radiation

4. Use combination treatment technologies.

a. A mechanical process (such as shredding) before treatment to facilitate treatment

b. A mechanical process (such as compaction) after treatment to facilitate subsequent handling

c. Sharps destruction as part of the treatment process to eliminate risk of physical injury

d. Rendering pathological wastes unrecognizable prior to disposal because of aesthetic considerations

5. Comply with state regulations requiring the following.

a. That treatment equipment be tested and certified

b. That medical wastes be unrecognizable at time of disposal

F. Disposal of untreated infectious waste

Two options may be available for disposal of untreated infectious waste depending on local ordinances.

1. Landfill disposal

Meet any restrictions imposed by the locality or the landfill operator (e.g., a requirement that the waste not be recognizable as medical waste)

2. Sewer disposal

a. Prevent employee exposure to splashing and aerosols that can be generated when liquid infectious wastes are poured down the drain.

b. Meet the parameters set by the local publically owned treatment works for wastewater constituents.

G. Off-site waste treatment

1. Consider the factors listed in item I.C.

2. Properly package the waste for transport to the facility.

a. Pack plastic bags in rigid containers (e.g., plastic barrels or heavy cartons).

b. Transport in closed leakproof dumpsters or trucks.

c. Mark containers with universal biohazard symbol.

H. Contingency planning

1. Plan for treatment alternatives before the need arises.

2. Develop contingency plans that can be implemented when necessary.

3. Consider various alternatives.

a. A mutual agreement with another generator

b. Provision for ample storage space

c. Arrangements for use of a vendor's services for off-site treatment on an as-needed basis

II. MANAGEMENT PLAN
(continued)

I. Emergency response

1. Incorporate emergency response procedures in the waste management plan.

2. Specify procedures for the following.

a. Delineation of the affected area

b. Use of PPE

c. Spill cleanup

d. Decontamination of personnel, area, and equipment

e. Reporting

f. Record keeping

g. Incident analysis

J. Training

1. Comply with the OSHA blood-borne-pathogen regulations that require appropriate training of all employees who handle wastes contaminated with blood-borne pathogens and other infectious materials (14).

2. Train all personnel who generate or handle any type of infectious waste. Include in the training the following.

a. The risks of exposure to infectious materials

b. The policies and procedures that have been adopted

c. Their own duties and responsibilities for implementation of the infectious-waste management plan

K. Record keeping

1. Comply with the various federal requirements for record keeping (e.g., OSHA requirements that pertain to medical records, exposures, and training [14]).

2. Comply with the particular record keeping requirements of your state. These may include quantities of waste generated, waste treatment procedures and validation testing, and tracking of untreated waste shipped off-site.

3. Use records of waste management activities (e.g., waste quantities, costs, and a record of accident and incidents) as a database for analysis and review of the effectiveness of the waste management plan.

4. Regard record keeping as documentation of the written policies and procedures, training sessions, medical activities (vaccinations, incident follow-up procedures), etc. Use it as an invaluable resource in contesting enforcement actions by regulatory agencies and in liability suits.

L. QA and QC

1. Institute QA and QC measures. Include, for example, the following.

a. Periodic validation testing of treatment protocols

b. Policy of collecting only properly packaged waste

2. Use QA and QC to gauge implementation and to evaluate the functioning of the waste management plan.

3. Use QA and QC for identifying problems, minimizing exposures, and preventing untreated or improperly treated waste from leaving the facility as treated waste.

M. Incident and accident analysis

1. Analyze periodically the data on incidents and accidents that result in employee exposure, patterns of occurrence, problems in the waste management system, etc.

2. Modify the waste management plan accordingly.

III. IMPLEMENTATION

Observe standard precautions.

A. Incorporate the following essential elements to ensure successful implementation of the waste management plan.
  1. Enlist the involvement and support of management.
  2. Grant authority for implementing the plan to appropriate personnel.
  3. Train all personnel who handle infectious waste in any way (in waste generation, collection, or treatment) so that they understand the following.
    a. All aspects of the plan and its importance
    b. The possible consequences to themselves and to the institution of not following procedures
    c. Individual responsibilities for plan implementation

B. Implement the waste management plan.

IV. REVIEW

A. Review the infectious-waste management plan annually.

B. Examine the data to determine the following.
  1. How well the system is functioning
  2. Whether it is current relative to wastes generated in the laboratory
  3. Whether it is current relative to regulatory requirements
  4. Which areas need improvement or change

V. BENEFITS

Realize the benefits of a comprehensive infectious-waste management plan, including the following.

A. Compliance with regulatory requirements
B. Meeting of standards required for professional certification
C. Minimization of hazards and occupational exposures
D. Greater efficiency and reduced costs
E. Minimization of risk and liability

REFERENCES

1. **Centers for Disease Control.** 1987. Recommendations for prevention of HIV transmission in health-care settings. *MMWR Morb. Mortal. Wkly. Rep.* **36**(Suppl. 2S)**:**1S–18S.
2. **Centers for Disease Control.** 1988. Update: universal precautions for prevention of transmission of human immunodeficiency virus, hepatitis B virus, and other blood-borne pathogens in health-care settings. *MMWR Morb. Mortal. Wkly. Rep.* **37:**377–387.
3. **Centers for Disease Control.** 1989. Guidelines for prevention of transmission of human immunodeficiency virus and hepatitis B virus to health-care and public-safety workers. *MMWR Morb. Mortal. Wkly. Rep.* **38**(Suppl. 6)**:**1–37.
4. **Centers for Disease Control and Prevention.** 1998. Recommendations for prevention and control of hepatitis C virus (HCV) infection and HCV-related chronic disease. *MMWR Morb. Mortal. Wkly. Rep.* **47**(RR-19)**:**1–39.
5. **Centers for Disease Control and Prevention and National Institutes of Health.** February 2007, posting date. *Biosafety in Microbiological and Biomedical Laboratories*, 5th ed. U.S. Government Printing Office, Washington, DC. http://www.cdc.gov/OD/ohs/biosfty/bmbl5/bmbl5toc.htm.
6. **Denys, G. A.** 2000. Infectious waste management, p. 782–796. *In* J. Lederberg (ed.), *Encyclopedia of Microbiology*, 2nd ed., vol. 2. Academic Press, Orlando, FL.
7. **Garner, J. S.** 1996. Hospital Infection Control Practices Advisory Committee. Guidelines for isolation precautions in hospitals. *Infect. Control Hosp. Epidemiol.* **17:**54–80.
8. **Gordon, J. G., and G. A. Denys.** 1995. Minimization of waste generation in medical laboratories, p. 163–193. *In* P. A. Reinhardt, K. L. Leonard, and P. C. Ashbrook (ed.), *Pollution Prevention and Waste Minimization in Laboratories.* Lewis Publishers, Chelsea, MI.
9. **Gordon, J. G., and G. A. Denys.** 2001. Infectious wastes: efficient and effective management, p. 1139–1157. *In* S. S. Block (ed.), *Disinfection, Sterilization, and Preservation*, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
10. **Gordon, J. G., P. A. Reinhardt, and G. A. Denys.** 2004. Medical waste management, p. 1773–1785. *In* C. G. Mayhall (ed.), *Hospital Epidemiology and Infection Control*, 3rd ed. Lippincott Williams & Wilkins, Philadelphia, PA.

REFERENCES *(continued)*

11. **Joint Commission on Accreditation of Healthcare Organizations.** 1998. *Hospital Accreditation Standards: Standards, Intents.* Joint Commission on Accreditation of Healthcare Organizations, Chicago, IL.
12. **Marsik, F. J., and G. A. Denys.** 1995. Sterilization, decontamination, and disinfection procedures for the microbiology laboratory, p. 86–98. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), *Manual of Clinical Microbiology,* 6th ed. American Society for Microbiology, Washington, DC.
13. **NCCLS.** 2002. *Clinical Laboratory Waste Management.* Approved guideline GP5-A2, 2nd ed. NCCLS, Wayne, PA.
14. **Occupational Safety and Health Administration.** 1991. Occupational exposure to bloodborne pathogens:—final rule. *Fed. Regist.* **56:**64004–64182. (Also CFR Title 29, Part 1910.1030: Bloodborne pathogens.)
15. **Reinhardt, P. A., and J. G. Gordon (ed.).** 1991. *Infectious and Medical Waste Management.* Lewis Publishers, Chelsea, MI.
16. **State and Territorial Association of Alternative Treatment Technologies.** 1998. *Technical Assistance Manual: State Regulatory Oversight of Medical Waste Treatment Technologies.* Report from meeting held in New Orleans, LA, 15–16 February 1998. STAATTII.
17. **U.S. Department of Transportation.** 1991. Performance-oriented packaging standards: revisions and response to petitions for reconsideration. *Fed. Regist.* **56:**66124–66287. (Also CFR Title 49, Parts 171–180, specifically Section 173.197: Regulated medical waste; Part 178: Specifications for packaging; and Section 172.101: Hazardous materials table.)
18. **U.S. Department of Transportation.** 1996. CFR Title 49, Section 173.134(a)(4): Regulated medical waste.
19. **U.S. Environmental Protection Agency.** 1986. *EPA Guide for Infectious Waste Management.* Publication EPA/530-SW-86-014. U.S. Environmental Protection Agency, Washington, DC.
20. **U.S. Environmental Protection Agency.** 1997. CFR Title 40, Section 60.51c: Definitions, medical/infectious waste.

SECTION 16 Bioterrorism

SECTION EDITOR: *James W. Snyder*

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16.1 General Introduction to Bioterrorism

I. PRINCIPLE

Since the events of 11 September 2001 and the subsequent release of anthrax in October 2001, bioterrorism preparedness has been a major priority for the nation. As an integral member of the "first-responder" team in recognizing or suspecting an act of bioterrorism, the clinical laboratory, especially the clinical microbiology laboratory, will serve as a sentinel in the detection, recovery, characterization, and identification of the targeted biological agent(s). In preparation for responding to a biological terrorism event, the clinical microbiologist is encouraged to participate in and apply the guidelines of the Laboratory Response Network (LRN). Members of the laboratory staff should be formally trained and knowledgeable in the following areas: (i) the BSL of their laboratory; (ii) principles of specimen collection, preservation, packaging, labeling, and shipment; (iii) criteria for recognizing or suspecting a potential bioterrorist activity and the institutional chain of communication; (iv) biothreat levels as designated by the LRN; (v) diagnostic testing according to consensus protocols; (vi) timely and accurate testing and reporting; and (vii) the chain of communication linking local, state, and federal agencies. Although it is not anticipated to be a major factor, members of the microbiology staff should have an understanding of the chain-of-custody guidelines being practiced in their institution. It is vital for a laboratory to be familiar with its role in response to a suspected or confirmed bioterrorism event and to develop formal standard operations procedures (SOPs), which describe how the laboratory will function in the event of a biological incident. The SOP should be part of an institution-wide SOP that is a multidisciplinary document comprised of policies from Infection Control, Public Relations, Risk Management, Pharmacy, Security, Medical Staff, and Administration. The primary role of the clinical microbiology laboratory in responding to a bioterrorism event will be no different from its present role: to detect, recover, and characterize or identify the etiological agent(s). Of utmost importance is maintaining awareness that an event may be occurring and raising suspicion that requires further investigation. Secondary roles include maintaining an active surveillance and a continuous monitoring program. The primary focus of this section and associated procedures is to provide the clinical microbiologist with guidance and information for use in preparing for and responding to a suspected or confirmed bioterrorism event. Critical issues addressed herein include the types of bioterrorism events; laboratory capacity; laboratory safety, including the packaging and shipping of biological materials, especially infectious substances; and diagnostic testing protocols for those biological agents targeted as being most likely to be released in an event because they can be easily disseminated or transmitted person to person, cause high mortality with the potential for major public health impact, cause public panic and social disruption, and require special action for public health preparedness (R. Timperi, personal communication). These agents, currently classified as category A agents, include (i) *Bacillus anthracis,* the agent of anthrax; (ii) botulinum toxin, produced by *Clostridium botulinum;* (iii) *Brucella* spp., the agents of brucellosis; (iv) *Yersinia pestis,* the agent of plague; (v) *Francisella tularensis,* the agent of tularemia; (vi) *Burkholderia mallei* and *Burkholderia pseudomallei,* the agents of glanders and melioidosis; (vii) staphylococcal enterotoxin B, produced by *Staphylococcus aureus;* and (viii) variola virus, the agent of smallpox. However, it is important to realize that any microbial agent can potentially be used in the commission of a biocrime or act of terrorism.

Since most microbiologists have not experienced or been involved with bioterrorist activity, the following definitions are provided as background information. Terms such as "bioterrorist agents," "biothreat agents," "biocrime," and "biothreat" are used interchangeably and are defined as follows: "bioterrorist agents" or "biothreat agents" refers to microbial pathogens and/or toxins which have been considered for or used in biological warfare and recent terrorist events, "biothreat" is the suspected but unconfirmed release of a biological agent(s), and "biocrime" is a criminal act involving the use of biological agents or weapons.

The LRN was revised in response to recommended laboratory designations and to reflect expansion to include additional specialty and international (England, Canada, and Australia) laboratories. Laboratories previously designated as level A are now designated as sentinel laboratories, level B and C are designated as reference laboratories, and level D laboratories are designated as federal laboratories. It should be noted that the definition of a sentinel laboratory was recently revised as follows. Depending on the level of diagnostic testing, the LRN currently classifies a sentinel laboratory as an **advanced** or **basic** laboratory. An advanced sentinel laboratory functions at the front line and has the most analytical capability. Laboratories with less analytical capability but

which are in a position to handle specimens that may contain agents of bioterrorism or emerging infectious diseases are designated basic sentinel clinical laboratories. Specific criteria used to classify a laboratory as advanced or basic are as follows.

A. Advanced sentinel laboratory

1. Is certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA) by the Centers for Medicare & Medicaid Services (CMS) for the applicable subspecialty within the specialty of microbiology and meets the requirements to perform high-complexity testing
2. Is inspected successfully by CMS, a CMS agent, a CMS-approved accreditation organization, or, for a CLIA-exempt laboratory, by that laboratory's state
3. Has policies and procedures for direct referral of suspicious specimens or isolates to the nearest LRN reference laboratory in its jurisdiction
4. Has a class II or higher certified biological safety cabinet
5. Complies with BSL 2 practices, *and*
6. Has policies and procedures in place for use of additional respiratory protection, including a definition of when such use is necessary, as well as documentation of safe use (e.g., N-95 fit-testing)
7. Has policies and procedures that include the LRN sentinel-level clinical microbiology laboratory guidelines that are available and can be downloaded from the American Society for Microbiology (ASM) website (http://www.asm.org/Policy/index.asp?bid = 6342)
8. All personnel have been trained, with demonstrated competency, and are fully aware of the details contained within each LRN sentinel-level clinical microbiology laboratory guideline.
9. Personnel have been trained and certified in packing and shipping of infectious substances guidelines.
10. Has procedures to track and account for decontamination of laboratory biological waste (specimens and cultures). At a minimum, the laboratory ensures that any contract or procedure for waste disposal is available for inspection in the laboratory safety or waste disposal manual.

It is further highly recommended that advanced sentinel laboratories comply with the following:

11. The microbiology laboratory operates under negative pressure as recommended by the American Institute of Architects (AIA) *Guidelines for Construction of Healthcare Facilities* (1). If a microbiology laboratory is planning to remodel or construct a new facility, it should be designed to operate under negative air pressure as recommended by the AIA. This only applies to new construction or remodeling.
12. There is on-site terminal decontamination capability, e.g., autoclaving, for disposing of wastes categorized as BSL 3 or select agent.

B. Basic sentinel clinical laboratory

1. The laboratory is certified under CLIA by the CMS for the applicable subspecialty within the specialty of microbiology.
2. The laboratory is inspected successfully by CMS, a CMS agent, a CMS-approved accreditation organization, or, for a CLIA-exempt laboratory, by that laboratory's state.
3. The laboratory has policies and procedures for referral of diagnostic specimens to an advanced sentinel or reference laboratory.
4. The laboratory has policies and procedures for direct referral of suspicious specimens or isolates to the nearest LRN reference laboratory in its jurisdiction.

The above designations and criteria for the respective basic and advanced sentinel laboratory were proposed by the ASM Professional Affairs Sentinel Level Guidelines Subcommittee and endorsed by the CDC and the Association of Public Health Laboratories (APHL); they became effective in October 2006. Each state health laboratory is responsible for designating a sentinel laboratory as either advanced or basic. The respective roles and responsibilities of these laboratories have not changed, i.e., "rule out and refer" (sentinel) and "rule in and confirm" (reference). The LRN has been expanded to include chemical, veterinary, water, radiological, environmental, and selected military laboratories (3, 6–8). Please note these revisions when reviewing this section.

II. TYPES OF BIOTERRORISM EVENTS

Bioterrorism events are classified as either overt or covert (3). In an overt event, the agent is announced. For example, the numerous reported hoaxes that have occurred since 1998 have involved letters or environmental samples in which it was stated that "you have just been exposed to anthrax." The letters that were sent to two senators and Tom Brokaw of NBC News during the 2001 anthrax outbreak were overt in nature. A covert event, which is most likely to occur, is unannounced and is much more of a challenge to detect. The initial case of anthrax reported from

II. TYPES OF BIOTERRORISM EVENTS *(continued)*

Florida represented a covert form of biocrime. Detection of a covert event is similar in many ways to the daily examination and interpretation of cultures in which the microbiologist attempts to determine if a pathogen is present in addition to the nature or type of pathogen. Therefore, the focus of preparation in the clinical microbiology laboratory should be on the recognition of the covert form of bioterrorism. Dissemination of a biological agent as an aerosol or through contamination of water, food supplies, and, most recently, the mail will not have an immediate impact because of the delay between exposure and the onset of illness (the incubation period [Table 16.1–1]). As a result, physicians and health care workers, including the staff of the microbiology laboratory, will be the first to identify patients or to suspect a bioterrorism-related event. Epidemiological clues should be used to assess whether a patient's presentation is typical of an endemic disease or is an unusual event that should raise concern. Laboratory and epidemiological features that should alert microbiologists and other health care providers to the possibility of a bioterrorism-related outbreak include the following.

A. A rapidly increasing disease incidence (e.g., within hours or days) in an otherwise healthy population
B. An epidemic curve that rises and falls during a short period
C. An unusual increase in the number of people seeking care, especially with fever or respiratory or gastrointestinal complaints
D. An endemic disease rapidly emerging at an uncharacteristic time or in an unusual pattern
E. Any patient presenting with a disease that is relatively uncommon and has bioterrorism potential (e.g., pulmonary anthrax, tularemia, or plague) who is without any risk factors for the disease (e.g., travel to the Southwest for plague)
F. A large number of rapidly fatal cases
G. An increase in the number of specimen types submitted for microbiological analysis (e.g., respiratory specimens and blood cultures)
H. Higher-than-normal clustering of a specific organism
I. Recovery of an unusual or rarely encountered microorganism
J. A higher-than-normal rate of patient admission to the emergency room or hospital
K. Receipt of information by the microbiology laboratory that a clinician suspects a specific agent (e.g., "I suspect tularemia in this patient")

Table 16.1–1 Incubation periods of select agents

Disease	Time to disease onset	
	Usual	Range
Anthrax	48 h	Hours to days
Botulism	18–36 h	Hours to days
Brucellosis	5–60 days	Several months
Plague	2–4 days	1–7 days
Tularemia	3–5 days	1–14 days
Glanders	7–14 days	1–14 days
Melioidosis	2–5 days	1–14 days
Q fever	2–3 wk	1–20 yr

III. LRN

In response to the threat of bioterrorism, a three-tiered LRN was established by the CDC in partnership with the APHL and Federal Bureau of Investigation (FBI) for the purpose of providing a network of public and private laboratories that can detect and identify biological agents and communicate information to the appropriate individuals or agency in time to treat cases and prevent the spread of disease, guide public health and safety interventions, and support planning and decision making for national preparedness and response to bioterrorism, as well as other epidemic and outbreak events that may occur, e.g., pandemic influenza or suspected Ebola virus infections (2–4). It has been designed in an effort to link all clinical laboratories to public health agencies in all states, districts, and territories with access to state-of-the-art facilities for the analysis of suspected biological agents. The LRN defines three laboratory levels (Fig. 16.1–1). As the front line in a bioterrorism event, the LRN must provide (i) early detection and identification of an event; (ii) accurate, credible, and timely test results; and (iii) communication of data and information through secure electronic reporting systems (2).

Sentinel laboratories include smaller public health laboratories and most private laboratories (in hospitals, clinics, and physicians' offices, as well as commercial laboratories) with low-level biosafety facilities (BSL 2). These laboratories will use clinical data and conventional microbiological tests to decide which specimens and isolates should be forwarded to higher-level biocontainment laboratories. Sentinel laboratory personnel will be trained in the safe collection, packaging, labeling, and shipping of samples that may contain dangerous pathogens (2). Although considerable expertise in microbiological analysis and diagnostic testing is available in the private sector, the LRN was also designed to minimize the hazards associated with the handling of biological agents and the realization that most clinical laboratories are limited to a containment capacity of BSL 2. In addition, with the emphasis on safety, a major objective of the LRN is to limit complex and sophisticated diagnostic testing of suspected agents, as well as access to reagents, QC strains,

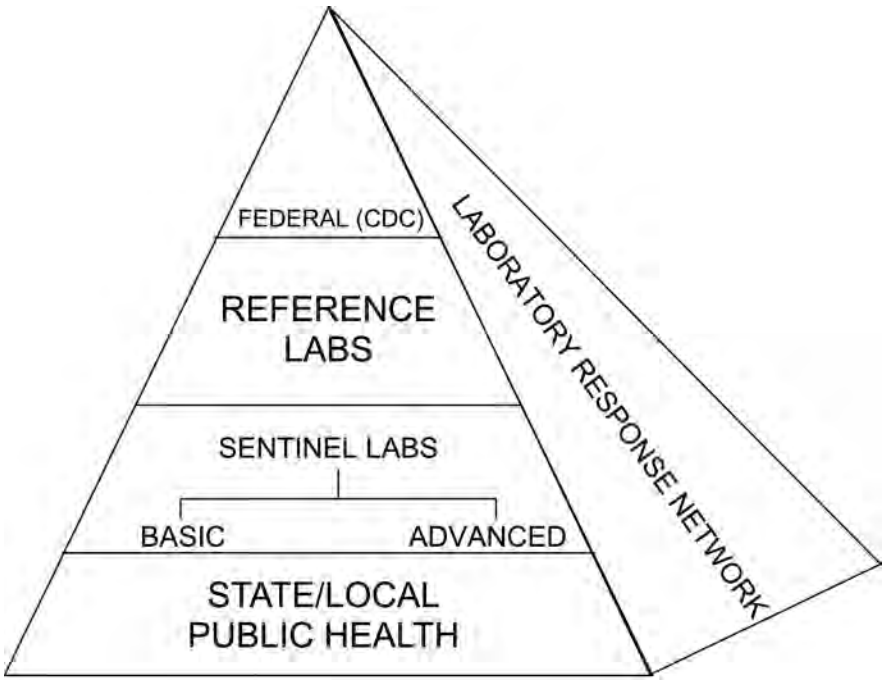

Figure 16.1–1 LRN.

III. LRN *(continued)*

and proficiency testing, to a few highly specialized laboratories. By restricting diagnostic testing to a small number of selected laboratories, security measures can be more easily controlled, and thus the access of potential terrorists or known enemies to information can be limited. If a sentinel laboratory wishes to function as a reference level laboratory, it must make application to the state health department and specify which agent(s) it wishes to attain reference status for testing. Furthermore, the applying laboratory must meet the physical requirements of a BSL 3 containment facility. The primary responsibility of a sentinel laboratory is to rule out and refer the suspected agent to the nearest LRN reference laboratory (usually a state health laboratory) or federal level laboratory, depending on the suspected agent. A major responsibility of sentinel laboratories is to raise suspicion that something unusual is occurring based on the recommended clues listed above. It is not the responsibility of the sentinel laboratory to confirm or announce that a bioterrorist event has taken place. *The laboratory should notify the institutional infection control or epidemiology personnel, who are responsible for assessing the situation and notifying local or state public health officials, who will make the final decision and notify federal officials.* Laboratory personnel should be familiar with the morphological, cultural, and identification characteristics of those agents that have been targeted for dispersal in a bioterrorism event, in addition to the clinical syndromes they produce. Standardized, consensus-based testing protocols that utilize conventional microbiological tests have been developed through a partnership consisting of the ASM, the CDC, and the APHL. The protocols have been designed to facilitate the ruling out of a potential or suspected agent prior to shipping a specimen to the next-higher level. The diagnostic procedures described in the preceding sections of this handbook represent the current sentinel-level testing protocols that can be accessed from the ASM website (http://www.asm.org). Access to testing protocols utilized by the respective reference and federal laboratories is restricted to designated public health laboratories and requires an assigned password issued by the CDC before admission to the CDC secure website can be obtained.

Reference-level laboratories are primarily state and local public laboratories that can test for specific agents and forward organisms or specimens to higher-level biocontainment laboratories. Reference laboratories will minimize false-positive results and protect federal laboratories from overload. Ultimately, reference laboratories will maintain the capacity to perform confirmatory and susceptibility testing. In addition, selected reference laboratories have the capability to perform toxicity testing (botulinum toxin) and employ advanced diagnostic technologies (e.g., nucleic acid amplification and molecular fingerprinting). Additionally, reference laboratories are responsible for participating in the evaluation and validation of new tests and reagents.

Federal laboratories have the highest level of containment (BSL 4) and expertise in the diagnosis of rare and dangerous biological agents. These are specialized laboratories with unique experience in the diagnosis of rare diseases (e.g., smallpox and Ebola virus disease). A federal laboratory will develop or evaluate new tests, reagents, and methods; archive organism isolates for future characterization and comparisons; identify chimeras (recombinant agents); detect genetically engineered agents; and perform advanced functions, such as identification of organisms deemed too hazardous for sentinel and reference laboratories.

If you are uncertain or have any questions regarding your laboratory's capacity or the disposition of samples and/or cultures suspected of being potential biological agents, contact your local or state health department for consultation and guidance.

IV. CHAIN-OF-CUSTODY GUIDELINES

Following confirmation by the FBI, a biocrime or act of bioterrorism is regarded as a criminal offense. The clinical microbiology laboratory may be required to save specimens or isolates suspected of being linked to or associated with the crime under investigation and thus must preserve what is regarded as evidence. Protection of evidence must adhere to the security process known as the chain of custody. Although sentinel laboratories will not handle or analyze environmental samples (e.g., soil, bone, hair, surface swabs, water, food, or letters) that are collected in an overt event in which chain of custody is required, it is important for the microbiology staff to be familiar with the process. Unless otherwise specified by the FBI, which is the ultimate authority, all environmental specimens should be transported directly to a reference laboratory. If the laboratory is called upon to handle an environmental sample, all activities, beginning with the receipt and examination of the sample, must be documented using a chain-of-custody form (Fig. 16.1–2). The key elements of this form include (i) the document number assigned and the date that the evidence was received, (ii) the specimen identification number, (iii) a brief description of the evidence (e.g., specimen type and biological agent), (iv) the date of final disposition, (v) signatures of all personnel who handled and examined the evidence, and (vi) remarks.

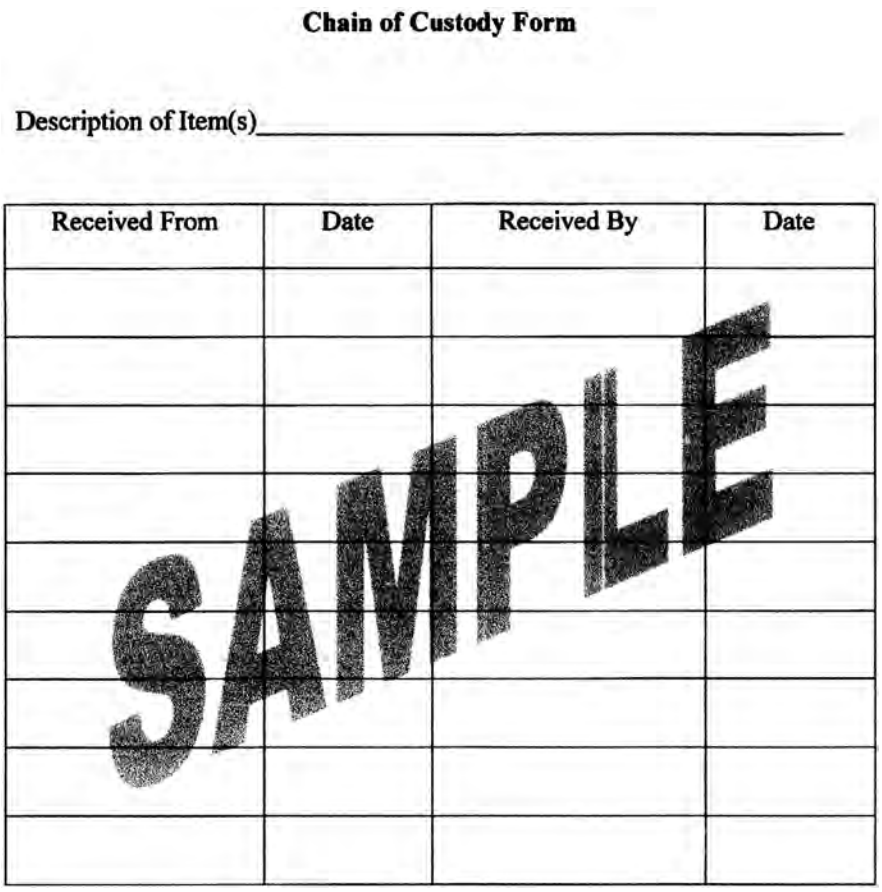

Figure 16.1–2 Chain-of-custody form.

V. RECOMMENDATIONS FOR THE SENTINEL LABORATORY

The following recommendations are provided as a checklist for use by the sentinel laboratory.

A. Refer all environmental samples to a reference laboratory (local or state health department).

B. Refer specimens suspected of containing botulinum toxin directly to the local or state health department. Prior notification and consultation with representatives at the local or state health department regarding disposition of the sample are advised.

C. Refer specimens suspected of containing the agent of smallpox to the CDC. Prior notification and consultation with the CDC are recommended before shipment of the specimen or tissue culture. The respective state and local health departments should be advised of the communication with the CDC.

D. Maintain an active system of surveillance and monitoring of microorganisms recovered in the laboratory—establish a baseline incidence for each organism.

E. Establish a line of communication within your institution for reporting suspicious, unusual, or known organisms encountered at higher-than-normal frequency. Microbiology laboratory personnel should notify Infection Control directly of such incidences; Infection Control is responsible for notifying local or state health department officials.

F. Establish a line of communication with other local and/or regional laboratories.

G. Develop and maintain a bioterrorism SOP. A model template can be found in procedure 16.13 of this handbook. Elements for inclusion in the SOP include (i) sentinel testing protocols, (ii) policies (e.g., chain of communication, internal contacts, surveillance processes, personnel safety, and current packaging and shipping guidelines), (iii) current literature references, and (iv) documentation of staff attendance at and participation in continuing-education activities.

H. Assess the BSL capacity of the laboratory.

I. Schedule periodic inservices and updates for the laboratory staff; participate in tabletop exercises and local mock exercises to evaluate laboratory preparedness.

J. Recognize that laboratory security is related to but different from laboratory safety (5).

K. The laboratory does not confirm or announce the occurrence of an act of bioterrorism; it only raises suspicion.

◩ **NOTE:** The U.S. government has deemed the agents discussed in this section to be select agents of bioterrorism. Select agents are microorganisms, biological agents, or biological toxins that have been deemed by the U.S. government to be major threats to public health and safety because they have been or could be used as agents of bioterrorism. If a select agent, specimen, or item suspected of containing a select agent must be shipped or transported from one facility to another, (i) both the shipper and the consignee must contact the appropriate state and federal authorities for guidance, instructions, and permission before such transfer occurs, and (ii) the shipper must confirm that the recipient is approved for receiving select agents.

REFERENCES

1. **American Institute of Architects.** 2001. *Guidelines for Construction of Healthcare Facilities.* American Institute of Architects, Washington, DC.
2. **Centers for Disease Control and Prevention.** 2000. Biological and chemical terrorism: strategic plan for preparedness and response. *MMWR Morb. Mortal. Wkly. Rep.* **49:**1–14.
3. **Gilchrist, M. J. R., W. P. McKinney, J. M. Miller, and A. S. Weissfeld.** 2000. *Cumitech 33, Laboratory Safety, Management, and Diagnosis of Biological Agents Associated with Bioterrorism.* Coordinating ed., J. W. Snyder. ASM Press, Washington, DC.

REFERENCES *(continued)*

4. **Klietmann, W. F., and K. L. Ruoff.** 2001. Bioterrorism: implications for the clinical microbiologist. *Clin. Microbiol. Rev.* **14:**364–381.
5. **Morse, S. A.** 2001. Bioterrorism: laboratory security. *Lab. Med.* **32:**303–306.
6. **Nauschuetz, W. F.** 2005. Straight talk on bioterror from the Army's LRN Gatekeeper. *MLO* **37:**10–16.
7. **Snyder, J. W.** 2003. Role of the hospital-based microbiology laboratory in preparation for and response to bioterrorism. *J. Clin. Microbiol.* **41:**1–4.
8. **Snyder, J. W.** 2005. The Laboratory Response Network: before, during, and after the 2001 anthrax incident. *Clin. Microbiol. Newsl.* **27:**171–175.

SUPPLEMENTAL READING

American Society for Microbiology. http://www.asm.org.

Centers for Disease Control and Prevention. http://www.bt.cdc.gov.

Federal Register. Part IV, Department of Transportation, 49 CFR Part 172, Hazardous Materials: Security Requirements for Offerors and Transporters of Hazardous Materials; Final Rule. http://www.gpoAccess.gov/FR/

Johns Hopkins University Center for Civilian Biodefense Studies. http://www.hopkins-biodefense.org.

U.S. Department of Health and Human Services. 2007. *Biosafety in Microbiological and Biomedical Laboratories*, 5th ed. U.S. Government Printing Office, Washington, DC.

16.2 Levels of Laboratory Safety

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

The importance of laboratory safety must be emphasized. Laboratory workers have always been shown to be at risk for laboratory-acquired infections. Such infections include typhoid fever; Q fever; cholera; glanders; brucellosis; tetanus; tuberculosis; tularemia; shigellosis; salmonellosis; infections caused by streptococci, *Chlamydia* spp., and *Neisseria meningitidis;* and viral infections, such as those caused by the hepatitis viruses, arboviruses, and many others (1, 2, 4, 6–13). Interestingly, these infections have occurred in association with documented laboratory accidents in only 16% of the cases (13), of which 9.5% resulted in death (12, 13). No national monitoring of laboratory-associated infections exists, but one estimate derived from recent surveys suggests a rate of one to five infections per 1,000 employees (5, 10, 14). Harding and Byers have reviewed laboratory-associated infections recently (3).

◩ **NOTE:** Current information reinforces the fact that a safer work environment requires strict adherence to the principles of biological safety (15). These principles rely on a combination of microbiological practices, laboratory facilities, and safety equipment which is designed to minimize exposure of laboratory workers, their contacts, and the outside environment to potentially hazardous organisms. The use of these principles can be customized for each laboratory according to its specific needs.

II. GOOD LABORATORY PRACTICES

Observe standard precautions.

It is imperative that these cultures be handled in a biological safety cabinet.

All specimens containing or suspected of containing microorganisms are regarded as potentially harmful. Contact with any biological specimen must occur under controlled conditions that emphasize protection of the laboratorian. The use of standard precautions will protect the laboratory worker when handling clinical specimens. Good laboratory practice includes strict adherence to sound microbiological techniques, including containment procedures. Persons working in a laboratory environment should be given instruction in the safe handling of biological agents, be required to follow standard operating procedures, and be aware of the potential danger associated with these agents. Good laboratory practice also includes the proper use of primary and secondary barriers within the laboratory. Primary barriers include biological safety cabinets (BSCs), enclosed centrifuge cups and carriers, and PPE (i.e., gloves, safety glasses or goggles, face shields, masks, gowns, and shoe covers). Secondary barriers include factors related to facility design and construction, which consist of the location of the work area in relation to public access and the availability of an autoclave, eyewash and hand-washing facilities, specialized ventilation systems, and controlled-access zones.

III. BSLs

The combination of safe laboratory practices and the use of various primary and secondary barriers constitutes the four levels of biosafety used in the laboratory environment (6, 15). BSLs are agent dependent, are designed for the specific procedures performed by the laboratory, and take into consideration the various routes of transmission of the infective agents.

A bioterrorism act may be either covert (unannounced) or overt (announced). A covert event may be first recognized in the clinical microbiology laboratory following the isolation of an organism that is not usually found in the geographical area (e.g., *Brucella* or *Francisella* in the southeastern United States) or when testing of a *Bacillus* organism isolated from a blood culture (normally regarded as a contaminant) shows that is it neither motile nor hemolytic, and thus is a possible *Bacillus anthracis* isolate. In the case of an overt, announced bioterrorism event, sentinel laboratories should be prepared to send specimens to a higher-level laboratory, such as a state facility.

A. BSL 1

BSL 1 is used for undergraduate and secondary educational training and teaching laboratories (Table 16.2–1). It represents a basic level of containment that relies on standard microbiological practices (as defined in Table 16.2–1) with no special need for primary or secondary barriers other than a sink for hand washing. Representative organisms that can be safely handled in a BSL 1 laboratory are *Bacillus subtilis, Naegleria gruberi,* and infectious canine hepatitis virus.

B. BSL 2

BSL 2 applies to most clinical diagnostic, teaching, research, and commercial laboratories that handle a wide variety of indigenous, moderate-risk microorganisms usually present in the community and associated with human diseases of various severities (Table 16.2–2), depending on the health and immune status of the individuals in the population. BSL 2 is appropriate for working with human body fluids where the presence of a potentially infectious agent is unknown. The activities at this level can be performed on the open bench without special primary or secondary barriers other than a sink for hand washing and waste decontamination facilities. At this level, overt splashing and aerosol for-

Table 16.2–1 Summary of BSL 1

Agent	Standard microbiological practices	Special practices	Primary barriers	Secondary barriers
Not known to cause disease in healthy individuals	Limit access to laboratory. Wash hands. Prohibit eating, drinking, smoking, applying cosmetics, or storing food. Prohibit mouth pipetting. Establish policies for safe handling of sharps. Minimize creation of splashes and aerosols. Decontaminate work surfaces daily and after spills. Waste is decontaminated or transported in durable, leakproof, closed containers to decontamination site.	None	Laboratory coats, gowns, or uniforms recommended. Wear gloves if hands are dry, chapped, or cut. Use protective eye wear if splashing is anticipated.	Doors are used for access control. Sinks are used for hand washing. Easily cleaned equipment and impervious bench tops are used. Windows are screened.

Table 16.2–2 Summary of BSL 2

Agents	Standard microbiological practices	Special practices	Primary barriers	Secondary barriers
Known to be associated with human disease	BSL 1 plus rodent and insect control program	Personnel are advised of potential hazards; must follow standard operating procedures. Biohazard sign is posted at laboratory entrance. Personnel have received appropriate immunizations. Baseline sera may be collected. Personnel are knowledgeable about biosafety manual. Personnel receive annual updates and training for hazards. High degree of precaution is used when handling sharps. All specimens are in leakproof containers for collection, handling, processing, storage, and transport. Spills and accidents are reported, and medical evaluation is given.	BSL 1 plus: BSC I or II and PPE used when creating aerosols or handling high concentrations and/or volumes of agents PPE removed prior to leaving the laboratory	BSL 1 plus: Location away from public areas Eyewash station readily available Autoclave available

III. BSLs *(continued)*

mation should be kept to a minimum. If splashing or aerosol formation is likely when working with a specimen or organisms in a BSL 2 laboratory, such procedures should be conducted in a class I or II BSC. BSCs are primary containment devices used in laboratories working with infectious agents. When used in conjunction with good microbiological practices, BSCs provide an effective containment system for the safe manipulation of moderate- and high-risk microorganisms. Safety centrifuge cups may be used if deemed necessary when centrifuging specimens or organisms. Other primary barriers that should be available for use if necessary include goggles, face masks and shields, gloves, and gowns. Specimens suspected of containing *B. anthracis*, *Francisella tularensis*, *Yersinia pestis*, and *Brucella* spp. can be safely processed under BSL 2 conditions. In addition, hepatitis B virus, human immunodeficiency virus, and *Salmonella* and *Toxoplasma* spp. are suitable for BSL 2 practices. The handling and manipulation of mould cultures, including *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Aspergillus* spp., should be performed at a minimum under BSL 2 conditions, although BSL 3 is preferred and recommended.

C. BSL 3

BSL 3 is followed by clinical diagnostic, teaching, research, or production facilities where procedures are performed with agents commonly transmitted by the respiratory route and known to cause significant morbidity and mortality (Table 16.2–3). All procedures at BSL 3 must be conducted in a BSC class I or II or other enclosed equipment fitted with HEPA particulate filters. Other mandatory primary barriers include goggles, particulate respirator masks, face shields, gloves, and gowns. Secondary barriers must include controlled access to the laboratory and a ventilation system that keeps potentially contaminated air from being released from the facility. Examples of cultured organisms that can be safely worked with at this level are *B. anthracis*, *F. tularensis*, *Y. pestis*, *Brucella* spp., *Mycobacterium tuberculosis*, *Coxiella burnetii*, and St. Louis encephalitis virus. This level is also utilized when manipulating moulds, espe-

Table 16.2–3 Summary of BSL 3

Agent	Standard microbiological practices	Special practices	Primary barriers	Secondary barriers
Associated with aerosol transmission to humans where disease may have significant morbidity and mortality	BSL 2	BSL 2 plus: Doors are closed when working. Persons at increased risk for acquiring infections are not allowed. No minors are allowed. Personnel are tested for exposure. Personnel must demonstrate technical proficiency. No procedures are conducted on open bench. Containment equipment must be decontaminated before removal. Decontamination of all waste is necessary. Clothing must be decontaminated before laundering or disposable laboratory coats that are autoclaved must be worn.	BSL 2 plus: Solid-front or wrap gowns, scrub suits or coveralls, and gloves are worn by personnel. All procedures must be performed in a BSC I or II. Respirators and face shields must be worn. Centrifuge safety cups or sealed containers must be used.	BSL 2 plus: Separation from public corridors is required. Passage through two lockable doors is required for entry. Walls, floors, ceilings are constructed for easy cleaning and decontamination. Windows are closed and sealed. Decontamination facilities are available in laboratory or institution. Ventilation system is used to create directional airflow from clean to contaminated areas. Exhaust air is not recirculated. Exhaust air is HEPA filtered or dispersed away from occupied areas. BSCs are inspected and certified at least annually. If BSC III are used, they should be directly connected to exhaust system. Facilities should be initially verified and reverified at least annually for proper functioning.

III. BSLs *(continued)*

cially the agents of systemic mycosis (e.g., *H. capsulatum, B. dermatitidis,* and *C. immitis*).

D. BSL 4

BSL 4 is used by clinical diagnostic, research, or production facilities where procedures are performed with agents that are commonly transmitted by the respiratory route and pose a high risk of life-threatening disease for which there is no vaccination or therapy (Table 16.2–4). All procedures at BSL 4 must be performed in a manner that provides complete isolation of the laboratory worker from aerosolized infectious agents. All procedures must be conducted in a BSC class III or in a full-body, air-supplied positive-pressurized suit. The BSL 4 laboratory should be located in a separate building or a remote isolated zone within a facility. This level also requires specialized secondary barriers for air ventilation and waste decontamination. Examples of agents that require handling in a BSL 4 environment include Ebola, Marburg, and Crimean-Congo hemorrhagic fever viruses.

Table 16.2–4 Summary of BSL 4

Agents	Standard microbiological practices	Special practices	Primary barriers	Secondary barriers
High risk of life-threatening disease by aerosol transmission to humans or unknown risk of human disease	BSL 3	BSL 3 plus: Logbook is signed upon entry into and exit from facility. Biohazard sign is posted listing agent(s), laboratory director, and special requirements for entry. Personnel enter and exit through clothing-changing and shower rooms, showering upon exit. Airlocks are used to enter and exit laboratory. Clothing is removed in changing room and left; only laboratory clothing is used for entry into laboratory; upon exit, laboratory clothing is left in inner changing room; soiled clothing is autoclaved before being laundered. Supplies enter through air locks, autoclaves, or fumigation chambers and are decontaminated after each use. Biological material or waste is removed through disinfectant dunk tanks, fumigation chambers, or airlocks. All materials are decontaminated prior to removal and disposal. Spills are handled by professional staff trained and equipped to work with concentrated infectious material. Laboratory accidents and absenteeism for medical surveillance are reported.	BSL 3 plus: All procedures done in class III BSC or class I or II BSC when used with one-piece positive-pressure suits ventilated with a life support system	BSL 3 plus: Location in a separate building or isolated zone Daily inspection of life support systems Sewer vents and service lines fitted with HEPA filters Hands-free or automatic hand-washing sink Self-closing access doors Break-resistant, sealed windows Double-door autoclaves Dedicated nonrecirculating ventilation system Cabinet air passed through HEPA filters HEPA filters tested and certified at least annually Communications system between laboratory and outside

REFERENCES

1. **Centers for Disease Control and Prevention.** 2000. Laboratory-acquired human glanders—Maryland. *MMWR Morb. Mortal. Wkly. Rep.* **49:**532–535.
2. **Hanson, R. P., S. E. Sulkin, E. I. Buescher, W. M. Hammon, R. W. McKinney, and T. H. Work.** 1967. Arbovirus infections of laboratory workers. *Science* **158:**1283–1286.
3. **Harding, A. L., and K. B. Byers.** 2000. Epidemiology of laboratory-associated infections, p. 35–54. *In* D. O. Fleming and D. L. Hint (ed.), *Biological Safety Principles and Practices*, 3rd ed. ASM Press, Washington, DC.
4. **Harrington, J. M., and H. S. Shannon.** 1976. Incidence of tuberculosis, hepatitis, brucellosis and shigellosis in British medical laboratory workers. *Br. Med. J.* **1:**759–762.
5. **Jacobson, J. T., R. B. Orlob, and J. L. Clayton.** 1985. Infections acquired in clinical laboratories in Utah. *J. Clin. Microbiol.* **21:**486–489.
6. **Jamison, R., M. A. Noble, E. M. Proctor, and J. A. Smith.** 1996. *Cumitech 29, Laboratory Safety in Clinical Microbiology.* Coordinating ed., J. A. Smith. ASM Press, Washington, DC.
7. **Meyer, K. F., and B. Eddie.** 1941. Laboratory infections due to Brucella. *J. Infect. Dis.* **68:**24–32.
8. **Pike, R. M.** 1976. Laboratory-associated infections: summary and analysis of 3921 cases. *Health Lab. Sci.* **13:**105–114.
9. **Pike, R. M., S. E. Sulkin, and M. L. Schulza.** 1965. Continuing importance of laboratory-acquired infections. *Am. J. Public Health* **55:**190–199.
10. **Sewell, D. L.** Laboratory-acquired infections. 2000. *Clin. Microbiol. Newsl.* **22:**73–77.
11. **Skinholj, P.** 1974. Occupational risks in Danish clinical chemical laboratories. II. Infections. *Scand. J. Clin. Lab. Investig.* **33:**27–29.

REFERENCES *(continued)*

12. **Sulkin, S. E., and R. M. Pike.** 1949. Viral infections contracted in the laboratory. *N. Engl. J. Med.* **241:**205–213.
13. **Sulkin, S. E., and R. M. Pike.** 1951. Survey of laboratory-acquired infections. *Am. J. Public Health* **41:**769–781.
14. **Wilson, M. L., and L. B. Reller.** 1998. Clinical laboratory-acquired infections, p. 343–355. *In* J. V. Bennett and P. S. Brachman (ed.), *Hospital Infections*, 4th ed. Lippincott-Raven, Philadelphia, PA.
15. **U.S. Department of Health and Human Services.** 2007. *Biosafety in Microbiological and Biomedical Laboratories*, 5th ed. U.S. Government Printing Office, Washington, DC.

SUPPLEMENTAL READING

Centers for Disease Control and Prevention. 2000. Biological and chemical terrorism: strategic plan for preparedness and response. *MMWR Morb. Mortal. Wkly. Rep.* **49:**1–14.

Gilchrist, M. J. R., W. P. McKinney, J. M. Miller, and A. S. Weissfeld. 2000. *Cumitech 33, Laboratory Safety, Management, and Diagnosis of Agents Associated with Bioterrorism.* Coordinating ed., J. W. Snyder. ASM Press, Washington, DC.

Klietman, W. F., and K. L. Ruoff. 2001. Bioterrorism: implications for the clinical microbiologist. *Clin. Microbiol. Rev.* **14:**364–381.

16.3 Packing and Shipping Select Agents

I. PRINCIPLE

The U.S. Department of Health and Human Services regulates and the CDC oversees the *possession, use,* and *transfer* of certain specifically listed biological agents and toxins (called "select agents") that have the potential to pose a severe threat to public health and safety. The Select Agent Program (http://www.cdc.gov/od/sap/) oversees all activities with select agents and registers all U.S. laboratories, persons, and other entities that possess, use, and/or transfer select agents.

II. SELECT AGENTS

Select agents are microorganisms, biological agents, or biological toxins that have been deemed by the U.S. government to be major threats to public health and safety because they could be used as agents of bioterrorism. Select agents are listed in item IV.

III. PACKING AND SHIPPING SELECT AGENTS

See procedure 15.6 of this handbook for complete details of packing and shipping infectious substances in general.

A select agent may be packaged and prepared for shipment as any other infectious substance is packed according to the guidelines in this document (procedure 15.6); i.e., packaging for select agents is the same as that for routine infectious substances: leakproof primary and secondary containers, absorbent, a sturdy and well-labeled outer container, shipper's declaration for category A substances, etc. However, the requirements regarding the *possession, use,* and *transfer* of select agents are intricate, strict, and aggressively enforced.

If a select agent, specimen containing a select agent, or a substance suspected of containing a select agent is intended to be shipped, both the sender and the recipient must (read, it is imperative to) (i) contact the appropriate federal authorities for guidance, instructions, and permission to ship or receive the select agent *before* such transfer occurs and (ii) apply for and receive a site registration number from the CDC *before* the transfer occurs. In addition, the shipper must confirm that the recipient is approved for receiving select agents. The aforementioned CDC website provides complete information about select agents, the strict regulations related to transferring select agents, possession and transfer application forms, and additional resources.

IV. LIST OF SELECT AGENTS SPECIFICALLY DESIGNATED BY THE U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES

A. Bacteria

1. *Bacillus anthracis*
2. *Brucella abortus*
3. *Brucella melitensis*
4. *Brucella suis*
5. *Burkholderia mallei*
6. *Burkholderia pseudomallei*
7. *Francisella tularensis*
8. Neurotoxin-producing strains of *Clostridium botulinum*
9. *Yersinia pestis*

B. Viruses

1. Eastern, western, and Venezuelan equine encephalitis viruses
2. Ebola virus
3. Handra virus
4. Hemorrhagic fever viruses (Crimean-Congo, Junin, Machupo, Sabia, Flexal, and Guanarito)
5. Herpes B virus
6. Lassa fever virus
7. Marburg virus
8. Monkeypox virus
9. Nipah virus
10. Reconstructed replication-competent forms of the 1918 pandemic influenza H1N1 virus
11. Rift Valley fever virus
12. Smallpox virus
13. Tick-borne encephalitis flaviviruses

C. Rickettsia

1. *Coxiella burnetii*
2. *Rickettsia prowazekii*
3. *Rickettsia rickettsii*

D. Fungi

1. *Coccidioides immitis*
2. *Coccidioides posadasii*

E. Toxins

1. Aflatoxins
2. *Clostridium botulinum* neurotoxin
3. Ricin
4. Shiga toxin
5. *Staphylococcus* enterotoxin
6. Others

SUPPLEMENTAL READING

Department of Health and Human Services. 2005. Possession, use, and transfer of select agents and toxins. 42 CFR Parts 72 and 73. *Fed. Regist.* **70:**13294–13325.

Department of Health and Human Services. 2008. Possession, use, and transfer of select agents and toxins. 42 CFR Parts 72 and 73. *Fed. Regist.* **70:**61363–61366.

International Air Transport Association. 2009. *Dangerous Goods Regulations*, 50th ed. International Air Transport Association, Montreal, Canada.

16.4 Anthrax—*Bacillus anthracis*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Bacillus anthracis, the etiologic agent of anthrax, is classified as a category A agent because of its suitability for and likelihood of use in an attack or biocrime. Disease occurs most frequently in herbivorous animals (e.g., cattle, sheep, and goats), which acquire the endospores from contaminated soil. Human disease is less common and results from contact with infected animals or with commercial products derived from them, such as wool and hides. Infection can occur in one of three forms, depending on the route of acquisition. (i) Cutaneous anthrax, responsible for >95% of naturally occurring cases, is initiated when spores of *B. anthracis* are introduced into the skin through cuts or abrasions, such as when handling contaminated wool, hides, leather, or hair products (especially goat hair) from infected animals (11, 19). There are a few case reports of transmission by insect bites, presumably after the insect fed on an infected carcass (1, 16, 17). This form is rarely fatal following appropriate antimicrobial therapy. (ii) Gastrointestinal anthrax may occur 1 to 7 days following the consumption of contaminated undercooked meat from infected animals. Pharyngeal lesions may also occur from ingestion of contaminated food. Mortality in both forms is high (16). (iii) Inhalation anthrax results from the inhalation of *B. anthracis* spores. Though treatable in its early prodromal stage, mortality remains extremely high if antimicrobial treatment is not initiated within 48 h of the onset of symptoms (20). A single case of inhalation anthrax should alert all health care workers to the possibility of a bioterrorism event (4). Person-to-person transmission of inhalational anthrax has not been confirmed (2, 7).

In the minds of most military and counterterrorism planners, the aerosolization of anthrax spores is among the most likely methods for a bioterrorism attack (6, 12). Though the minimum infectious inhaled dose has not been determined, the U.S. Department of Defense estimates that the 50% lethal dose for humans is between 8,000 and 10,000 spores (3). Following inhalation, the endospores enter pulmonary macrophages and are carried to the mediastinal lymph nodes. Germination and vegetative growth result in the production of an antiphagocytic capsule and a toxin consisting of three proteins, edema factor, lethal factor, and protective antigen, which play major roles in virulence and the associated infectious and clinical manifestations (5). Following an incubation period of 1 to 6 days, the initial symptoms of inhalation anthrax are mild and relatively nonspecific, including fever, malaise, a mild cough, and chest pain. A brief interval of improvement commonly occurs over the next 2 to 3 days, followed by rapid deterioration (13). Regional lymph nodes become overwhelmed, followed by entry of the organism into the systemic circulation. Fatal sepsis with generalized hemorrhage, massive hemorrhagic mediastinitis, and necrosis ensue. Death is universal in untreated cases and may occur in as many as 95% of treated cases if therapy is not started within 48 h following the onset of symptoms (3, 5, 13, 20).

The primary responsibility of the sentinel level laboratory is to rule out *B. anthracis* (and other suspected bioterrorism agents) based on results generated from the sentinel level testing protocols (see below) and then refer specimens or isolates to the nearest Laboratory Response Network (LRN) reference level laboratory (usually the state health department) for rapid confirmation. It is important to retain a portion of the specimen or a subculture of the suspicious isolate before shipping the material to the reference laboratory in the event that additional material is needed for retrospective studies. As indicated in procedure 16.1, a chain of custody is unlikely to be needed at the sentinel level laboratory. However, retention of the specimen(s) and/or isolate(s) is highly recommended when it is encountered under suspicious conditions.

II. SAFETY CONSIDERATIONS

Observe standard precautions.

Members of the laboratory staff must always remember to follow the safety guidelines associated with standard precautions when handling and examining human clinical specimens and maintain the attitude that all specimens are suspect. BSL 2 practices, containment equipment, and facilities are permissible for activities using clinical materials and diagnostic quantities of culture material (*see* procedure 16.2). Strict adherence to standard microbiological practices and techniques in conjunction

II. SAFETY CONSIDERATIONS *(continued)*

It is imperative that these cultures be handled in a biological safety cabinet.

with a class II biological safety cabinet and PPE (e.g., lab coat and gloves are required; safety glasses or a face shield is recommended) will provide sufficient protection. In addition, contaminated items, such as pipettes, needles, plastic loops, and microscope slides, should be soaked in 10% bleach or 10 to 30% formalin for 24 h before being autoclaved. Phenolics are not sporicidal at the usual working dilutions (10, 18).

Environmental samples, including powders, such as those received in envelopes (hoax letters) and the mail during the 2001 anthrax epidemic, should be examined in a BSL 3 (reference) laboratory and should not be accepted or handled by the sentinel level laboratory. In many instances, the closest reference laboratory will be the state health department laboratory. Extreme caution should be used during specimen handling to avoid the creation of aerosols. Vaccination is not recommended unless frequent work with infected clinical specimens or diagnostic cultures is anticipated (3, 18). If, when working in a sentinel level laboratory, there is a strong suspicion that a specimen or culture may consist of *B. anthracis,* all manipulations should be performed in a biological safety cabinet.

III. SPECIMEN COLLECTION

Observe standard precautions.

The specimen of choice depends on the disease presentation and the stage of the illness. Advice and information on specimen collection should be shared with emergency medical personnel, including clinicians, nurses, and supporting staff.

A. Cutaneous (19)

1. Vesicular (early) stage
Unroof vesicle and collect fluid on two sterile swabs for culture and Gram staining (if possible, obtain the fluid sample via aspiration with a needle and syringe).

2. Eschar stage
Without removing the eschar, insert a swab beneath the edge of the eschar, rotate the swab or obtain an aspirate as previously described, and collect lesion material for culture and Gram staining.

◩ **NOTE:** Removing the eschar could hasten dissemination (7).

B. Gastrointestinal (19)

1. Stool—best if collected in the early stage of disease. Collect 5 to 10 g in a clean, leakproof container.

2. Blood—later stage of disease. Collect in accordance with the institution's protocol for routine blood cultures.

3. Any hemorrhagic fluid from nose, mouth, or anus should be cultured and Gram stained (15).

4. Postmortem tissues.

C. Inhalation

The inhalational form of anthrax is the most likely to occur following the intentional release of spores in the commission of a bioterrorist attack. In a covert event, the laboratory may be the first to raise suspicion that *B. anthracis* has been recovered based on clues listed in procedure 16.1.

1. Early postexposure (0 to 24 h) following overt aerosolization of spores (11, 14)

a. Nasal and throat swabs for culture (recommended only for epidemiological screening following the confirmation that a release has occurred; not to be used for diagnostic purposes)

b. Induced respiratory secretions for culture. For optimal specimens, collection should be performed by respiratory therapy personnel. Patient preparation and collection should also be described in the institution's microbiology specimen collection manual.

III. SPECIMEN COLLECTION
(continued)

2. Prodromal phase (early nonspecific symptomatology) (19)
   Sputum culture and Gram stain, particularly if the patient has a productive cough; however, pneumonia is not a usual feature of inhalation anthrax.
3. Late phase (2 to 8 days postexposure) (19)
   - **a.** Blood culture and direct Gram stain of blood
   - **b.** CSF for culture and Gram staining, particularly if signs of meningitis occur (7)
4. Postmortem tissues

D. Environmental specimens—as directed by the Federal Bureau of Investigation (FBI) and public health authorities (9)
Soil, bone, hair, environmental swabs, swabs or material from a potential spore aerosolization device, water, or envelopes containing a powdered substance are among the possible environmental sources that may be examined for *B. anthracis.* These specimens would normally be collected by the FBI during the course of a criminal investigation and transported to an LRN reference laboratory (usually the state health department) for examination. As previously indicated, sentinel laboratories should not accept or process any form of environmental specimen and should restrict diagnostic testing to human specimens. Prior to the shipment of human specimens, cultures, or isolates that cannot be ruled out as *B. anthracis*, notify the state health laboratory and follow their instructions (2).

IV. CULTURE MATERIALS
(See reference 19.)

- **A. BAP**
- **B. MAC**
- **C. CHOC**
- **D. Phenylethyl alcohol agar (PEA)**
- **E. TSB**
- **F. Clean glass slides**
- **G. Sterile swabs and transport media**
- **H. Clinical centrifuge with appropriate biocontainment tube holder**
- **I. Sporicidal disinfectant (0.5% sodium hypochlorite)**
- **J. Formalin (10 to 30%)**
- **K. Sterile disposable bacteriological inoculating loops**

V. SPECIMEN TRANSPORT
(See reference 8.)

Observe standard precautions.

A. Vesicular fluid and eschar swabs (Dacron) may be transported at room temperature if there is no more than a 24-h delay.

B. Stool specimens should be transported at room temperature if the delay is 1 h or less; transport at 4°C if there is a >1-h delay.

C. Sputum specimens may be transported at room temperature if there is no more than a 2-h delay or at 4°C if there is a 2- to 24-h delay.

D. Blood should be inoculated directly into blood culture bottles in accordance with the institution's protocol for routine blood cultures (19).

VI. SPECIMEN PROCESSING
(See reference 19.)

It is imperative that these cultures be handled in a biological safety cabinet.

A. Cutaneous and tissue specimens (swab/biopsy specimens) should be plated on BAP, MAC, and TSB or equivalent (refer to ASM/CDC Sentinel Level Protocol; http://www.asm.org or http://www.cdc.gov). A smear for Gram staining should also be made.

B. Stool specimens should be plated to BAP, MAC, and PEA.

C. Sputum specimens should be inoculated onto BAP, CHOC, and MAC. A smear for Gram staining should also be made.

D. Blood culture methods routinely performed in the laboratory are sufficient. A direct smear of the blood for Gram staining should be made.

E. CSF specimens should be centrifuged at 1,500 × *g* for 15 min using a clinical centrifuge equipped with appropriate biocontainment tube holders. Open the biocontainment tube holders only in a biological safety cabinet. Inoculate the culture sediment onto BAP, CHOC, and TSB. Prepare a smear for Gram staining (19).

VII. INCUBATION AND EXAMINATION OF CULTURES

(See reference 19.)

It is imperative that these cultures be handled in a biological safety cabinet.

A. All cultures should be incubated at 35 to 37°C under ambient conditions.

B. Cultures should be examined within 18 to 24 h of incubation. However, growth of *B. anthracis* may be observed as early as 8 h after inoculation. This can be helpful when looking for *B. anthracis* from mixed cultures (i.e., stool or sputum).

C. TSB cultures may be Gram stained and/or subcultured after incubation.

ANALYTICAL CONSIDERATIONS

VIII. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Routine QC procedures are used to check media, stain procedures, and reagents unless otherwise noted (19).

B. An avirulent, nonencapsulated strain of *B. anthracis* (Sterne strain) from which the anthrax vaccine is derived is commercially available (Colorado Serum Company, Denver, Colo.) and does not require registration or compliance with the Select-Agent Rule. This can be used for QC as well as for training purposes. Retaining a 24-h-old BAP culture at room temperature for 2 to 3 days promotes the production of endospores. Comparing wet mounts of these spores to similar preparations, e.g., talc and cornstarch, serves as an acceptable training tool and a source for use in competency testing.

IX. IDENTIFICATION

It is imperative that these cultures be handled in a biological safety cabinet.

A. Colony morphology (19)

At 16 to 24 h, colonies of *B. anthracis* are 2 to 5 mm in diameter. They are irregularly round and flat or slightly convex, with a ground-glass appearance and edges that are slightly undulate. There are often comma-shaped projections from the edge of the colony, producing the "Medusa head" shape. The colonies are nonhemolytic on BAP and have a tenacious consistency, so that when teased with a loop, the growth will stand up like beaten egg white. *B. anthracis* does not grow on MAC or PEA.

B. Gram stain morphology (19)

B. anthracis is a large (1- to 1.5- by 3- to 5-μm) gram-positive rod that forms oval central to subterminal endospores that do not swell the vegetative cell. Endospores are not seen on direct smears of clinical samples unless they have been exposed to atmospheric levels of CO_2. Vegetative cells seen on direct Gram-stained smears of clinical samples often occur in short chains of two to four cells that are encapsulated. An India ink preparation can be performed on blood and CSF specimens to enhance visualization of the capsule (see below). The India ink preparation should only be performed by an individual who has experience and knowledge in performing and interpreting test results and can differentiate true-positive and false-positive results. It is very rare for species other than *B. anthracis* to produce capsules. Gram stains from colonies grown on BAP show long chains of nonencapsulated gram-positive bacilli.

Include QC information on reagent container and in QC records.

C. India ink stain (19)

India ink is useful for enhancing the visualization of encapsulated *B. anthracis* in clinical specimens, such as blood and CSF.

1. Materials

- **a.** Microscope slides
- **b.** Coverslips
- **c.** India ink (may be purchased commercially prepared in droppers)

2. Controls

Use an 18- to 24-h-old stock strain of *Klebsiella pneumoniae* on BAP as a positive encapsulated control and *Escherichia coli* ATCC 25922 as the negative nonencapsulated control.

IX. IDENTIFICATION
(continued)

3. Procedure
- **a.** For the controls, transfer a small amount of growth from each control BAP into 0.5 ml of whole EDTA-treated blood or serum. Mix.
- **b.** Transfer 5 to 10 μl each of the clinical specimen (blood or CSF) and controls to clean glass microscope slides. Place a coverslip on the drop, and add 5 to 10 μl of India ink to the edge of the coverslip. Allow the ink to diffuse under the coverslip.
- **c.** View the cells at 100× using oil immersion.
- **d.** Discard the slides in 0.5% hypochlorite solution.

4. Interpretation

The capsule appears as a well-defined clear zone around the rod forms for the positive control. No zone is observed surrounding the negative control (19).

5. Limitations

Capsules may be seen on direct smears from blood and/or CSF specimens; however, cells from growth on BAP under ambient conditions do not produce capsules and often occur as long chains of bacilli.

D. Motility test (19)

B. anthracis is nonmotile, which is rather unusual among *Bacillus* spp. and is useful in the preliminary identification.

1. Materials
- **a.** Microscope slides
- **b.** Coverslips
- **c.** Sterile distilled water
- **d.** Sterile disposable bacteriological inoculating loops
- **e.** Sterile glass tubes
- **f.** Light microscope at ×400

2. Controls
- **a.** Positive control: 18- to 24-h culture of *Pseudomonas aeruginosa* ATCC 35032 or equivalent
- **b.** Negative control: *Acinetobacter* sp. strain ATCC 49139, Sterne (vaccine) strain of *B. anthracis* (optional), or equivalent

3. Procedure
- **a.** Place 2 drops of sterile distilled water into a sterile glass tube.
- **b.** Using an inoculating loop, suspend a small amount of the colony in a test tube.

  ◩ **NOTE:** A loopful of a fresh broth culture can be used.
- **c.** Transfer 1 drop of the suspension to a microscope slide and overlay it with a coverslip.
- **d.** Examine the slides under ×400 magnification.
- **e.** Discard the slides in 0.5% hypochlorite solution.

4. Interpretation
- **a.** Motile organisms can be observed moving randomly and independently throughout the suspension.
- **b.** Nonmotile organisms may show no movement at all or move with Brownian motion.

5. Limitations

True motility must be distinguished from Brownian movement.

E. Presumptive identification of *B. anthracis* (19)

1. Direct Gram staining shows large gram-positive rods in blood, CSF, lesion material, or blood cultures. Spores are not seen on direct smears from clinical specimens, as the CO_2 levels in the body inhibit sporulation.

2. Nonhemolytic, broad, spore-forming gram-positive rods isolated on BAP. Colonies have a ground-glass appearance. Spores are oval and nonswelling

IX. IDENTIFICATION
(continued)

and do not exceed the diameter of vegetative cells. Cells are nonmotile and catalase positive.

3. Be aware that most naturally occurring strains of *B. anthracis* are susceptible to penicillin; however, it is probable that a strain used for bioterrorism activity will be penicillin resistant.

◩ **NOTE:** The most common *Bacillus* sp. submitted to the CDC to rule out *B. anthracis* is *Bacillus megaterium* (nonmotile isolates).

F. RedLine Alert test

A commercially available, FDA-approved, culture-dependent method, known as the RedLine Alert (Tetracore Inc., Gaithersburg, MD) test, may be used as an adjunct to the current sentinel diagnostic tests (Gram stain, hemolysis, catalase, and motility) for the rapid and presumptive identification of *B. anthracis.* The test is a lateral flow immunoassay contained in a plastic cassette and is intended to screen for nonhemolytic colonies of *Bacillus* growing on sheep blood agar. The principle target of the assay is a cell surface protein commonly found in *B. anthracis* vegetative cells, and the presence or absence of this protein can be used to differentiate *B. anthracis* from other nonhemolytic *Bacillus* spp. A positive internal control, a lyophilized antigen derived from the avirulent Sterne strain of *B. anthracis,* combined with reconstitution buffer (colony isolation buffer) is provided in the test kit. A non-*Bacillus* sp., such as *Staphylococcus aureus,* or a nonhemolytic *Bacillus* sp., such as *B. megaterium,* can be used as external negative controls. Test results are generated within 15 min. If the test is positive and the bacterial colony is nonhemolytic, *B. anthracis* cannot be ruled out, and the sample or isolate should be sent to the nearest LRN reference laboratory for confirmation. The Sterne strain (avirulent, devoid of plasmid XO2) of *B. anthracis* has been excluded from the Select Agent Rule, and it may be used as an additional external positive control. To ensure test accuracy and performance, external controls should be performed with each new test lot. Test limitations include the following: (i) testing should be restricted to colonies growing on sheep blood agar only and should not be performed on suspicious powders, other environmental samples, or spore preparations; (ii) false-negative results may occur if the levels of specific antigen are below the sensitivity of the test; (iii) the test cassette cannot be reused; (iv) hemolytic colonies, including those produced by *Bacillus* spp., should not be tested; (v) isolates that yield negative results but are suspicious of being *B. anthracis,* based on other criteria, e.g., colonies that are flat or slightly convex and irregularly rounded, with edges that are slightly undulate, have a ground glass appearance, or exhibit a tenacious consistency resembling beaten egg white, should be forwarded to the nearest LRN reference laboratory for confirmatory testing.

G. Actions following the presumptive identification of *B. anthracis* (2, 19)

1. Preserve original specimens pursuant to a potential criminal investigation or at the request of the next-higher-level laboratory.
2. Contact institutional Infection Control, which in turn contacts the local health department, which is then responsible for contacting higher levels of public health laboratories within the LRN. The original laboratory contacts the state health laboratory only when planning to send an isolate for confirmation.
3. In conjunction with the FBI, implement a chain-of-custody system so that samples and laboratory results are protected and controlled to maintain their admissibility as evidence in a court of law.
4. Local FBI agents will forward samples to the state health department as necessary. Consultation with the state health department as soon as *B. anthracis* is suspected is strongly encouraged. Remember that the sentinel laboratory rules out *B. anthracis* and does not perform confirmatory testing.

POSTANALYTICAL CONSIDERATIONS

X. SUMMARY

A. Basis for presumptive identification of *B. anthracis* (Fig. 16.4–1)

1. Large gram-positive rods seen on direct smears from clinical specimens
2. Large gram-positive rods that are nonhemolytic on BAP, catalase positive, and nonmotile

B. Actions

1. Preserve all specimens and isolates.
2. Notify the institutional Infection Control and state public health laboratory (or the nearest LRN reference laboratory to send the specimen).
3. Implement chain of custody for specimens, isolates, and laboratory results if requested by the FBI.
4. Work in conjunction with the local or state public health laboratory to ensure that isolates and specimens are properly handled and transported to the next level (reference or federal) laboratory.

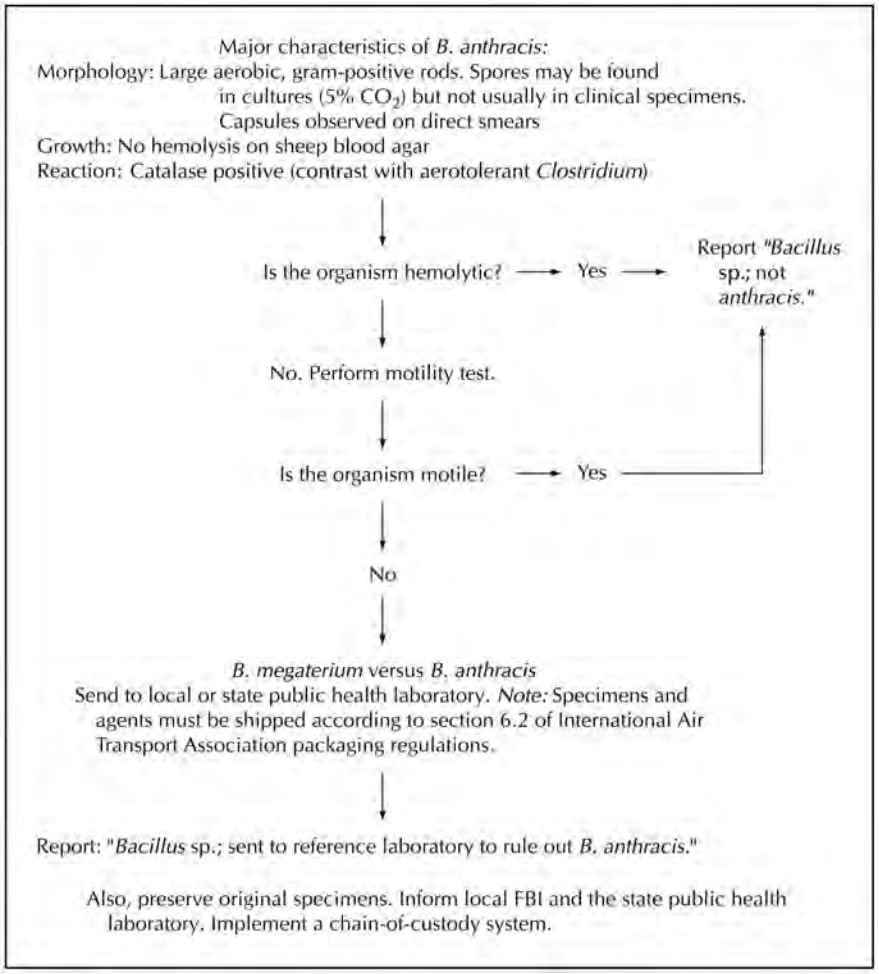
Major characteristics of B. anthracis:
Morphology: Large aerobic, gram-positive rods. Spores may be found in cultures (5% CO_2) but not usually in clinical specimens. Capsules observed on direct smears
Growth: No hemolysis on sheep blood agar
Reaction: Catalase positive (contrast with aerotolerant Clostridium)
Is the organism hemolytic?
Yes
Report "Bacillus sp.; not anthracis."
No. Perform motility test.
Is the organism motile?
Yes
No
B. megaterium versus B. anthracis
Send to local or state public health laboratory. Note: Specimens and agents must be shipped according to section 6.2 of International Air Transport Association packaging regulations.
Report: "Bacillus sp.; sent to reference laboratory to rule out B. anthracis."
Also, preserve original specimens. Inform local FBI and the state public health laboratory. Implement a chain-of-custody system.

Figure 16.4–1 *B. anthracis* sentinel level laboratory flowchart.

REFERENCES

1. **Bradaric, N., and V. Punda-Polic.** 1992. Cutaneous anthrax due to penicillin-resistant *B. anthracis* transmitted by an insect bite. *Lancet* **340:**306–307.
2. **Centers for Disease Control and Prevention.** 1998. Bioterrorism alleging use of anthrax and interim guidelines for management—United States, 1998. *MMWR Morb. Mortal. Wkly. Rep.* **48:**69–74.
3. **Centers for Disease Control and Prevention.** 2000. Use of anthrax vaccine in the United States. *MMWR Morb. Mortal. Wkly. Rep.* **49**(RR-15):1–20.
4. **Chin, J.** 2000. Anthrax, p. 20–25. *In Control of Communicable Diseases Manual*, 17th ed. American Public Health Association, Washington, DC.
5. **Cieslak, T. J., and E. M. Eitzen.** 1997. Clinical and epidemiologic principles of anthrax. *Emerg. Infect. Dis.* **5:**552–555.
6. **Departments of the Army, Navy, and Air Force.** 1996. *NATO Handbook on the Medical Aspects of NBC Defensive Operations.* Departments of the Army, Navy, and Air Force, Washington, DC.
7. **Dixon, T. C., M. Meselson, J. G. Guillemin, and P. C. Hanna.** 1999. Anthrax. *N. Engl. J. Med.* **341:**815–826.
8. **Eitzen, E., J. Pavlin, T. Cieslak, G. Christopher, and R. Culpepper (ed.).** 1999. *Medical Management of Biological Casualties Handbook*, 3rd ed. U.S. Army Medical Research Institute for Infectious Diseases, Fort Detrick, MD.
9. **English, J. F., M. Y. Cundiff, J. D. Malone, J. A. Pfeiffer, M. Bell, L. Steele, and M. Miller.** 1999. APIC Bioterrorism Task Force and CDC Working Group, p. 8–9. *In Bioterrorism Readiness Plan: a Template for Healthcare Facilities.* Association for Professionals in Infection Control and Epidemiology, Washington, DC.
10. **Fleming, D. O., J. H. Richardson, J. J. Tullis, and D. Vesley (ed.).** 1995. *Laboratory Safety Principles and Practices*, 2nd ed. American Society for Microbiology, Washington, DC.
11. **Franz, D. R., P. B. Jahrling, A. M. Friedlander, D. J. McClain, D. L. Hoover, W. R. Bryne, J. A. Paulin, G. W. Christopher, and E. M. Eitzen, Jr.** 1997. Clinical recognition and management of patients exposed to biological warfare agents. *JAMA* **278:**399–411.
12. **Franz, D. R., and R. Zajtchuk.** 2000. Biological terrorism: understanding the threat, preparation, and medical response. *Dis. Mon.* **46:**125–192.
13. **Friedlander, A. M.** 1997. Anthrax, p. 467–478. *In* R. Zajtchuk (ed.), *Textbook of Military Medicine: Medical Aspects of Chemical and Biological Warfare.* Department of the Army, Washington, DC.
14. **Hail, A. S., C. A. Rossi, G. V. Ludwig, B. E. Ivans, R. F. Tammariello, and E. A. Henchall.** 1999. Comparison of noninvasive sampling sites for early detection of *Bacillus anthracis* spores from rhesus monkeys after aerosol exposure. *Mil. Med.* **164:**833–837.
15. **Logan, N. A., and P. C. B. Turnbull.** 1999. *Bacillus* and recently derived genera, p. 357–363. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 7th ed. ASM Press, Washington, DC.
16. **Shafzand, S., R. Doyle, S. Ruoss, A. Weinacker, and T. Rafin.** 1995. Inhalational anthrax. *Chest* **116:**1369–1376.
17. **Turell, M. J., and G. B. Knudson.** 1987. Mechanical transmission of *B. anthracis* by stable flies (*Stomoxys calcitrans*) and mosquitoes (*Aedes aegypti* and *Aedes taeniorhynchus*). *Infect. Immun.* **55:**1859–1861.
18. **U.S. Department of Health and Human Services.** 1999. *Biosafety in Microbiological and Biomedical Laboratories*, 4th ed. U.S. Government Printing Office, Washington, DC.
19. **Weyant, R. S., J. W. Ezzell, T. Popovic, K. Q. Lindsay, and S. A. Morse.** 1999. Basic laboratory protocols for the presumptive identification of Bacillus anthracis. *In* Bioterrorism Preparedness and Response. http://www.bt.cdc.gov.
20. **Wiener, S. L., and J. Barret,** 1986. Biological warfare defense. *In Trauma Management for Civilian and Military Physicians.* W. B. Saunders, Philadelphia, PA.

SUPPLEMENTAL READING

Centers for Disease Control and Prevention. 2000. Biological and chemical terrorism: strategic plan for preparedness and response. *MMWR Morb. Mortal. Wkly. Rep.* **49**(RR-4):1–14.

Gilchrist, M. J. R., W. P. McKinney, J. M. Miller, and A. S. Weissfeld. 2000. *Cumitech 33, Laboratory Safety, Management, and Diagnosis of Biological Agents Associated with Bioterrorism.* Coordinating ed., J. W. Snyder. ASM Press, Washington, DC.

Klietmann, W. F., and K. L. Ruoff. 2001. Bioterrorism: implications for the clinical microbiologist. *Clin. Microbiol. Rev.* **14:**364–381.

16.5 Botulinum Toxin—*Clostridium botulinum*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Clostridium botulinum is an anaerobic gram-positive rod that produces spores and is ubiquitous in soil and marine sediments throughout the world. Botulism is a neuroparalytic illness resulting from the action of a toxin produced by strains of *C. botulinum*. These toxins are extremely hazardous to humans, requiring only a minute quantity to cause profound intoxication and death. There are four distinct forms of botulism: (i) food borne, (ii) wound, (iii) infant, and (iv) adult or child. Food-borne botulism, although the most common form, is relatively rare but often fatal. This form of botulism results from the ingestion of food items containing the preformed toxin. The clinical diagnosis of food-borne botulism can be confirmed by isolating the organism from the feces of the patient. Isolation of the organism from remnants of the consumed food item does not provide confirmatory evidence of botulism in the absence of other supporting laboratory data. *The demonstration of botulinum toxin in patient feces or serum or in the suspected food item supports the clinical diagnosis.* Wound botulism occurs following the colonization of the wound by *C. botulinum*. Multiplication of the organism may result in the production of toxin. Confirmation requires demonstration of the organism and toxin in serum, feces, or material from the wound. Infant botulism results when ingested spores germinate within the intestinal tract, causing toxin production. Confirmation of infection is dependent on the demonstration of botulinum toxin in the feces.

Adult or child botulism is represented by those cases in which no food source has been identified and there is no evidence of wound botulism but there is evidence of intestinal colonization in a person over the age of 1 year.

It is anticipated that in a bioterrorism-related exposure, botulinum toxin will most likely be transmitted by ingestion of toxin-contaminated food or possibly by aerosolization (2, 3). With the aerosolized form, symptoms usually begin within 36 h but may take longer, depending on the magnitude of the dose. Initial symptoms involve abnormalities of cranial nerve function, leading to blurred and double vision, light-induced pain, speech disorders, difficulty in swallowing, and a descending, symmetrical skeletal-muscle paralysis (3, 4). Person-to-person transmission of botulism does not occur.

The neurologic signs and symptoms resulting from toxin production dominate the clinical syndrome of botulism, regardless of the form. There are seven recognized antigenic types of *C. botulinum*, all with similar symptomatologies. The antigenic type of *C. botulinum* is determined by the complete neutralization of its toxin by the homologous antitoxin.

II. SAFETY CONSIDERATIONS

Observe standard precautions.

It is imperative that these cultures be handled in a biological safety cabinet.

Botulinum toxins are extremely poisonous. Exposure to the toxin represents the primary laboratory hazard. The 50% lethal dose of this toxin is 0.001 g/kg of body weight; therefore, acquisition by ingestion, inhalation, or absorption can cause intoxication and death. All materials suspected of containing toxin must be handled using standard precautions and BSL 2 containment criteria. Additional primary containment and personnel precautions, such as those recommended for BSL 3, are indicated for activities with a high potential for aerosol or droplet production (5, 6). A class II biological safety cabinet should be used when processing specimens to prevent the release of aerosols in the laboratory. *C. botulinum* is inactivated by a 1:10 dilution of household bleach. The disinfection solution must be in contact with the toxin or organism for 15 to 20 min to ensure complete inactivation. If a spill is suspected of containing both the toxin and the organism, it should be treated sequentially with bleach and 1 M sodium hydroxide.

◩ **NOTE:** Exposure to bleach does not inactivate the spores of *C. botulinum*.

ANALYTICAL CONSIDERATIONS

III. SPECIMEN COLLECTION AND TRANSPORT

The diagnosis of botulism is made clinically, based on patient history and physical findings. Routine laboratory tests are of limited value in the diagnosis of botulism. Laboratory criteria for diagnosis include detection of botulinum toxin in serum, feces, or food or isolation of *C. botulinum* from fecal cultures (1).

Sentinel laboratories should be familiar with and provide consultation on the proper selection, collection, transport, and shipping of appropriate specimens.

◩ **NOTE:** Due to the dangers inherent in working with *C. botulinum*, sentinel laboratories are responsible only for the collection, packaging, and shipment of specimens suspected of containing botulinum toxin. Refrain from performing cultures and smears for microscopy.

Observe standard precautions.

A. Specimens

1. Serum

Specimens should be obtained as soon as possible after the onset of symptoms.

a. Specimens should be obtained prior to antitoxin treatment.

b. Obtain 10 to 15 ml of serum for toxin analysis (equivalent to 20 to 30 ml of whole blood).

c. Less than 3 ml of serum may result in an inconclusive examination.

2. Feces

a. Collect 25 to 50 g of feces for testing.

b. Specimens should be collected prior to antitoxin treatment.

c. In cases of constipation, an enema may be given using sterile water or saline.

d. Intestinal contents obtained at autopsy may be submitted for analysis.

3. Food samples

a. Remnants of suspected foods may be tested.

b. Specimens should be submitted in the original containers.

4. Vomitus or gastric contents

Collect 20 ml into a sterile container.

5. Wounds

Specimens include exudates, debrided tissue, or swabs (least preferred) of the wound.

6. Autopsy specimens

a. Serum

b. Gastric and intestinal contents

B. Specimen transport and shipping

1. Wound and fluid specimens should be shipped in a commercial anaerobic transport system.

2. Wound specimens, including swabs containing transport medium, should be transported without refrigeration.

3. All other specimens should be transported with a refrigerant (cold packs, ice, or dry ice per International Air Transport Association regulations).

4. All specimens should be transported in leakproof containers.

5. Specimens should be shipped by the most rapid means possible.

6. Packaging and shipping containers must conform to guidelines of the U.S. Department of Transportation.

7. Fecal and serum specimens may be frozen if a delay in testing is anticipated.

◩ **NOTE:** Freezing will not affect the ability to detect toxin; however, it will compromise the isolation and recovery of the organism.

◩ **NOTE:** When botulism is suspected, sentinel laboratories *must* contact their local or state health department laboratory before shipping specimens for testing

III. SPECIMEN COLLECTION AND TRANSPORT *(continued)*

and confirmation. Since botulism is a public health emergency even when criminal activity is not suspected, prior contact with these agencies is recommended for diagnostic consultation, epidemiological assistance, and diagnostic laboratory services. The Food-Borne and Diarrheal Diseases Branch of the CDC can be contacted 24 h a day, 365 days a year, at (404) 639-2206 (Monday to Friday, 8:30 a.m. to 4:30 p.m. eastern standard time) or (404) 639-2888 (after hours and on weekends) (4). The CDC Emergency Response Hotline is available 24 h a day at (770) 488-7100. Always notify your institutional infection control officer or nurse in the event of a suspected case(s) of botulism.

POSTANALYTICAL CONSIDERATIONS

Although the microbiology staff does not have a direct role in the detection and confirmation of botulinum toxin, it can provide consultation to the infection control team and the risk management program. For example, a fact sheet such as is available from the CDC (http://www.bt.cdc.gov/agent/botulism/factsheet.asp) that highlights (but is not restricted to) the following information can be provided: (i) an explanation that people who are exposed to botulinum toxin are not contagious, (ii) a description of symptoms produced by botulinum toxin (blurred vision, drooping eyelids, and shortness of breath), and (iii) a note that if such symptoms develop or are observed, the affected individuals should report for evaluation.

REFERENCES

1. **Allen, S. D., C. L. Emery, and D. M. Lyerly.** 2003. *Clostridium*, p. 835–856. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 8th ed. ASM Press, Washington, DC.
2. **Arnon, S. S., et al.** 2001. Botulism toxin as a biological weapon. Medical and public health management. *JAMA* **285:**1059–1070. (Erratum, **285:**2081.)
3. **Franz, D. R., P. B. Jahrling, A. M. Friedlander, D. J. McClain, D. L. Hoover, W. R. Bryne, J. A. Pavlin, G. W. Christopher, and E. M. Eitzen, Jr.** 1997. Clinical recognition and management of patients exposed to biological warfare agents. *JAMA* **278:**399–411.
4. **Gilchrist, M. J. R., W. P. McKinney, J. M. Miller, and A. S. Weissfeld.** 2000. *Cumitech 33, Laboratory Safety, Management, and Diagnosis of Biological Agents Associated with Bioterrorism.* Coordinating ed., J. W. Snyder. ASM Press, Washington, DC.
5. **Miller, J. M.** 2000. The laboratory response to agents of bioterrorism. American Society for Microbiology Audioconference Series. American Society for Microbiology, Washington, DC.
6. **U.S. Department of Health and Human Services.** 2007. *Biosafety in Microbiological and Biomedical Laboratories*, 5th ed. U.S. Government Printing Office, Washington, DC.

SUPPLEMENTAL READING

Holden, J. 2004. Collection and transport of clinical specimens for anaerobic culture, p. 4.2.1–4.2.7. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook*, 2nd ed. ASM Press, Washington, DC.

Isenberg, H. D. 2004. Specimen collection, transport, and acceptability, p. 2.1.1–2.1.25. *In* H. D. Isenberg (ed.), *Clinical Microbiology Procedures Handbook*, 2nd ed. ASM Press, Washington, DC.

Klietmann, W. F., and K. L. Ruoff. 2001. Bioterrorism: implications for the clinical microbiologist. *Clin. Microbiol. Rev.* **14:**364–381.

16.6 Brucellosis—*Brucella* spp.

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Brucella is a fastidious, intracellular, aerobic, small gram-negative coccobacillus. Brucellosis is a zoonotic disease caused by four species that are recognized as human pathogens: *Brucella abortus* (cattle), *B. melitensis* (goats, sheep, and camels), *B. suis* (pigs), and *B. canis* (dogs). There have been rare reports of naturally acquired infections of humans with a marine mammal-associated species of *Brucella* (11). These isolates are most closely related to *B. suis.*

Approximately 50 to 100 cases of brucellosis are diagnosed each year in the United States, with the majority occurring in two patient populations. The first group includes people who work with unvaccinated animals. This group comprises farmers, veterinarians, and slaughterhouse workers who become infected by direct contact with or aerosolization of infected tissues. *B. abortus* and *B. suis* are the most common agents seen in this group. The second group of individuals includes those that ingest unpasteurized dairy products contaminated with *Brucella* spp. These patients usually have a history of travel to or have migrated from Latin America or the Middle East, where the disease is endemic in dairy animals, especially goats and camels. *B. melitensis* is the primary agent in these cases. Infections due to *B. canis* are very rare (4).

Aerosolization of *Brucella* is predicted to be the most likely form of dispersal in the commission of a biocrime or act of terrorism. In 1954, *B. suis* was the first biological agent to be weaponized by the United States in its biological-warfare program. The infective dose for *Brucella* spp. is approximately 10 to 100 organisms (6), which makes *Brucella* a potentially effective bioterrorism agent as well as a hazard in the clinical microbiology laboratory (1).

Person-to-person transmission does not occur, with the exception of very rare cases reported following blood exposure or exposure to infected tissues or after sexual contact (1). The incubation period is highly variable, ranging from 5 days to 2 months. Clinically, *Brucella* can cause both acute and chronic infections. Acute disease is characterized by generalized symptoms, including fever, profuse sweating, headache, muscle pain, anorexia, and weight loss. The chronic form of disease resembles miliary tuberculosis, with lesions in the liver, bone, and spleen (3). Because of this, brucellosis is commonly included in the differential diagnosis of fever of unknown origin. The mortality rate ($\leq$5%) is low, and mortality is primarily due to untreated endocarditis or meningitis (1, 3, 15).

II. SAFETY CONSIDERATIONS

It is imperative that these cultures be handled in a biological safety cabinet.

Brucellosis is the most commonly reported laboratory-acquired infection. Cases have occurred in clinical laboratory settings by sniffing of cultures, direct skin contact with cultures, and aerosol-generating procedures. Clinical specimens that are suspected of containing *Brucella* should be handled using BSL 2 procedures. BSL 3 practices are recommended when manipulating growth from cultures believed to contain *Brucella* spp., and all plates should be sealed (12, 13, 14, 15). It is recommended that household bleach (1:10 dilution) be utilized to decontaminate work surfaces. It is also recommended that laboratories develop a policy to utilize BSL 3 practices for handling cultures with a Gram stain that shows very, very tiny gram-negative bacilli.

III. SPECIMEN COLLECTION

Observe standard precautions.

Recommended specimens include the following.

A. Blood* (20 ml)
B. Bone marrow* (0.5 to 1.0 ml collected from the iliac crest)
C. Other (lymph node, aspirates from abscesses, liver and spleen biopsy specimens, and CSF)
D. Serum: acute-phase (at the onset of disease) and convalescent-phase (3 to 4 weeks) specimens

*Diagnostic specimen of choice

IV. MATERIALS

A. Commercial blood culture system
B. Medium
- **1.** Sheep blood agar
- **2.** CHOC
- **3.** MAC
- **4.** Modified Thayer-Martin agar (for contaminated specimens)
- **5.** *Brucella* base agar or heart infusion agar

C. Tissue-grinding apparatus
- **1.** Mortar and pestle with sterile sand or alundum
- **2.** Commercial tissue homogenizer system

D. Gram stain reagents

ANALYTICAL CONSIDERATIONS

V. SPECIMEN PROCESSING

Observe standard precautions.

Clinical specimens should be processed within 2 h following collection. In the event of delays, refrigerate specimens at 4°C (*see* section 3 of this handbook).

A. Blood
- **1.** Inoculate blood into a continuously monitored blood culture system and incubate it at 35°C for 7 to 10 days. Automated systems, such as BACTEC 9240 and BacT/Alert, have been reported to detect *Brucella* within 10 days (2, 7). Commercial, nonautomated systems which utilize a biphasic culture system, such as Septi-Chek and Opticult, are acceptable but may require incubation for up to 21 days, with blind subculturing every 7 days. Terminal subcultures and incubation of sealed plates for 7 days are required if using nonautomated blood culture systems. Lysis-centrifugation methods (ISOLATOR) have also been used for the recovery of *Brucella* spp. but may be less sensitive than broth-based systems.
- **2.** On automated systems, blood culture bottles that flag as positive but for which organisms are not seen on Gram stain should have another aliquot examined by a different stain, such as acridine orange (4).
- **3.** If *Brucella* is suspected, terminal subcultures should be performed, if using nonautomated blood culture systems, before the broth is discarded, and all subculture plates should be incubated for a minimum of 1 week (2, 5, 7).

B. Bone marrow and other fluids

Process as described for blood specimens or use standard inoculation procedures and plate directly onto sheep blood agar, CHOC, MAC, Thayer-Martin (Martin-Lewis) agar, a *Brucella* base agar, or heart infusion agar.

C. Tissue, wound specimens, and other specimens

Homogenize tissues aseptically in a mortar using sterile sand or alundum and a small quantity (1 ml) of sterile broth or saline.

Caution: *Homogenization produces aerosols and should be performed only in a biological safety cabinet (*see *section 3 of this handbook). PPE, including a face shield, is recommended!*

- **1.** Inoculate tissue homogenate and wound specimens onto sheep blood agar or *Brucella* base agar, CHOC, and MAC. If contamination is suspected, inoculate Thayer-Martin (Martin-Lewis) agar.
- **2.** Incubate plates for 7 days at 35 to 37°C in 5 to 10% CO_2.

Table 16.6–1 Differentiation of *Brucella* spp. from other similar organisms

Test	Result[a]			
	Brucella spp.	*Oligella* spp.	*Haemophilus influenzae*	*Francisella tularensis*
Gram stain	Small coccobacilli	Small coccobacilli	Small coccobacilli	Small coccobacilli
Oxidase	Positive	Positive	Variable	Negative
Urea hydrolysis	Positive	Positive	Variable	Negative
Growth on blood agar	Positive	Positive	Negative	Positive[b]

[a] Specimen sources are as follows: *Brucella* spp., blood and bone marrow; *Oligella* spp., urinary tract; *H. influenzae* and *F. tularensis*, various.

[b] Enhanced by cysteine (some strains fail to grow on blood agar when subcultured).

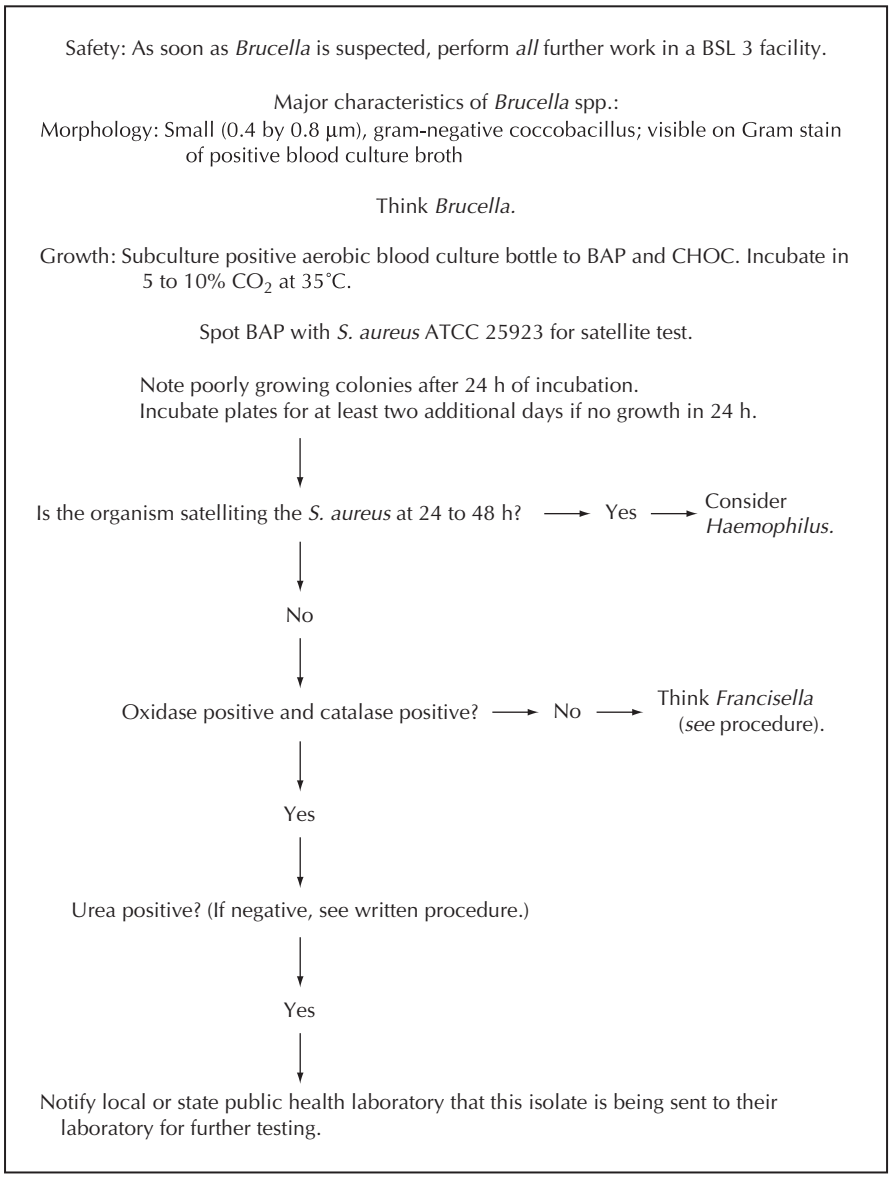
Safety: As soon as *Brucella* is suspected, perform *all* further work in a BSL 3 facility.
Major characteristics of *Brucella* spp.:
Morphology: Small (0.4 by 0.8 μm), gram-negative coccobacillus; visible on Gram stain of positive blood culture broth
Think *Brucella.*
Growth: Subculture positive aerobic blood culture bottle to BAP and CHOC. Incubate in 5 to 10% CO_2 at 35°C.
Spot BAP with *S. aureus* ATCC 25923 for satellite test.
Note poorly growing colonies after 24 h of incubation.
Incubate plates for at least two additional days if no growth in 24 h.
Is the organism satelliting the *S. aureus* at 24 to 48 h?
Yes
Consider *Haemophilus.*
No
Oxidase positive and catalase positive?
No
Think *Francisella* (*see* procedure).
Yes
Urea positive? (If negative, see written procedure.)
Yes
Notify local or state public health laboratory that this isolate is being sent to their laboratory for further testing.

Figure 16.6–1 *Brucella* species sentinel-level laboratory flowchart.

VI. IDENTIFICATION

(See reference 12.)

A. Colony morphology

◩ **NOTE:** Visible growth of *Brucella* spp. on CHOC or blood agar incubated in 5 to 10% CO_2 may require 48 h or longer (1 week). *Brucella* spp. rarely grow on MAC. *B. canis* is known to grow on MAC. Colonies of *Brucella* spp. are pinpoint, raised, white to cream, glistening, and nonhemolytic; young colonies are slightly yellow and opalescent and may darken, becoming brownish with age (3, 7).

B. Gram stain characteristics

Brucella spp. are very small, faintly staining, gram-negative coccobacilli (0.5 to 0.7 by 1.5 μm); the microscopic appearance is that of fine sand (3, 7, 8).

■ *Organisms demonstrating this type of morphology should be handled with extreme caution until* Brucella *spp. and* Francisella *spp. have been ruled out.*

C. Presumptive identification (Table 16.6–1)

D. Sentinel laboratory tests for ruling out *Brucella* spp. (Fig. 16.6–1)

1. Oxidase (Kovacs' modification) (9)

a. Perform test on fresh isolates grown on noninhibitory media.

b. A positive test is indicated by the production of a blue color within 10 s; a blue color that develops after 10 s should be disregarded and considered a false positive.

c. *B. abortus, B. melitensis,* and *B. suis* are oxidase positive; *B. canis* is oxidase variable.

2. Catalase

All *Brucella* spp. are catalase positive.

3. Satellite test

◩ **NOTE:** *Brucella* spp. may be confused with *Haemophilus* spp.

Inoculate a blood agar plate with the suspect organism, followed by spotting with *Staphylococcus aureus* ATCC 25923 in each quadrant. After 24 to 48 h of incubation in 5 to 10% CO_2, the absence of growth is suggestive of *Brucella;* growth only around *Staphylococcus aureus* is suggestive of *Haemophilus* spp.

4. Urea hydrolysis (10)

a. Inoculate a urea agar slant heavily with growth from a fresh culture.

b. Incubate it at 35°C under aerobic conditions.

c. Observe it for color change (pink) within 1 h.

d. Most *Brucella* spp. will hydrolyze urea within 1 h.

e. Other organisms that are urease positive and may be confused with *Brucella* spp. include *Oligella ureolytica, Psychrobacter phenylpyruvicus, Psychrobacter immobilis,* and *Bordetella bronchiseptica.*

Caution: *Do not attempt to identify* Brucella *spp. with commercial identification systems due to the high probability of misidentification.*

E. Antimicrobial susceptibility testing

1. Although Clinical and Laboratory Standards Institute (CLSI) guidelines are available, antimicrobial susceptibility testing *should not* be performed in sentinel laboratories due to the hazards associated with this process.

2. QC ranges have been established and approved for 48-h incubation for MIC testing using the broth dilution method (5).

POSTANALYTICAL CONSIDERATIONS

F. Confirmatory identification

1. Preserve original specimens and isolates as potential criminal evidence in a secure location.
2. Contact the infection control officer, who is responsible for notifying the local health department that the laboratory cannot rule out *Brucella* and that the isolate is being forwarded to the state health department laboratory for confirmatory identification.
3. Consultation with the local or state health department laboratory prior to shipping is recommended as soon as *Brucella* is suspected.
4. Federal Bureau of Investigation officials may forward the specimens and cultures for confirmatory testing in the event of overt activity.
5. Download Select Agents Form 4 from the website http://www.cdc.gov/od/sap/forms/APHIS-CDC-Form4.pdf, complete it, and send it to the CDC and the state health department within 7 days. Keep a copy of this form and all related printed materials.

REFERENCES

1. **Bossi, P., A. Tegnell, A. Baka, F. Van Loock, J. Hendriks, A. Werner, H. Maidhof, and G. Gouvras.** 2004. Bichat Guidelines for the Clinical Management of Brucellosis and Bioterrorism-Related Brucellosis. *Eurosurveillance* **9:**1–5.
2. **Centers for Disease Control and Prevention.** 2000. *Bioterrorism Preparedness and Response: Basic Laboratory Protocols for the Presumptive Identification of* Brucella *Species.* Centers for Disease Control and Prevention, Atlanta, GA.
3. **Chu, M. C., D. Lindquist, and W. S. Probert.** 2007. *Francisella* and *Brucella*, p. 815–834. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and M. L. Landry (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
4. **Chusid, M. J., R. W. Perzigian, W. M. Dunne, and E. A. Gecht.** 1989. Brucellosis: an unusual cause of a child's fever of unknown origin. *Wis. Med. J.* **88:**11–13.
5. **Clinical and Laboratory Standards Institute.** 2008. *Performance Standards for Antimicrobial Susceptibility Testing.* Eighteenth informational supplement. CLSI document M100-S18. Clinical and Laboratory Standards Institute, Wayne, PA.
6. **Gilchrist, M. J. R., W. P. McKinney, J. M. Miller, and A. S. Weissfeld.** 2000. *Cumitech 33, Laboratory Safety, Management, and Diagnosis of Biological Agents Associated with Bioterrorism.* Coordinating ed., J. W. Snyder. ASM Press, Washington, DC.
7. **Hoover, D., and A. Friedlander.** 1997. Brucellosis, p. 513–521. *In Textbook of Military Medicine: Medical Aspects of Chemical and Biological Warfare.* U.S. Department of the Army, Surgeon General, and the Borden Institute, Washington, DC.
8. **Laboratory Response Network.** 2004. *Sentinel Laboratory Guidelines for Suspected Agents of Bioterrorism:* Brucella *Species.* American Society for Microbiology, Washington, DC.
9. **MacFaddin, J. F.** 2000. Oxidase test, p. 368–378. *In Biochemical Tests for Identification of Medical Bacteria*, 3rd ed. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.
10. **MacFaddin, J. F.** 1980. Urease test, p. 298. *In Biochemical Tests for Identification of Medical Bacteria*, 2nd ed. Waverly Press, Inc., Baltimore, MD.
11. **McDonald, W. L., R. Jamaludin, G. Mackereth, M. Hansen, S. Humphrey, P. Short, T. Taylor, J. Swingler, C. E. Dawson, A. M. Whatmore, E. Stubberfield, L. L. Perrett, and G. Simmons.** 2006. Characterization of a *Brucella* sp. strain as a marine-mammal type despite isolation from a patient with spinal osteomyelitis in New Zealand. *J. Clin. Microbiol.* **44:**4363–4370.
12. **Miller, J. M.** 2000. The laboratory response to agents of bioterrorism. American Society for Microbiology Audioconference Series. American Society for Microbiology, Washington, DC.
13. **U.S. Department of Health and Human Services.** 2007. *Biosafety in Microbiological and Biomedical Laboratories*, 5th ed. U.S. Government Printing Office, Washington, DC.
14. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 1996. *Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultative Anaerobic Bacteria*, 2nd ed. Williams and Wilkins, Baltimore, MD.
15. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar.** 2001. *Laboratory Protocols for Bioterrorism Response Laboratories for the Identification of Brucella Species.* Association for Public Health Laboratories, Washington, DC.

SUPPLEMENTAL READING

Centers for Disease Control and Prevention. 2000. Biological and chemical terrorism: strategic plan for preparedness and response. *MMWR Morb. Mortal. Wkly. Rep.* **49**(RR-4)**:**1–14.

Klietmann, W. F., and K. L. Ruoff. 2001. Bioterrorism: implications for the clinical microbiologist. *Clin. Microbiol. Rev.* **14:**364–381.

16.7 Plague—*Yersinia pestis*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Yersinia pestis is the causative agent of plague, an acute febrile infectious disease with a high fatality rate (16). Plague may present in three forms: bubonic, pneumonic, and septicemic (13). Bubonic plague is characterized by sepsis that is accompanied by the sudden onset of fever, chills, weakness, headache, and the formation of buboes and swelling of regional lymph nodes of the groin, axilla, or neck. Septicemic plague is basically the same as bubonic plague but without the swelling of the lymph nodes. Pneumonic plague, the most deadly form of the disease and the form that can spread rapidly among susceptible individuals, presents as fever and lymphadenopathy with cough, chest pain, and often hemoptysis. Secondary pneumonia from hematogenous spread of the organisms can occur, or the organism can occasionally be passed by aerosols from human to human as primary pneumonic plague.

◩ **NOTE:** Primary pneumonic plague is the form that would be seen if *Y. pestis* was used as a bioterrorism agent and would also be transmitted in aerosolized form.

A supportive diagnosis of plague is most readily made by a direct stained smear of bubo aspirates, blood, and respiratory specimens or of CSF samples in plague meningitis (2). In addition, direct fluorescent-antibody testing of clinical samples can provide a more rapid presumptive diagnosis of plague (2, 13). This testing is usually available through state laboratories. However, recovery of the organism in culture is the most rapid and definitive means to confirm diagnosis. Serologic diagnosis is often used to verify or confirm suspected cases of plague. Alternative methods for diagnosing plague include PCR analysis and DNA hybridization studies; however, neither of these methods is generally available in the routine diagnostic laboratory (10, 11).

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. The proper specimens to collect for the diagnosis of plague include aspirations of buboes, blood, respiratory tract secretions (sputum, bronchoalveolar lavage fluid, bronchial washes, tracheal or lung aspirates, and lung tissue biopsy specimens), and CSF for staining and culture (2, 13). Suitable autopsy specimens include lymphoid tissue, lung tissue, and bone marrow.

B. Transport of these specimens using routine laboratory procedures will ensure the viability of the organisms.

C. Paired serum samples, acute and convalescent or convalescent and postconvalescent phase, are best used to establish a diagnosis of plague by serologic means. However, a single high-titer serum sample can be used to provide a presumptive diagnosis of plague (13).

III. MATERIALS

It is imperative that these cultures be handled in a biological safety cabinet.

A. Clinical material should be processed according to normal laboratory protocols in a biological safety cabinet, prepared for staining, and inoculated onto routine laboratory isolation medium.

B. Blood agar, MAC, and CHOC should be plated.

C. For blood cultures, process in accordance with normal laboratory protocol. If blood cultures are signaled as positive, they should be stained and subcultured as described above.

D. Serum should be separated and stored frozen until it is tested.

ANALYTICAL CONSIDERATIONS

IV. PROCEDURES

A. Stains

1. Fix prepared, air-dried slide with 100% methanol and stain with Gram or Wright-Giemsa stain (2, 9, 13).
2. Staining with a fluorescent antibody specific for the F1 capsular antigen may be performed at a reference laboratory (13).
3. Gram stains must be performed to characterize the Gram reaction and morphology of the organism.

B. Culture

1. Inoculate properly obtained clinical material onto laboratory medium and streak for isolation.
2. Nonselective media, such as BAP or BHI agar, can be used for sterile sites; enrichment broth may also be included for these sites. MAC or cefsulodin-irgasan-novobiocin selective agar can be added to the above and used for contaminated specimens.
3. All media should be incubated aerobically with increased CO_2 at 28 to 35°C for several days.
4. The optimal temperature for growth of *Y. pestis* is reported to be 28°C (2).

C. Serology

1. Serological diagnosis of *Y. pestis* infection is most commonly performed at the CDC or other large reference laboratories (6).
2. The classic serological technique used for the diagnosis of plague is the passive hemagglutination assay (PHA) (2, 3, 5, 6, 13).
3. Other assays have also been developed for testing sera for antibodies to the plague bacillus; these include ELISA (4), latex agglutination (15), and solid-phase radioimmunoassay (8).

V. RESULTS

A. Stains

1. Staining of direct specimens that contain *Y. pestis* will reveal small gram-negative bacilli, from 1 to 2 μm in length, mostly in singles and pairs and which may stain bipolar (darker on the ends than in the middle).
2. Bipolar staining characteristics are more readily seen on a Wright-Giemsa stain and may not be apparent on a Gram stain of the specimen (7).
3. Bipolar-staining gram-negative bacilli on a routine blood film or buffy coat smear should heighten suspicion of *Y. pestis.*

B. Culture

1. *Y. pestis,* a member of the family *Enterobacteriaceae,* is a catalase-positive, oxidase-negative, non-lactose-fermenting, aerobic gram-negative bacillus that does not form spores.
2. It grows more slowly than other enterobacteriaceae on solid media and may only form visible colonies after 24 to 48 h of incubation (2, 13).
3. Colonies measure 1 to 2 μm in diameter, are gray-white, smooth, and opaque; and may have a "beaten-copper" (9) or "hammered-metal" (13) appearance.
4. The organism may grow better at 28°C than at higher temperatures.
5. If a suspect organism is grown in culture, routine biochemical testing will confirm the identification of the organism.
6. The organisms will be alkaline or acid in triple-sugar–iron agar with the key biochemical reactions shown in Table 16.7–1 (14).
7. Any isolate showing the characteristics in Table 16.7–1 should immediately be submitted to the appropriate state public health laboratory or other reference laboratory for further identification.
8. Lysis by a specific bacteriophage can be used by the CDC to confirm the identification of cultured *Y. pestis* (1, 12).

Table 16.7–1 Key biochemical reactions for *Y. pestis*

Positive	Negative
Catalase	Oxidase
Glucose	Urea
Trehalose	Indole
	Lactose
	Citrate
	Motility (25 and 37°C)

V. RESULTS *(continued)*

C. Serology

1. A fourfold rise in paired acute- and convalescent-phase serum samples or a fall in titer between convalescent- and postconvalescent-phase serum samples is considered confirmatory for the diagnosis of plague.
2. A single serum sample with a PHA titer of >10 is presumptive evidence of recent infection with *Y. pestis* (13).

◩ **NOTE:** A flowchart depicting an identification scheme for *Y. pestis* is shown in Fig. 16.7–1.

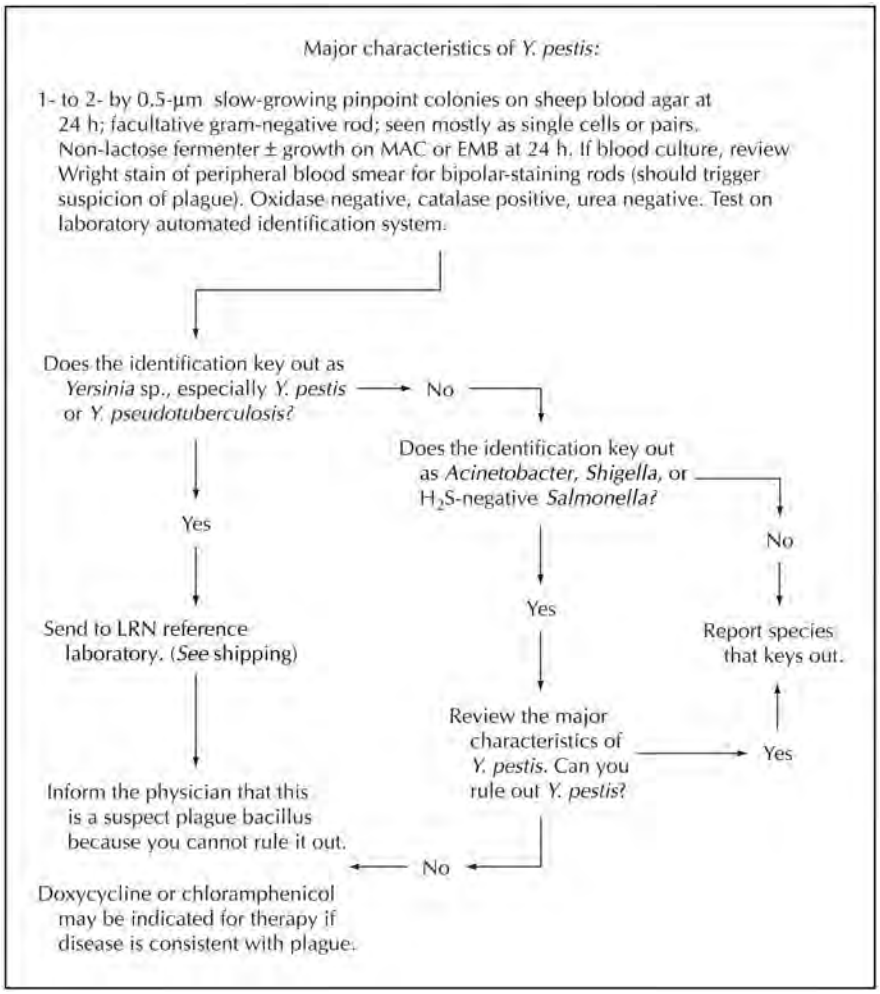
Major characteristics of Y. pestis:
1- to 2- by 0.5-µm slow-growing pinpoint colonies on sheep blood agar at 24 h; facultative gram-negative rod; seen mostly as single cells or pairs. Non-lactose fermenter ± growth on MAC or EMB at 24 h. If blood culture, review Wright stain of peripheral blood smear for bipolar-staining rods (should trigger suspicion of plague). Oxidase negative, catalase positive, urea negative. Test on laboratory automated identification system.
Does the identification key out as Yersinia sp., especially Y. pestis or Y. pseudotuberculosis?
No
Does the identification key out as Acinetobacter, Shigella, or H2S-negative Salmonella?
Yes
No
Yes
Send to LRN reference laboratory. (See shipping)
Report species that keys out.
Review the major characteristics of Y. pestis. Can you rule out Y. pestis?
Yes
Inform the physician that this is a suspect plague bacillus because you cannot rule it out.
No
Doxycycline or chloramphenicol may be indicated for therapy if disease is consistent with plague.

Figure 16.7–1 *Y. pestis* sentinel-level laboratory flowchart. LRN, Laboratory Response Network.

VI. QUALITY CONTROL

Include QC information on reagent container and in QC records.

A. Stains

Routine QC for stains is recommended as outlined in the appropriate section of this handbook.

B. Culture

Routine QC for media and biochemicals are recommended as outlined elsewhere in this handbook.

C. Serology

Serological testing is usually performed only at the CDC or other large reference laboratories.

POSTANALYTICAL CONSIDERATIONS

VII. LIMITATIONS

A. Stains

1. Gram and Wright-Geimsa stains are nonspecific for plague, and the fluorescent-antibody stain on direct specimens is only presumptive evidence of *Y. pestis* infection (13).

2. *In addition, fluorescent-antibody stains can only be performed on samples that have been refrigerated for <30 h. Longer refrigeration times can result in false-negative results* (13).

B. Culture

1. Not all automated identification systems have a database that will allow the identification of *Y. pestis* (12), and some may actually misidentify the organism. In addition, due to the organism's low growth rate, initial negative results in routine biochemical testing may also be suspect (13).

2. Organisms that might be confused with *Y. pestis* include *Yersinia pseudotuberculosis*, *Shigella* spp., H_2S-negative *Salmonella*, and *Acinetobacter* spp. (17).

C. Serology

Detection of specific antibodies, while not a rapid means for diagnosing plague, is normally used retrospectively to confirm suspected cases (13).

VIII. NOTIFICATIONS

A. Notify your state health department of any suspected isolate of *Y. pestis.*

B. Consult with the state health laboratory prior to shipment of a specimen or isolate for confirmation.

C. If *Y. pestis* cannot be ruled out or is confirmed, infection control personnel should be informed for institution of infection control measures.

REFERENCES

1. **Baltazard, M. D., H. S Davis, R. Devignat, G. Girard, M. A. Gohar, L. Kartman, K. F. Meyer, M. T. Parker, T. Pollitzer, F. M. Prince, S. F. Quan, and P. Wagle.** 1956. Recommended laboratory methods for the diagnosis of plague. *Bull. W. H. O.* **14:**457–509.
2. **Butler, T.** 1983. Plague and other *Yersinia* infections, p. 163–188. *In* W. B. Greenough III and T. C. Merigan (ed.), *Current Topics in Infectious Disease.* Plenum Medical Book Company, New York, NY.
3. **Cavanaugh, D. C., P. J. Deoras, D. H. Hunter, J. D. Marshall, D. V. Quy, J. H. Rust, S. Purnaveja, and P. E. Winter.** 1970. Some observations on the necessity for serological testing of rodent sera for *Pasteurella pestis* antibody in a plague control program. *Bull. W. H. O.* **42:**451–459.
4. **Cavanaugh, D. C., M. K. Fortier, D. M. Robinson, J. E. Williams, and J. H. Rust.** 1979. Application of the ELISA technique to the problems in the serologic diagnosis of plague. *Bull. Pan Am. Health Org.* **13:**399–402.
5. **Chen, T. H., and K. F. Meyer.** 1966. An evaluation of *Pasteurella pestis* fraction-1-specific antibody for the confirmation of plague infections. *Bull. W. H. O.* **34:**911–918.
6. **Forbes, B. A., D. F. Sahm, and A. S. Weissfeld (ed.).** 1998. *Bailey and Scott's Diagnostic Microbiology*, 10th ed., p. 509–526. Mosby, St. Louis, MO.

REFERENCES *(continued)*

7. **Gilchrist, M. J. R., W. P. McKinney, J. M. Miller, and A. S. Weissfeld.** 2000. *Cumitech 33, Laboratory Safety, Management, and Diagnosis of Biological Agents Associated with Bioterrorism.* Coordinating ed., J. W. Snyder. ASM Press, Washington, DC.
8. **Hudson, B. W., K. Wolff, and T. Butler.** 1980. The use of solid-phase-radioimmunoassay techniques for serodiagnosis of human plague infection. *Bull. Pan Am. Health Org.* **14:**244–250.
9. **Koneman, E. W., S. D. Allen, W. M. Janda, P. C. Schreckenberger, and W. C. Winn, Jr. (ed.).** 1997. *Color Atlas and Textbook of Diagnostic Microbiology,* 5th ed., p. 171–252. Lippincott, Philadelphia, PA.
10. **McDonough, K. A., T. G. Schwan, R. E. Thomas, and S. Falkow.** 1988. Identification of *Yersinia pestis*-specific DNA probe with potential for use in plague surveillance. *J. Clin. Microbiol.* **26:**2515–2519.
11. **Norkina, O. V., A. N. Kulichenko, A. L. Gintsberg, I. V. Tuchkov, Y. A. Popov, M. U. Aksenor, and I. G. Drosdov.** 1994. Development of a diagnostic test for *Yersinia pestis* by the polymerase chain reaction. *J. Appl. Bacteriol.* **76:**240–245.
12. **Nunes, P. M., and I. Suassuna.** 1978. Bacteriophage specificity in the identification of *Yersinia pestis* as compared with other Enterobacteriaceae. *Rev. Bras. Pesqui Med. Biol.* **11:**359–363.
13. **Perry, R. D., and J. D. Fetherston.** 1997. *Yersinia pestis*—etiologic agent of plague. *Clin. Microbiol. Rev.* **10:**35–66.
14. **Sharp, S. E.** 2008. The genus *Yersinia,* p. 603–608. *In* E. Goldman and L. H. Green (ed.), *Practical Handbook of Microbiology,* 2nd ed. CRC Press, Boca Raton, FL.
15. **Suzuki, S., H. Sakakibara, and S. Horta.** 1977. Latex agglutination tests for measurements of antiplague antibodies. *J. Clin. Microbiol.* **6:**332–336.
16. **Titball, R. W., and S. E. Leary.** 1998. Plague. *Br. Med. Bull.* **54:**625–633.
17. **Wilmoth, B. A., M. C. Chu, and T. J. Quan.** 1996. Identification of *Yersinia pestis* by BBL Crystal Enteric Nonfermenter identification system. *J. Clin. Microbiol.* **43:**2829–2830.

SUPPLEMENTAL READING

Centers for Disease Control and Prevention. 2000. Biological and chemical terrorism: strategic plan for preparedness and response. *MMWR Morb. Mortal. Wkly. Rep.* **49**(RR-4):1–14.

Klietmann, W. F., and K. L. Ruoff. 2001. Bioterrorism: implications for the clinical microbiologist. *Clin. Microbiol. Rev.* **14:**364–381.

16.8 Tularemia—*Francisella tularensis*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Francisella tularensis is a tiny (0.2- to 0.5-by 0.7- to 1.0-μm), pleomorphic, nonmotile, fastidious, gram-negative, facultative intracellular coccobacillus. *F. tularensis* can be divided into two subspecies, *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *holarctica* (type B), based on virulence testing, 16S sequencing, biochemical reactions, and epidemiological features. Type A and type B strains are highly infectious (e.g., require only 50 to 100 organisms to cause disease) and are the principal agents of tularemia, a zoonotic plague-like disease distributed only in the northern hemisphere (3, 4). In the United States, the principal reservoir is the cottontail rabbit (lagomorph), but the disease may also be carried and transmitted by a variety of terrestrial and aquatic mammals, such as beavers, ground squirrels, muskrats, and other rodents (3, 4). Transmission to humans can occur through the handling of infected animals; through the bites of ticks, mosquitoes, or deerfly vectors; or by ingestion of contaminated stream water. *F. tularensis* subsp. *novicida* is infrequently identified as a cause of human disease.

Tularemia presents in the human population in one of several forms depending on the route of entry of the organism into the body. The most common form is ulceroglandular disease (45 to 80%), indicating an insect bite as the mode of entry (2, 4). Glandular, oculoglandular, typhoidal, septic, oropharyngeal, and pneumonic forms are rarer. The incubation period is 1 to 14 days (average, 3 to 5 days) (3). Symptoms are not unique. Onset is abrupt, typically with fever, chills, headache, generalized body aches, pharyngitis, cough, and chest pain or tightness. There may be an ulcer at the inoculation site. Bacteria are transported from the site(s) of penetration by the lymphatic system to the lymph nodes and then disseminated to the major target organs—lungs, spleen, liver, and kidneys (2). Untreated, nonspecific symptoms may persist for several weeks, characterized by progressive weakness, sweats, and weight loss (1, 2, 4). Any of the principal forms of tularemia may be complicated by hematogenous spread, leading to pneumonia (most common), sepsis (uncommon), and meningitis (rare) (2, 4). The pneumonic form of tularemia may result in a mortality rate of 30% or greater if treatment is not initiated. Person-to-person transmission has not been documented.

II. SAFETY CONSIDERATIONS

It is imperative that these cultures be handled in a biological safety cabinet.

This organism is highly virulent and must be handled with extreme caution. Laboratory infections can be acquired through any manipulation in which aerosols or droplets are produced.

Handle all clinical specimens suspected of containing *F. tularensis* using BSL 2 procedures. BSL 3 conditions are recommended for all culture manipulations as soon as *F. tularensis* is suspected (1, 4).

F. tularensis is a select agent. Sentinel laboratories must rule it out or refer potential isolates to a Laboratory Response Network (LRN) reference-level laboratory. Research or other laboratories require registration with the CDC and/or U.S.Department of Agriculture for possession, use, storage, and/or transfer. See the following website for additional information: http://www.selectagents.gov/securitydoc.htm.

III. SPECIMEN COLLECTION AND PROCESSING

Observe standard precautions.

◩ **NOTE:** When this organism is suspected, the physician must communicate this information to the laboratory because of its danger to laboratory personnel and need for special diagnostic procedures (2).

A. Specimens of choice (1–4)

1. Lymph node aspirate
2. Sputum, bronchial washings, and gastric washings
3. Ulcer material—scraping preferable; swab of ulcer acceptable
4. Blood—recovery requires optimum volume (10 ml) per bottle
5. CSF
6. Urine

B. Specimen transport and storage

Clinical specimens should be inoculated onto culture medium as soon as possible.

1. If it is necessary to hold specimens for more than a few hours, refrigerate at 4°C after moistening with sterile broth or saline.
2. If a specimen needs to be shipped to a distant laboratory for processing, it should be kept cold but not frozen (1, 4).

C. Direct examination

1. Gram staining of biopsy material may be of little value. The Gram stain may be difficult to interpret because the bacterial cells are minute and faintly staining and may not be readily distinguished from background material (1, 4).
2. Bipolar staining is not a feature of this organism (1, 2).

It is imperative that these cultures be handled in a biological safety cabinet.

D. Inoculation of specimen onto media

1. Streak the specimen for isolation onto the following media.
  a. CHOC
  b. BAP or equivalent
  c. MAC or EMB
  d. Modified Thayer-Martin agar for contaminated specimens (2)
2. Inoculate the specimen into THIO broth and into BHI or TSB supplemented with 1% IsoVitaleX (4).
3. Incubate at 35 to 37°C in ambient air. Use of 5% CO_2 is acceptable but not required.
4. Incubate primary plates for at least 10 days before discarding.
5. Inoculate blood specimens into the appropriate broth bottles as per individual institutional policy using optimum volumes of blood. *F. tularensis* is only occasionally isolated from blood cultures (2). It may grow in commercial blood culture media (1).

ANALYTICAL CONSIDERATIONS

IV. ORGANISM IDENTIFICATION

It is imperative that these cultures be handled in a biosafety cabinet.

A. Culture characteristics

1. At 24 h on BAP, CHOC, or Thayer-Martin agar
  a. Gray-white, translucent colonies
  b. Usually too small to be seen individually
2. At 48 h
  a. BAP: 1- to 2-mm diameter, gray-white to bluish gray, opaque, no hemolysis or a tiny alpha-hemolytic zone; will not grow on subculture to BAP due to lack of cysteine in medium (1)
  b. Thayer-Martin agar or CHOC: 2- to 4-mm diameter, gray, raised, smooth, moist (4)
  c. No growth on MAC or EMB

IV. ORGANISM IDENTIFICATION *(continued)*

d. Oxidase negative
e. Catalase weakly positive
f. Beta-lactamase (cefinase) positive (1)
g. Satellite test negative using *Staphylococcus aureus* ATCC 25923 (1)
h. Urease test negative

3. Broth growth characteristics (3)
Broth tubes may require 10 days of incubation due to slow growth of the organism.
a. THIO: growth is seen first as a dense band near the top that diffuses throughout the medium as the culture grows
b. Blood culture bottles: hold bottles for 7 to 10 days. If growth is detected, a sample should be removed for subculture on appropriate agar and for Gram staining.

B. Staining characteristics
The organisms are very, very tiny, pleomorphic, faintly staining gram-negative coccobacilli that are hard to visualize even at a magnification of ×1,000.

C. Identification (Fig. 16.8–1) (1)
1. Presumptive identification of *F. tularensis* includes the following.
a. Gram stain morphology
b. Isolates from blood, respiratory specimens, or tissue grow slowly on CHOC but poorly or not at all on BAP.
c. Negative oxidase reaction
d. Weakly positive catalase reaction

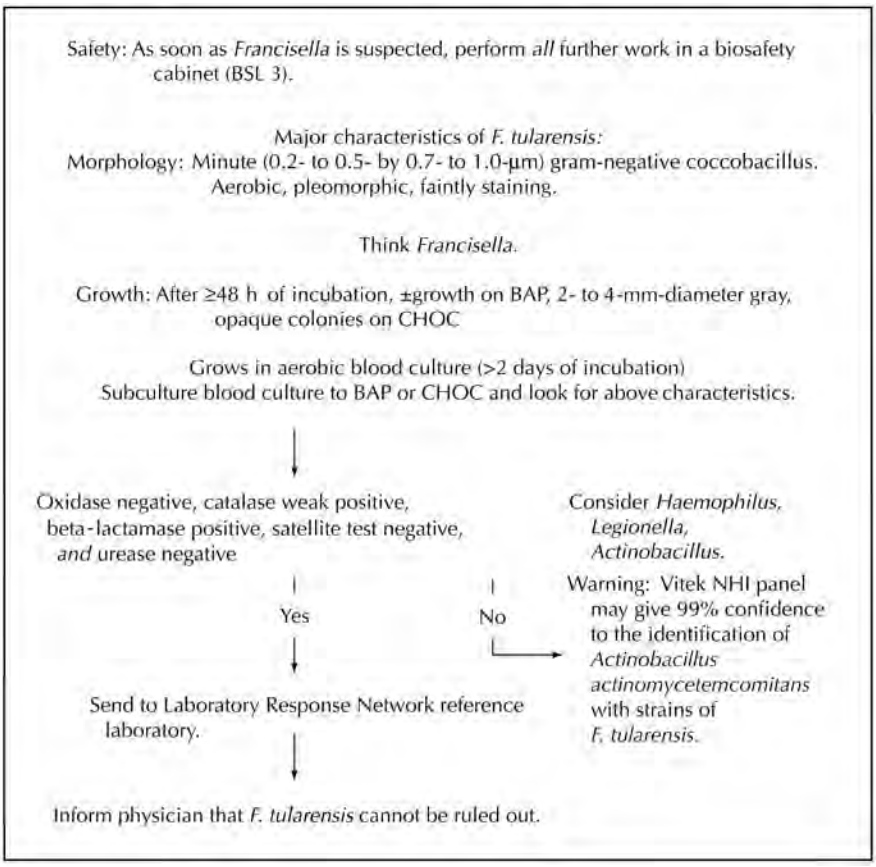
Safety: As soon as Francisella is suspected, perform all further work in a biosafety cabinet (BSL 3).
Major characteristics of F. tularensis:
Morphology: Minute (0.2- to 0.5- by 0.7- to 1.0-µm) gram-negative coccobacillus. Aerobic, pleomorphic, faintly staining.
Think Francisella.
Growth: After ≥48 h of incubation, ±growth on BAP, 2- to 4-mm-diameter gray, opaque colonies on CHOC
Grows in aerobic blood culture (>2 days of incubation)
Subculture blood culture to BAP or CHOC and look for above characteristics.
Oxidase negative, catalase weak positive, beta-lactamase positive, satellite test negative, and urease negative
Consider Haemophilus, Legionella, Actinobacillus.
Yes
No
Warning: Vitek NHI panel may give 99% confidence to the identification of Actinobacillus actinomycetemcomitans with strains of F. tularensis.
Send to Laboratory Response Network reference laboratory.
Inform physician that F. tularensis cannot be ruled out.

Figure 16.8–1 *F. tularensis* sentinel-level laboratory flowchart.

IV. ORGANISM IDENTIFICATION *(continued)*

e. Beta-lactamase (cefinase) positive
f. Negative satellite test
g. Negative urease

■ *Do not attempt identification using commercial identification systems due to the probability of misidentification.*

2. Refer isolate to an LRN reference laboratory for confirmatory identification.
3. Antimicrobial susceptibility testing
a. Although Clinical and Laboratory Standards Institute (CLSI) guidelines are available, antimicrobial susceptibility testing *should not* be performed in sentinel laboratories due to the hazards associated with this process.
b. All isolates of *F. tularensis* produce beta-lactamase and therefore are resistant to the beta-lactam classes of antimicrobial agents.
c. Isolates are usually susceptible to gentamicin, tetracycline, doxycycline, ciprofloxacin, levofloxacin, streptomycin, and chloramphenicol (5).

REFERENCES

1. **American Society for Microbiology.** 2001. *Basic Protocols for Sentinel Laboratories for the Presumptive Identification of* Francisella tularensis. American Society for Microbiology, Washington, DC. http://www.asm.org.
2. **Dennis, D. T., T. V. Inglesby, D. A. Henderson, J. G. Bartlett, M. S. Ascher, et al.** 2001. Tularemia as a biological weapon: medical and public health management. *JAMA* **285:**2763–2773.
3. **Ellis, J., P. C. F. Oyston, M. Green, and R. W. Titball.** 2002. Tularemia. *Clin. Microbiol. Rev.* **15:**631–646.
4. **Lindquist, D., M. C. Chu, and W. S. Probert.** 2007. *Francisella* and *Brucella*, p. 815–834. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
5. **Urich, S. K., and J. M. Petersen**. 2008. In vitro susceptibility of isolates of *Francisella tularensis* types A and B from North America. *Antimicrob. Agents Chemother.* **52:**2276–2278.

SUPPLEMENTAL READING

Centers for Disease Control and Prevention. 2000. Biological and chemical terrorism: strategic plan for preparedness and response. *MMWR Morb. Mortal. Wkly. Rep.* **49:**1–14.

Department of Health and Human Services. 2007. *Biosafety in Microbiological and Biomedical Laboratories*, 5th ed. U.S. Government Printing Office, Washington, DC.

Gilchrist, M. J. R., W. P. McKinney, J. M. Miller, and A. S. Weissfeld. 2000. *Cumitech 33, Laboratory Safety, Management, and Diagnosis of Biological Agents Associated with Bioterrorism.* Coordinating ed., J. W. Snyder. ASM Press, Washington, DC.

Klietmann, W. F., and K. L. Ruoff. 2001. Bioterrorism: implications for the clinical microbiologist. *Clin. Microbiol. Rev.* **14:**364–381.

16.9 Melioidosis *(Burkholderia pseudomallei)* and Glanders *(Burkholderia mallei)*

I. PRINCIPLE

Burkholderia mallei and *Burkholderia pseudomallei* are considered category B select agents since they are not as easily spread as category A organisms, nor do infections due to these organisms have mortality rates as high as those of category A organisms.

Burkholderia mallei is the etiologic agent of glanders, a febrile illness typically seen in equines, i.e., horses, mules, and donkeys (2, 4). It is a nonmotile, aerobic gram-negative coccobacillus, which may or may not be oxidase positive or grow on MAC. Infections with this organism can be either cutaneous or systemic (2). The incubation period is typically 1 to 14 days. Patients with the cutaneous infection have nodules with accompanying localized lymphadenitis. The systemic illness usually manifests itself as either bronchopneumonia or lobar pneumonia. Bacteremia may also occur, resulting in lesions being seen in the liver and spleen. Infection in humans with *B. mallei* is often fatal without antimicrobial treatment (10). Because of a program of compulsory slaughter of infected or seropositive horses or other animals, *B. mallei* has been eradicated from the United States and Western Europe. The last naturally acquired human case of glanders in the United States was seen in 1945, but a case of laboratory-acquired glanders occurred in 2000 (2). Equines are the primary reservoir of the rare cases of glanders still seen in Eastern Europe, the Middle East, Asia, and Africa (2).

Burkholderia pseudomallei was thought to cause a glanders-like illness in humans (27) and was called *"pseudomallei"* by Stanton and Fletcher (8). The disease in humans due to *B. pseudomallei* is now referred to in the medical literature as "melioidosis," from the Greek word *"melis,"* which was the term for distemper in donkeys (19). Melioidosis is a disease endemic in the tropical regions of the world, with the majority of cases in the medical literature being reported from rice-growing regions of Southeast Asia and the tropical, northern regions of Australia (7, 15). The organism has been detected in very high concentrations in water found in rice paddies in both Vietnam and Thailand (11). There are data to suggest that this organism is also endemic in both the Philippines and the Indian subcontinent (8). There is little known about the prevalence of this organism in tropical regions of Africa and the Americas, although cases have been recently documented in both Brazil and Honduras (3, 18). Infections with this organism in the United States and Western Europe are almost certainly imported from regions of endemicity (3, 11). Since the Vietnam War, melioidosis been referred to as the "Vietnam time bomb" because the disease, much like tuberculosis, can reactivate in returning soldiers after remaining latent for decades (6, 8, 20).

The recognition of multiple cases of melioidosis in North America or Western Europe in patients without an appropriate travel history requires a thorough investigation of the possibility of a bioterrorism attack.

B. pseudomallei is an oxidase-positive, aerobic gram-negative bacillus that is straight or slightly curved. The organism will grow on most standard laboratory media, such as sheep blood agar, CHOC, and MAC, and it produces a characteristic musty odor (11). The recent sequencing of the genomes suggests that *B. mallei* is a recently evolved clone of *B. pseudomallei. B. mallei* has a smaller genome, which makes it much less environmentally adaptable (14). Both species were classified as members of the genus *Pseudomonas*, but in 1992 they were reclassified into the genus *Burkholderia* (28).

Two organisms which are very similar to *B. pseudomallei* phenotypically have recently been described in the literature (9, 12). *Burkholderia thailandensis* is an environmental organism found in rice paddy water and soil in Thailand. It has been shown to be of comparatively low virulence in animal models and has been infrequently reported to be a cause of human disease (9, 12). The second organism, *Burkholderia oklahomensis*, has been reported from two cases in the United States and has been found to be essentially avirulent in animal models (9). These two organisms are not differentiated from *B. pseudomallei* by the algorithm supplied in this protocol. However, given the rarity of isolation of these two organisms in clinical settings, it is unlikely that they will be encountered in critically ill individuals.

The primary responsibility of the sentinel laboratory is to rule out these two organisms using the laboratory procedure that follows. Because travellers to regions of endemicity in Southeast Asia and northern Australia may acquire *B. pseudomallei* from the environment, finding of this organism may not represent an intentional release of the organism. Regardless, organisms suspected of being either *B. mallei* or *B. pseudomallei* should be referred to the appropriate Laboratory Response Network (LRN) reference laboratory, typically the city, regional, or state public health laboratory, for confirmation.

II. SPECIMEN COLLECTION

The primary tool for the diagnosis of both *B. mallei* and *B. pseudomallei* is blood culture. All of the following specimens should be collected in accordance with the institution's standard protocols.

Sentinel laboratories should not accept environmental or animal specimens; such specimens should be forwarded directly to the appropriate public health laboratory.

A. Specimens

1. Blood or bone marrow
   - **a.** Standard automated blood culture system
   - **b.** Lysis centrifugation system
2. Sputum or bronchoscopically obtained specimens
3. Tissue specimens (biopsy samples and abscess aspirates) and wound swabs
4. Urine
5. Serum (1 ml)

   Both acute-phase and convalescent-phase (obtained 14 days after the acute-phase specimen) specimens should be collected if serologic diagnosis of *B. pseudomallei* infection is being considered. Currently, no serology for *B. mallei* is available in the United States.

B. Specimen transport

1. Blood should be inoculated directly into blood culture bottles in accordance with the institution's protocol for routine blood cultures.
2. Abscess material, wound swabs, urine, sputum, or bronchoscopically obtained specimens can be transported at ambient temperature if there will be less than a 2-h delay and at 4°C if the delay will be between 2 and 24 h.

III. MATERIALS

A. Media

1. Blood culture bottles
2. CHOC
3. BAP
4. MAC
5. Selective agars for *Burkholderia pseudomallei*, including Ashdown and *Burkholderia cepacia* selective agars (11, 25; see also procedure 3.11.3)
6. Standard enrichment broth (thioglycolate or brain heart infusion broth; may substitute others if they support the growth of environmental gram-negative rods)

B. Supplies

1. Sterile disposable bacteriological inoculating loops
2. Glass slides
3. Disinfectants (phenolic acid-based or hypochlorite-based ones are appropriate)
4. Sterile swabs and transport devices

Caution: *All patient specimens and culture isolates should be handled while wearing gloves and gowns in a BSL 2 cabinet. Plates should be taped shut when incubating them. Alternatively, BSL 2 practices utilizing standard PPE (masks, gloves, and gowns) with safety glasses or face shields will provide sufficient protection. Any activity that may generate aerosols, such as preparing standard inoculum for identification systems, should be done using BSL 2 safety principles, preferably in a BSL 2 biological safety cabinet. Infections following laboratory exposure have been documented with both organisms (2, 26). For both, breaks in standard laboratory safety protocols were reported, emphasizing the importance of adhering to standard BSL 2 safety practices with these organisms. It is recommended if* B. pseudomallei *or* B. mallei *is suspected that it be transferred to a BSL 3 facility as soon as possible (26).*

IV. QUALITY CONTROL

Refer to the appropriate procedures in section 3 of this handbook and local and state regulations for QC requirements for media, stains, and reagents. Document QC per standard laboratory procedure/protocol.

V. PROCEDURE

A. Specimen processing

1. Tissue specimens, wound swabs, sputum, and bronchoscopically obtained specimens should be plated on BAP or CHOC and MAC. Enrichment broth should be inoculated for tissue specimens and wound swabs.
   - **a.** Ashdown agar or *Burkholderia cepacia* selective agar should be used if available and a high pretest probability exists (i.e., other cases have been reported) for the isolation of *B. pseudomallei* but *not B. mallei.*
   - **b.** For improved isolation, a colistin disk or polymyxin B disk may be placed in the initial inoculation area of the BAP if isolation of *Burkholderia* spp. is specifically requested.
   - **c.** A smear for Gram stain should be made.
2. Urine cultures should be performed according to the institution's standard quantitative urine streaking technique on BAP and MAC.

B. Incubation and examination of cultures

1. All cultures should be incubated at 35 to 37°C under ambient conditions. Incubation in CO_2 is acceptable.
2. Primary plates should be incubated for a minimum of 5 days and should be examined daily. *B. pseudomallei* reliably grows with 5 days of incubation from blood cultures, so extended incubation of either broth or plated blood cultures (lysis-centrifugation) is not necessary. *B. mallei* does not grow as rapidly as *B. pseudomallei* and may require extended incubation to as long as 14 days.
3. Recovery of *B. pseudomallei* from blood culture within the first 24 h of incubation indicates fulminant sepsis, which has a very high (90%) mortality rate.

C. Identification

1. The procedures described below are to be used to rule out the presence of *B. mallei* and *B. pseudomallei* in clinical specimens. These procedures do not differentiate *B. pseudomallei* from *B. thailandensis* or *B. oklahomensis.* Because the two species share >99% homology at the nucleotide level, differentiation between them at the molecular level is challenging (1). Biochemical differentiation can be more productive since they have unique characteristics.

   ◩ **NOTE:** Alternately, API 20NFT, also called 20NE (bioMérieux, Durham, NC), or Vitek 1 gram-negative identification panels (bioMérieux) can be used for preliminary identification of *B. pseudomallei* but not *B. mallei* with reasonable accuracy. For a review of the performance of commercial systems, see reference 24.

   While only the API 20NFT and the Vitek systems have been studied extensively (16, 22), other systems that have arginine as one of the biochemical tests may work well. *B. pseudomallei* is in the database of the MicroScan overnight system but not the rapid, gram-negative rod panel, although the sensitivity and specificity of the overnight product have not been studied extensively. *B. pseudomallei* is not in the Phoenix (BD) database and has been reported to call the isolates *B. cepacia* (21). Isolates identified by any commercial system as *B. pseudomallei* should be confirmed by an LRN reference laboratory before a final report is issued to a clinician.
2. Colony morphology
   - **a.** On BAP, *B. mallei* shows smooth, gray, translucent colonies in 2 days, without pigment or distinctive odor. *B. mallei* grows without any inhibition around the colistin or polymyxin B disk. Colonies may or may not be present on MAC.

V. PROCEDURE *(continued)*

b. On BAP, *B. pseudomallei* forms small, smooth, creamy colonies in the first 1 to 2 days, which gradually change after a few days to dry, wrinkled colonies similar to *Pseudomonas stutzeri*. Colonies are neither yellow nor violet pigmented. *B. pseudomallei* grows without any inhibition around the colistin or polymyxin B disk. Colonies are present on both BAP and MAC (Fig. 16.9–1 and 16.9–2). *B. pseudomallei* often produces a distinctive musty or earthy odor that is very pronounced on opening a petri dish growing the microorganism or even opening an incubator door when a positive plate is present. *"Sniffing" of plates containing* B. pseudomallei *is dangerous and should not be done.* However, the odor will be apparent without sniffing.

3. Gram stain morphology

a. *B. mallei* is a small, straight or slightly curved gram-negative coccobacillus. Cells are arranged in pairs, parallel bundles, or the Chinese-letter form.

b. *B. pseudomallei* is a small, straight, or slightly curved gram-negative rod; it may demonstrate bipolar morphology in direct specimens.

c. The organisms may be observed in direct Gram stain from respiratory specimens or abscess material/wounds. They can also be seen in smears of positive blood culture bottles.

4. Perform the following tests.

a. Catalase test. (procedure 3.17.10)

(1) If the isolate is *not* growing well on MAC in 48 h, perform the catalase test.

(2) Both *B. pseudomallei* and *B. mallei* are catalase positive.

b. Oxidase test. (procedure 3.17.39)

(1) *B. mallei* may be oxidase positive or negative.

(2) *B. pseudomallei* is oxidase positive.

c. Spot indole test (procedure 3.17.23)

B. mallei and *B. pseudomallei* are spot indole negative.

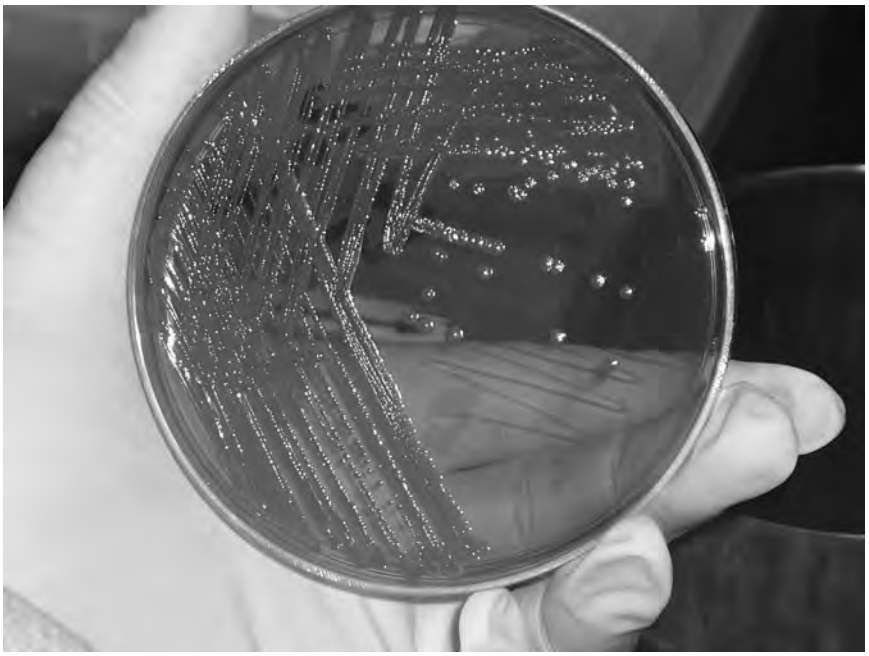

Figure 16.9–1 *B. pseudomallei* on MAC after 48 h. (Courtesy of Robert Sautter.)

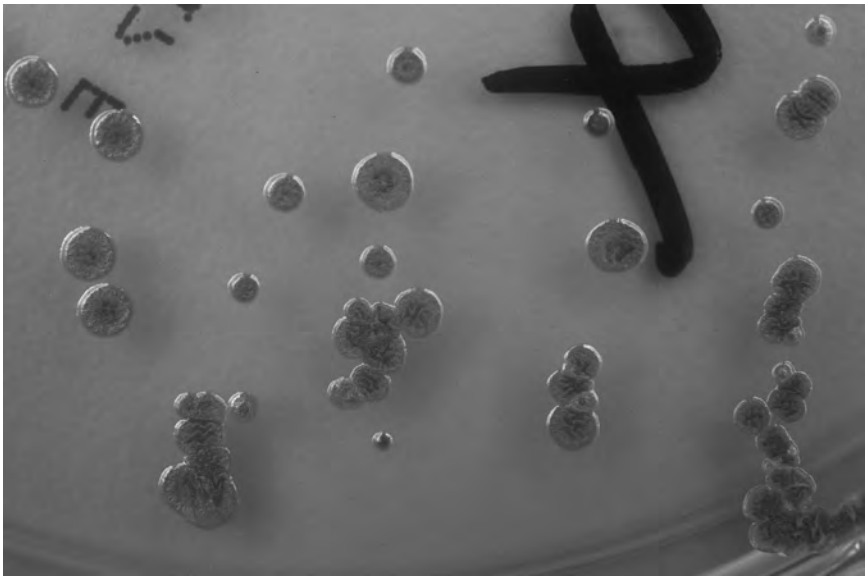

Figure 16.9–2 *B. pseudomallei* on a MAC plate. (Courtesy of Matthias Maiwald.)

V. PROCEDURE *(continued)*

d. Colistin or polymyxin B resistance (procedure 3.17.4)
- (1) *B. mallei* and *B. pseudomallei* are resistant to polymyxin B and colistin.
- (2) Other *Burkholderia* species, *Chromobacterium violaceum*, some *Vibrio* species, and *Ralstonia* are resistant; most *Pseudomonas* species are susceptible.
- (3) As an alternative, growth on *B. cepacia* selective agars or modified Thayer-Martin agar may substitute for the disk test, because these media contain polymyxin B or colistin. However, the lack of growth on these media should be confirmed by the disk test.

e. Motility test (procedure 3.17.31)
- (1) The motility test should be performed if the isolate has the colony morphology and Gram stain reaction of *B. mallei* and is resistant to colistin or polymyxin B. Because of the danger of laboratory-acquired infection, the wet-mount motility test should not be performed; the tube test is recommended.
- (2) Use motility semisolid medium with 2,3,5-triphenyltetrazolium chloride (TTC) indicator.
- (3) A diffusible red growth spreading away from the stab line indicates motility.
- (4) *B. mallei* is nonmotile, and *B. pseudomallei* is motile.

f. Additional screening tests

If available, triple sugar ion (TSI), arginine dihydrolase, and nitrate may be performed to further exclude similar organisms.
- (1) TSI (procedure 3.17.9)

  B. mallei and *B. pseudomallei* are non-glucose-fermenting rods or coccobacilli, and the results observed will be an alkaline (red or no change) in tube butt/no H_2S (no black color). *B. pseudomallei* may or may not produce an acid (yellow) slant, due to oxidation of lactose.
- (2) Arginine dihydrolase with Moeller's base control (procedure 3.17.15)

  B. mallei and *B. pseudomallei* are positive for arginine dihydrolase. If positive the arginine tube will be clearly purple, while Moeller base control will not change color or will have become yellow.

V. PROCEDURE *(continued)*

(3) Nitrate reduction with Durham tube for gas production (procedure 3.17.35)

B. mallei typically reduces nitrate without gas and will give a red color with the addition of reagents 1 and 2 and is therefore nitrate reductase positive without gas. *B. pseudomallei* typically reduces nitrate to nitrogen gas and is therefore nitrate reductase positive with gas. *B. pseudomallei* often produces only one small bubble of gas. The test may not be positive at 24 h and should be incubated for 5 days, with samples removed for testing daily.

VI. PRESUMPTIVE IDENTIFICATION OF *B. MALLEI* AND *B. PSEUDOMALLEI*

A. Isolates suspected to be *B. mallei* based on colony and Gram stain morphology should have the following biochemical test characteristics: catalase positive, oxidase variable, spot indole negative, colistin or polymyxin B resistant, and nonmotile. If supplemental biochemical tests are used, *B. mallei* will be K/N without H_2S production on TSI, arginine dihydrolase positive, and nitrate reductase negative without gas production. Isolates having this profile should be referred to an LRN reference laboratory. Supplemental biochemical testing will increase the specificity of the presumptive identification but is not required for ruling out *B. mallei* in an LRN sentinel laboratory.

There is only one study that describes the reliability of commercial systems for the identification of *B. mallei.* Both API 20 NE and RapID NF Plus (Remel, Lenexa, KS) either incorrectly identified or failed to identify 23 *B. mallei* isolates (13). Therefore, isolates that screen as potentially *B. mallei* and are not identified by commercial systems should be referred to an LRN reference laboratory for identification (Fig. 16.9–3).

B. Isolates suspected to be *B. pseudomallei* based on Gram stain, colonial morphology, and musty odor have the following biochemical test characteristics: catalase positive, oxidase positive, spot indole negative, colistin or polymyxin B resistant, and motility positive. If supplemental biochemical tests are used, *B. pseudomallei* will be K/N without H_2S production on TSI, arginine dihydrolase positive, and nitrate reductase negative with gas production. Isolates having this profile should be referred to an LRN reference laboratory (Fig. 16.9–4). Supplemental biochemical testing will increase the specificity of the presumptive identification but is not required for ruling out *B. pseudomallei* in an LRN sentinel laboratory.

Initial studies suggested that the API 20E and 20NE systems and Vitek 1 (but not Vitek 2) (bioMérieux) were reasonably reliable systems for the identification of *B. pseudomallei* (16, 22) (Fig. 16.9–4). More recent studies suggest that the API 20NE is not as reliable for identification of *B. pseudomallei* when a greater variety of isolates are tested (13, 17). In a recent study, when a colorimetric rather than fluorometric identification system was used in the Vitek 2, the identification of *B. pseudomallei* was still not particularly accurate (23). These data suggest that organisms identified as *B. pseudomallei* by commercial systems should be referred to an LRN reference laboratory for confirmation (Fig. 16.9–5). In addition, non-glucose fermenters which screen as potential *B. pseudomallei* isolates using the above-listed screening tests and give "no identification" by commercial systems should also be referred. Finally, some strains of *B. pseudomallei* have been misidentified by the API 20E and 20NE systems as *C. violaceum* (16, 22). Therefore, strains that are not violet but are identified as *C. violaceum* by the API system should also be referred to an LRN reference laboratory for confirmation.

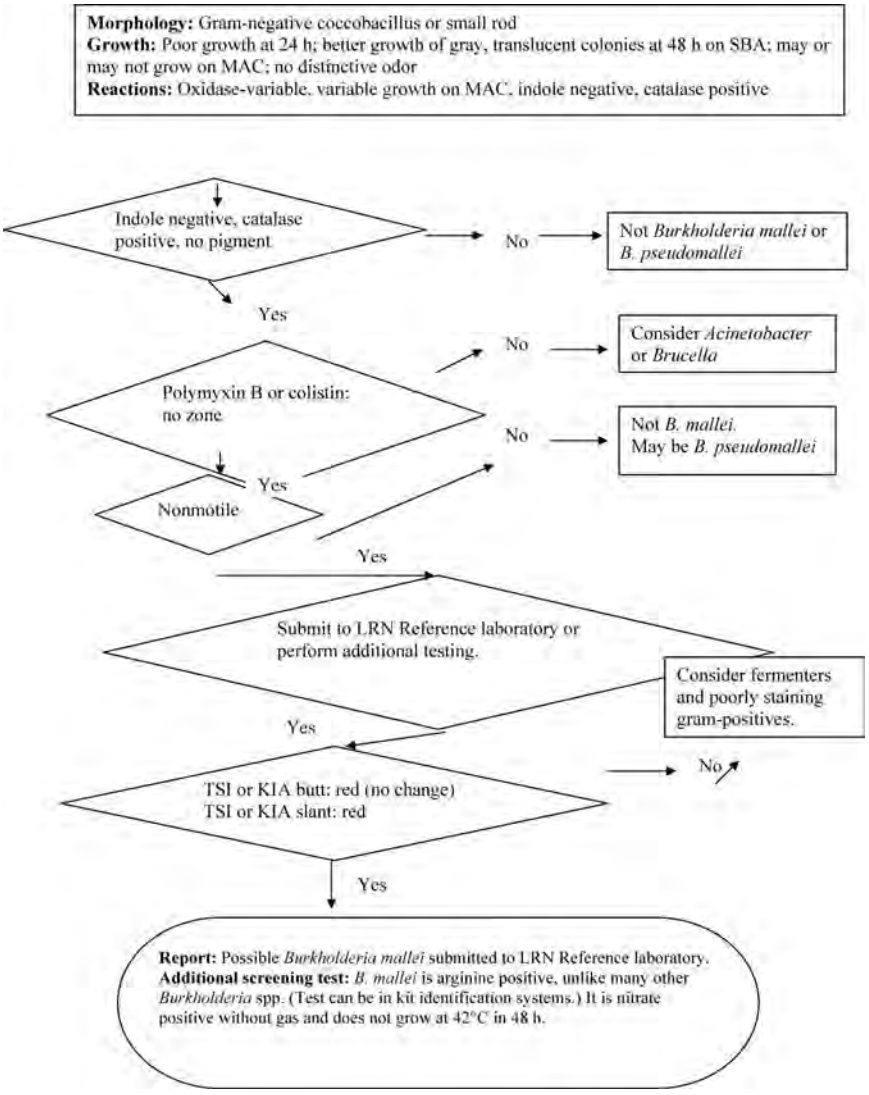
Morphology: Gram-negative coccobacillus or small rod
Growth: Poor growth at 24 h; better growth of gray, translucent colonies at 48 h on SBA; may or may not grow on MAC; no distinctive odor
Reactions: Oxidase-variable, variable growth on MAC, indole negative, catalase positive
Indole negative, catalase positive, no pigment
No
Not *Burkholderia mallei* or *B. pseudomallei*
Yes
No
Consider *Acinetobacter* or *Brucella*
Polymyxin B or colistin: no zone
No
Not *B. mallei*. May be *B. pseudomallei*
Yes
Nonmotile
Yes
Submit to LRN Reference laboratory or perform additional testing.
Consider fermenters and poorly staining gram-positives.
Yes
No
TSI or KIA butt: red (no change)
TSI or KIA slant: red
Yes
Report: Possible *Burkholderia mallei* submitted to LRN Reference laboratory.
Additional screening test: *B. mallei* is arginine positive, unlike many other *Burkholderia* spp. (Test can be in kit identification systems.) It is nitrate positive without gas and does not grow at 42°C in 48 h.

Figure 16.9–3 Flowchart for the presumptive identification of *B. mallei* using conventional phenotypic tests (sentinel laboratory).

VII. REPORTING RESULTS

A. Referral of presumptive *B. mallei* or *B. pseudomallei* isolates to LRN reference laboratory

1. When to refer

a. Naturally occurring cases of *B. mallei* are extremely rare in humans and should be referred to LRN reference laboratories in all cases.

b. Naturally occurring cases of *B. pseudomallei* may be observed in any of the following situations. Confirmation of the identification of these isolates may be requested from an LRN reference laboratory, but they are unlikely to represent a bioterrorism event. Patients who cannot be classified into any of the following patient populations may represent a bioterrorism event.

(1) Patients with acute infection who have a recent history of travel to the region of endemicity. This includes Southeast Asia (in particular Thailand, Vietnam, Myanmar, or Taiwan), the Philippines, the Indian subcontinent, or the northern coast of Australia. Recent U.S. cases

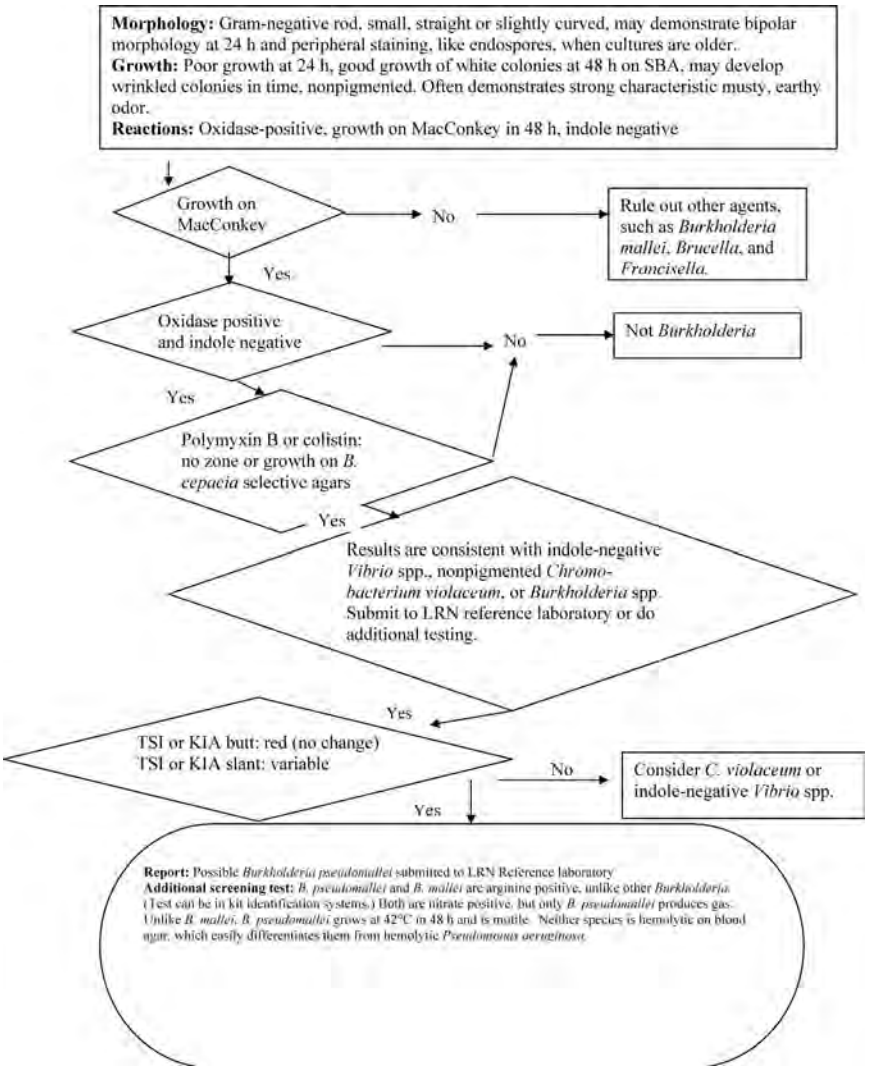
Morphology: Gram-negative rod, small, straight or slightly curved, may demonstrate bipolar morphology at 24 h and peripheral staining, like endospores, when cultures are older.
Growth: Poor growth at 24 h, good growth of white colonies at 48 h on SBA, may develop wrinkled colonies in time, nonpigmented. Often demonstrates strong characteristic musty, earthy odor.
Reactions: Oxidase-positive, growth on MacConkey in 48 h, indole negative
Growth on MacConkey
No
Rule out other agents, such as Burkholderia mallei, Brucella, and Francisella.
Yes
Oxidase positive and indole negative
No
Not Burkholderia
Yes
Polymyxin B or colistin: no zone or growth on B. cepacia selective agars
Yes
Results are consistent with indole-negative Vibrio spp., nonpigmented Chromobacterium violaceum, or Burkholderia spp. Submit to LRN reference laboratory or do additional testing.
Yes
TSI or KIA butt: red (no change)
TSI or KIA slant: variable
No
Consider C. violaceum or indole-negative Vibrio spp.
Yes
Report: Possible Burkholderia pseudomallei submitted to LRN Reference laboratory
Additional screening test: B. pseudomallei and B. mallei are arginine positive, unlike other Burkholderia. (Test can be in kit identification systems.) Both are nitrate positive, but only B. pseudomallei produces gas. Unlike B. mallei, B. pseudomallei grows at 42°C in 48 h and is motile. Neither species is hemolytic on blood agar, which easily differentiates them from hemolytic Pseudomonas aeruginosa.

Figure 16.9–4 Flowchart for the presumptive identification of *B. pseudomallei* using conventional phenotypic tests.

VII. REPORTING RESULTS
(continued)

have also been imported from Honduras, and cases have been reported in Brazil, so *B. pseudomallei* may also be considered, for biothreat reasons, to be endemic in tropical regions of Central and South America.

(2) Recent immigrants or visitors from the region of endemicity
(3) Patients with recent onset of diabetes, renal failure, or immunosuppressed states who have travelled in the region of endemicity mentioned above, even if that travel occurred decades before
(4) Individuals who work with animals (such as zoo employees) that have recently been imported from regions of endemicity
(5) Individuals who work in laboratories where they may be exposed to this organism

2. Whom to notify and when to notify them

a. Further epidemiological investigation is needed whenever a presumptive identification of *B. mallei* or *B. pseudomallei* is made.

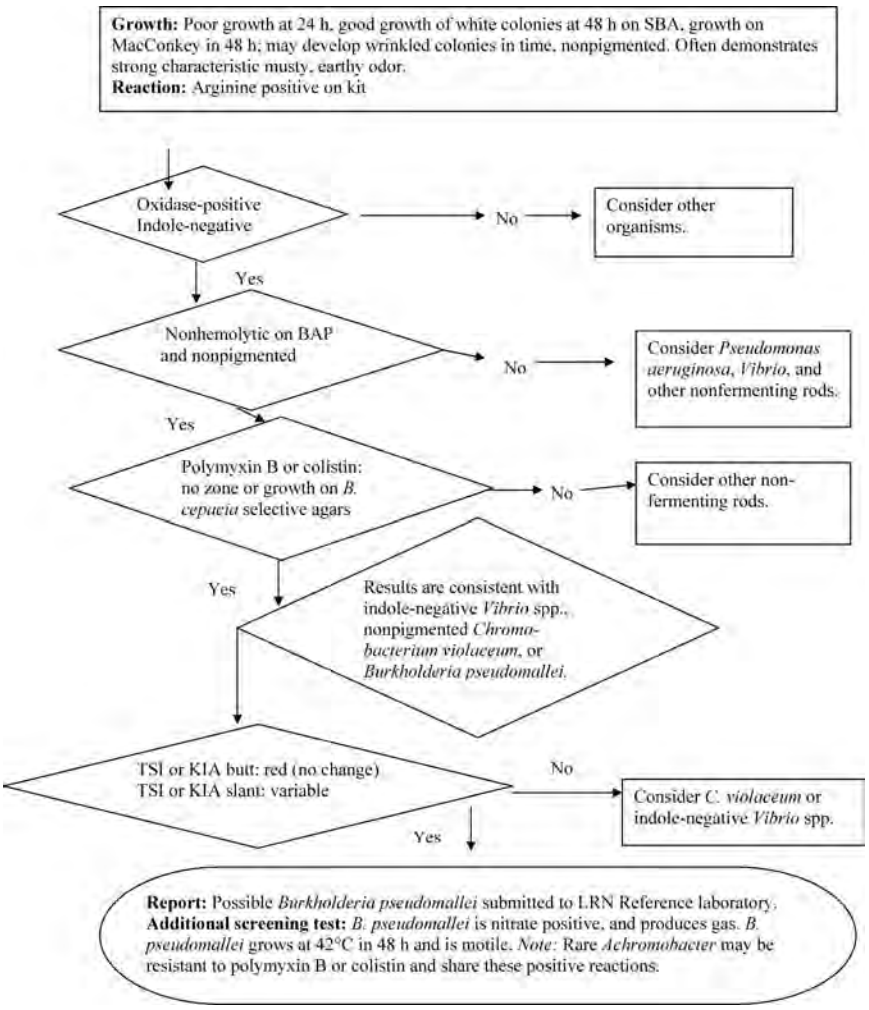
Growth: Poor growth at 24 h, good growth of white colonies at 48 h on SBA, growth on MacConkey in 48 h; may develop wrinkled colonies in time, nonpigmented. Often demonstrates strong characteristic musty, earthy odor.
Reaction: Arginine positive on kit
Oxidase-positive Indole-negative
No
Consider other organisms.
Yes
Nonhemolytic on BAP and nonpigmented
No
Consider *Pseudomonas aeruginosa*, *Vibrio*, and other nonfermenting rods.
Yes
Polymyxin B or colistin: no zone or growth on *B. cepacia* selective agars
No
Consider other nonfermenting rods.
Yes
Results are consistent with indole-negative *Vibrio* spp., nonpigmented *Chromobacterium violaceum*, or *Burkholderia pseudomallei*.
TSI or KIA butt: red (no change)
TSI or KIA slant: variable
No
Consider *C. violaceum* or indole-negative *Vibrio* spp.
Yes
Report: Possible *Burkholderia pseudomallei* submitted to LRN Reference laboratory.
Additional screening test: *B. pseudomallei* is nitrate positive, and produces gas. *B. pseudomallei* grows at 42°C in 48 h and is motile. *Note:* Rare *Achromobacter* may be resistant to polymyxin B or colistin and share these positive reactions.

Figure 16.9–5 Flowchart for the presumptive identification of *B. pseudomallei* for laboratories using selected commercial identification systems.

VII. REPORTING RESULTS *(continued)*

b. Within the hospital setting, the infectious disease service and/or infection control department should be notified so further investigation of the patient's history can be made in order to rule out naturally occurring infections.

c. The state laboratory director (or designate) should be notified of the presumptive identification of *B. mallei* or *B. pseudomallei.*

d. When referring the isolate to the LRN reference laboratory, the reference laboratory should provide the referring laboratory with guidance and recommendations for retaining the specimen or isolate. Once the identification is confirmed, and the isolate is confirmed as either *B. mallei* or *B. pseudomallei*, the sentinel laboratory is required to destroy it (e.g., by autoclaving). In particular, the appropriate material, including blood culture bottles, tubes and plates, and actual clinical specimens (aspirates, biopsy samples, and sputum specimens), should be saved until the reference laboratory confirms the identification.

e. Any environmental specimens collected for detection of *B. mallei* or *B. pseudomallei* as part of an epidemiological or criminal investigation should be referred directly to the state public health laboratory.

VII. REPORTING RESULTS
(continued)

f. The state public health laboratory director will coordinate notification of the CDC and state and federal law enforcement agencies.

B. Susceptibility testing

Susceptibility testing is performed only by the broth method and should be performed only in an appropriate BSL 2 laboratory, or in a BSL 3 laboratory if there is a danger of aerosol (5).

C. Action summary

1. Preserve all specimens and isolates.

2. Notify institutional infection control and the closest LRN reference laboratory, which typically will be the state public health laboratory when either organism is isolated.

3. Understand that *B. pseudomallei* infections may be naturally occurring and may not represent a biocrime. The infected patient's potential exposure to naturally occurring *B. pseudomallei* due to either travel or workplace exposure should be determined.

4. Refer potential *B. pseudomallei* and *B. mallei* isolates to the closest LRN reference laboratory for complete identification of the suspicious isolates. Work with the LRN reference laboratory to establish a chain of custody for the referred isolates.

REFERENCES

1. **Bauernfeind, A., C. Roller, D. Meyer, R. Jungwirth, and I. Schneider.** 1998. Molecular procedure for rapid detection of *Burkholderia mallei* and *Burkholderia pseudomallei*. *J. Clin. Microbiol.* **36:**2737–2741.
2. **Centers for Disease Control and Prevention.** 2000. Laboratory-acquired human glanders—Maryland, May 2000. *MMWR Morb. Mortal. Wkly. Rep.* **49:**532–535.
3. **Centers for Disease Control and Prevention.** 2006**.** Imported melioidosis—South Florida, 2005. *MMWR Morb. Mortal. Wkly. Rep.* **55:**873–876.
4. **Christopher, G. W., T. J. Cieslak, J. A. Pavlin, and E. M. Eitzen, Jr.** 1997. Biological warfare: a historical perspective. *JAMA* **278:**412–417.
5. **Clinical and Laboratory Standards Institute.** 2009. *Performance Standards for Antimicrobial Susceptibility Testing,* p. 88–90. Nineteenth informational supplement. CLSI document M100-S19. Clinical and Laboratory Standards Institute, Wayne, PA.
6. **Currie, B. J., D. A. Fisher, N. M. Anstey, and S. P. Jacups.** 2000. Melioidosis: acute and chronic disease, relapse and re-activation. *Trans. R. Soc. Trop. Med. Hyg.* **94:**301–304.
7. **Currie, B. J., D. A. Fisher, D. M. Howard, J. N. Burrow, D. Lo, S. Selva-Nayagam, N. M. Anstey, S. E. Huffam, P. L. Snelling, P. J. Marks, D. P. Stephens, G. D. Lum, S. P. Jacups, and V. L. Krause.** 2000. Endemic melioidosis in tropical northern Australia: a 10-year prospective study and review of the literature. *Clin. Infect. Dis.* **31:**981–986.
8. **Dance, D. A. B.** 1991. Melioidosis: the tip of the iceberg? *Clin. Microbiol. Rev.* **4:**52–60.
9. **DeShazer, D.** 2007. Virulence of clinical and environmental isolates of *Burkholderia oklahomensis* and *Burkholderia thailandensis* in hamsters and mice. *FEMS Microbiol. Lett.* **277:**64–69.
10. **Gilligan, P. H.** 2002. Therapeutic challenges posed by bacterial bioterrorism threats. *Curr. Opin. Microbiol.* **5:**489–495.
11. **Gilligan, P. H., G. Lum, P. Vandamme, and S. Whittier.** 2003. *Burkholderia, Stenotrophomonas, Ralstonia, Brevundimonas, Comamonas,* and *Acidovorax,* p. 729–748. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), *Manual of Clinical Microbiology,* 8th ed. ASM Press, Washington, DC.
12. **Glass, M. B., A. G. Steigerwalt, J. G. Jordan, P. P. Wilkins, and J. E. Gee.** 2006. *Burkholderia oklahomensis* sp. nov., a *Burkholderia pseudomallei*-like species formerly known as the Oklahoma strain of *Pseudomonas pseudomallei***.** *Int. J. Syst. Evol. Microbiol.* **56:**2171–2176.
13. **Glass, M. B., and T. Popovic.** 2005. Preliminary evaluation of the API 20NE and RapID NF Plus systems for rapid identification of *Burkholderia pseudomallei* and *B. mallei. J. Clin. Microbiol.* **43:**479–483.
14. **Holden, M. T., R. W. Titball, S. J. Peacock, A. M. Cerdeño-Tárraga, T. Atkins, L. C. Crossman, T. Pitt, C. Churcher, K. Mungall, S. D. Bentley, M. Sebaihia, N. R. Thomson, N. Bason, I. R. Beacham, K. Brooks, K. A. Brown, N. F. Brown, G. L. Challis, I. Cherevach, T. Chillingworth, A. Cronin, B. Crossett, P. Davis, D. DeShazer, T. Feltwell, A. Fraser, Z. Hance, H. Hauser, S. Holroyd, K. Jagels, K. E. Keith, M. Maddison, S. Moule, C. Price, M. A. Quail, E. Rabbinowitsch, K. Rutherford, M. Sanders, M. Simmonds, S. Songsivilai, K. Stevens, S. Tumapa, M. Vesaratchavest, S. Whitehead, C. Yeats, B. G. Barrell, P. C. Oyston, and J. Parkhill.** 2004. Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei. Proc. Natl. Acad. Sci. USA* **101:**14240–14245.

REFERENCES *(continued)*

15. **Hsueh, P. R., L. J. Teng, L. N. Lee, C. J. Yu, P. C. Yang, S. W. Ho, and K. T. Luh.** 2001. Melioidosis: an emerging infection in Taiwan? *Emerg. Infect. Dis.* **7:**428–433.
16. **Inglis, T. J. J., D. Chiang, G. S. H. Lee, and L. Chor-Kiang.** 1998. Potential misidentification of *Burkholderia pseudomallei* by API 20NE. *Pathology* **30:**62–64.
17. **Inglis, T. J. J., A. Merritt, G. Chidlow, M. Aravena-Roman, and G. Harnett.** 2005. Comparison of diagnostic laboratory methods for identification of *Burkholderia pseudomallei. J. Clin. Microbiol.* **43:**2201–2206.
18. **Inglis, T. J. J., D. B. Rolim, and A. De Queiroz Sousa.** 2006. Melioidosis in the Americas. *Am. J. Trop. Med. Hyg.* **75:**947–954.
19. **Ip, M., L. G. Osterberg, P. Y. Chau, and T. A. Raffin.** 1995. Pulmonary melioidosis. *Chest* **108:**1420–1424.
20. **Jones, A. L., T. J. Beveridge, and D. E. Woods.** 1996. Intracellular survival of *Burkholderia pseudomallei. Infect. Immun.* **64:**782–790.
21. **Koh, T. H., L. S. Yong Ng, J. L. Foon Ho, L.-H. Sng, G. C. Y. Wang, and R. Valentine Tzer Pin Lin.** 2003. Automated identification systems and *Burkholderia pseudomallei. J. Clin. Microbiol.* **41:**1809.
22. **Lowe, P., C. Engler, and R. Norton.** 2002. Comparison of automated and nonautomated systems for identification of *Burkholderia pseudomallei. J. Clin. Microbiol.* **40:**4625–4627.
23. **Lowe, P., H. Haswell, and K. Lewis.** 2006. Use of various common isolation media to evaluate the new VITEK 2 colorimetric GN card for identification of *Burkholderia pseudomallei. J. Clin. Microbiol.* **44:**854–856.
24. **O'Hara, C. M.** 2005. Manual and automated instrumentation for identification of *Enterobacteriaceae* and other aerobic gram-negative bacilli. *Clin. Microbiol. Rev.* **18:**147–162.
25. **Peacock, S. J., G. Chieng, A. C. Cheng, D. A. B. Dance, P. Amornchai, G. Wongsuvan, N. Teerawattanasook, W. Chierakul, N. P. J. Day, and V. Wuthiekanun.** 2005. Comparison of Ashdown's medium, *Burkholderia cepacia* medium, and *Burkholderia pseudomallei* selective agar for clinical isolation of *Burkholderia pseudomallei. J. Clin. Microbiol.* **43:**5359–5361.
26. **Peacock, S. J., H. P. Schweizer, D. A. B. Dance, T. L. Smith, J. E. Gee, V. Wuthiekanun, D. DeShazer, I. Steinmitz, P. Tan, and B. J. Currie.** 2008. Management of accidental laboratory exposure to *Burkholderia pseudomallei* and *B. mallei. Emerg. Infect. Dis.* **14:**e2.
27. **Whitmore, A.** 1913. An account of a glanders-like disease in Rangoon. *J. Hyg.* **13:**1–34.
28. **Yabuuchi, E., Y. Kosako, H. Oyaizu, I. Yano, H. Hotta, Y. Hashimoto, T. Ezaki, and M. Arakawa.** 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol. Immunol.* **36:**1251–1275.

16.10 Smallpox—Variola Major

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Variola virus, the etiologic agent of smallpox, is considered among the highly virulent microorganisms likely to be used in bioterrorism activities. If a community exposure to variola virus occurs, the challenge for the clinical microbiology laboratory will be threefold: (i) management of clinical specimens and/or viral isolates safely and appropriately, (ii) recognition of the agent within the limitations of routine testing, and (iii) rapid notification of a potential outbreak to the proper authorities.

Variola virus is transmitted directly from person to person via inhalation of infective aerosols or by direct contact with contaminated materials. Transmission occurs at the onset of viral lesions and continues for 7 to 10 days. Infectivity diminishes with the onset of scab formation. Symptoms develop 12 to 14 days after a primary exposure. A period of approximately 2 weeks may ensue before detection of visible symptoms and confirmation of the diagnosis. The symptoms are typical of many viral infections and include fever and severely aching muscles. Virus titers are concentrated in saliva during the first week of illness due to the initial presentation of vesicles in the oropharynx within 72 h of the advent of symptoms. Lesions then spread first to the face and then to the arms, hands, and feet during the next 7 to 14 days and are more abundant on the face, forearms, and lower legs and sparser on the trunk. Death may occur within 5 to 7 days in rapidly progressing infection or within 10 to 14 days in the more classical presentation of the infection.

The early appearance of the rash is identical to chickenpox; however, unlike chickenpox lesions, in which vesicles and older crusted lesions may occur together (crops), smallpox lesions are uniform in appearance during each stage of development. The rash is initially represented by erythematous papules (small round bumps), which progress to form vesicles, then pustules, and finally scabs. Two severe variations of smallpox can also occur which may confuse the initial recognition of infected individuals. Hemorrhagic smallpox is characterized by a shorter incubation period followed by more severe prodromal symptoms and formation of petechiae and hemorrhages into the skin and mucous membranes. Death often occurs within 5 to 6 days of the onset of the petechial rash. Malignant smallpox is characterized not only by an abrupt onset of severe prodromal symptoms but also by the development of a vesicular rash that coalesces into soft, flattened, sometime hemorrhagic lesions which have the appearance of crepe rubber. The rash does not progress to the formation of pustules or scabs. The documentation of a carefully obtained clinical history will provide invaluable information that will help both the physician and the laboratory to recognize a smallpox infection (Table 16.10–1).

ANALYTICAL CONSIDERATIONS

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. Smallpox may remain unrecognized in a patient at the time of initial specimen collection, and medical personnel may attempt to obtain samples from the skin lesions, as well as blood or other specimen sources, to verify the agent of infection. *Adherence to standard (universal) precautions is mandatory during the specimen collection and transport process. Medical and laboratory personnel must consider every specimen as a potential source of a highly infective agent.*
2. If smallpox is suspected, do not attempt to collect specimens for diagnostic testing. Immediately inform the appropriate personnel designated by the hospital's bioterrorism readiness protocol so that they may contact the local or

Table 16.10–1 Clinical conditions with symptoms compatible with smallpox

Preexanthem	Early exanthem	Late exanthem
Influenza	Measles, rubella	Chickenpox
Sepsis	Miscellaneous viral exanthem[a]	Erythema multiforme
Central nervous system infection	Drug eruptions	Stevens-Johnson syndrome
Appendicitis	Syphilis	Scabies
Pneumonia	Erythema multiforme	Impetigo
Leukemia	Insect bites	Drug eruptions
Enteric fever		Pemphigus
		Miscellaneous viral exanthem

[a] A miscellaneous viral exanthem (skin rash) would include infections caused by herpes simplex virus, varicella-zoster virus, enteroviruses, or other poxviruses, such as the agents of vaccinia or molluscum contagiosum or poxviruses that can be passed from animals to humans. A careful clinical history, including travel and animal exposure, along with the appropriate diagnostic tests will aid in differentiation of the zoonotic poxvirus infections, which include monkeypox, cowpox, buffalopox, camelpox, orf, bovine papular stomatitis, sealpox, yabapox, and tanapox.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

state health department for further instructions. If no protocol exists, medical personnel should initiate direct communication with the local or state department of health.

3. Individuals must be vaccinated before engaging in specimen collection from patients with suspected or confirmed cases of smallpox. Standard precautions, including gloves and mask, are mandatory.

B. Specimen transport and storage

1. Store specimens from patients with suspected smallpox in a tightly sealed protective container under refrigeration prior to receiving transport instructions. Retain frozen specimens in the freezer, preferably at $-70°C$. Retain tissue culture tubes suspected of containing the virus at 35°C.
2. Package each patient's specimen separately to avoid cross-contamination.

C. Transport

Clinical material collected for confirmation of a suspected case of smallpox must be transported to the CDC for analysis.

III. PROCEDURES

It is imperative that these cultures be handled in a biological safety cabinet.

A. Biosafety

A class II biological safety cabinet which meets BSL 2 specifications is adequate protection for processing of any specimens not initially recognized as harboring smallpox virus. As soon as preliminary information is received concerning the nature of the infection, any remaining specimens and/or culture isolates must be transported to a BSL 4 facility for further evaluation.

B. Methods of identification

Although a sentinel clinical laboratory should not attempt diagnostic testing for smallpox virus, the following methods are valuable for confirmation of infection.

1. Electron microscopy using negative staining is the most rapid and sensitive method of detection for variola virus from lesions or scabs. Although all orthopoxviruses will look similar when viewed by electron microscopy, a presumptive identification can be made based on the patient's clinical history.
2. Definitive identification requires growth of the virus in cell culture or on chorioallantoic egg membrane or by characterization of strains by newer molecular techniques, including PCR and measurement of restriction fragment length polymorphisms.

III. PROCEDURES *(continued)*

3. PCR has been described for identification of vaccinia virus and other types of poxviruses. Discrimination of different orthopoxvirus species by PCR is possible using primers directed against the gene sequences encoding the hemagglutinin protein. Electrophoretic analysis of viral proteins or viral DNA endonuclease cleavage profiles, restriction maps, and nucleotide sequences are definitive methods for differentiation of poxvirus genera and species.

4. Assays for measurement of the antibody response are only valuable for seroepidemiological surveys and not for diagnosis of acute infection.

Observe standard precautions.

C. Recognition of smallpox in the routine clinical laboratory

1. Specimens sent for routine viral or bacterial culture, electron microscopy, or histopathology may harbor virus from a clinically unrecognized case of smallpox. Laboratorians are advised against initiating diagnostic testing for smallpox without the express consent of public health officials; however, differentiation of smallpox virus from other infectious agents in clinical specimens that may be discovered during routine testing procedures is mandatory for protection of the employee and rapid detection of an infection.

2. Refer to Table 16.10–2 for differential characteristics.

D. Reporting

In the event of an occurrence of smallpox, laboratories may be limited in their ability to confirm the presence of the virus in clinical samples without assistance from government reference laboratory facilities. Immediate communication with local, state, and federal health officials in the event of a suspected infection is mandatory in order to expedite the identification of smallpox virus and the initiation of outbreak protocols.

E. Interpretation of results

The documentation of a carefully obtained clinical history will provide invaluable information that will help both the physician and the laboratory in determining the etiologic agent of an orthopoxvirus infection. Acquisition of infection by members of the genus other than variola virus requires animal exposure. Additionally, the travel history of the patient is important in determining the source of zoonosis. This information, in conjunction with the sequence of prodromal symptoms and the location of vesicles, will allow rapid presumptive differentiation of these infections.

Table 16.10–2 Differentiation of smallpox virus from other agents of infection

Diagnostic test	Smallpox virus result	Differentiation
Electron microscopy	Brick-shaped particle 140–230 by 210–390 nm with rows of surface tubules arranged as parallel surface ridges	Large particle size limits identification to *Poxviridae.* Further distinction between viruses within *Poxviridae* is based on shape and surface morphology. Electron microscopy cannot differentiate variola virus from other members of the orthopoxviruses, such as whitepox, cowpox, tanapox, and molluscum contagiosum viruses, but can aid in differentiation of variola virus from members of other viral families, such as varicella-zoster virus.
Light microscopy	Visible as featureless unstained particles	Differentiation from bacteria and fungi based on size, morphology, and staining characteristics
	Hematoxylin-eosin-stained histological section or direct smear; round-oval basophilic or acidophilic intracytoplasmic bodies spreading from the vicinity of the nucleus (Guarnieri's bodies; B-type inclusions)	Histopathology of other causes of viral exanthems[b]
Tissue culture[a]	Growth in HEL, IHF, CEF, and MK cell lines at 35°C; CPE, 1–3-mm-diam multinucleated plaques; cytoplasmic bridging; growth rate is variable; promotion of CPE may require several passages.	CPE caused by other agents of viral exanthems.[b] Initiate bioterrorism protocols if the identity of the viral agent is not confirmed using antigen detection or electron microscopy. Findings must be correlated with clinical symptoms.

[a] Tissue cultures that will support the growth of smallpox include human embryonic lung (HEL) cells, infant human fibroblasts (IHF) or other human fibroblast lines, chicken embryonic fibroblasts (CEF), and various types of monkey kidney cell lines (MK). CPE, cytopathic effect.

[b] See the footnote to Table 16.10–1.

SUPPLEMENTAL READING

Behbehani, A. B. 1995. Poxviruses, p. 511–520. *In* E. H. Lennette, D. A. Lennette, and E. T. Lennette (ed.), *Viral, Rickettsial, and Chlamydial Infections,* 7th ed. American Public Health Association, Washington, DC.

Centers for Disease Control and Prevention. 2000. Biological and chemical terrorism: strategic plan for preparedness and response. *MMWR Morb. Mortal. Wkly. Rep.* **49**(RR-4)**:**1–14.

Esposito, J. J., and J. H. Nakano. 1992. Human poxviruses, p. 643–668. *In* E. H. Lennette (ed.), *Laboratory Diagnosis of Viral Infections,* 2nd ed. Marcel Dekker, Inc., New York, NY.

SUPPLEMENTAL READING
(continued)

Gilchrist, M. J. R., W. P. McKinney, J. M. Miller, and A. S. Weissfeld. 2000. *Cumitech 33, Laboratory Safety, Management, and Diagnosis of Biological Agents Associated with Bioterrorism.* Coordinating ed., J. W. Snyder. ASM Press, Washington, DC.

Henderson, D. A., T. V. Inglesby, J. G. Bartlett, M. S. Ascher, E. Eitzen, P. B. Jahrling, J. Hauer, M. Layton, J. McDade, M. T. Osterholm, T. O'Toole, G. Parker, T. Perl, P. Russell, and K. Tonat. 1999. Smallpox as a biological weapon: medical and public health management. *JAMA* **281:**2127–2137.

Kleitmann, W. F., and K. L. Ruoff. 2001. Bioterrorism: implications for the clinical microbiologist. *Clin. Microbiol. Rev.* **14:**364–381.

Moss, B. 1996. Poxviridae: their replication, p. 2637–2671. *In* B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus (ed.), *Fields Virology*, 3rd ed. Raven Press, New York, NY.

Nakano, J. H., and J. J. Esposito. 1989. Poxviruses, p. 224–265. *In* N. J. Schmidt and R. W. Emmons (ed.), *Diagnostic Procedure for Viral, Rickettsial, and Chlamydial Infections*, 6th ed. American Public Health Association, Washington, DC.

Ropp, S. L., Q. Jin, J. C. Knight, R. F. Massung, and J. J. Esposito. 1995. PCR strategy for identification and differentiation of smallpox and other orthopoxviruses. *J. Clin. Microbiol.* **33:**2069–2076.

Ropp, S. L., J. J. Esposito, V. N. Loparev, and G. J. Palumbo. 1999. Poxviruses infecting humans, p. 1137–1144. *In* P. R. Murray, F. Tenover, E. J. Baron, M. A. Pfaller, and R. H. Yolken (ed.), *Manual of Clinical Microbiology*, 7th ed. ASM Press, Washington, DC.

16.11 Unknown Virus

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

If a community exposure to a virulent unknown virus occurs, the challenge for the clinical microbiology laboratory is threefold: (i) management of clinical specimens and/or viral isolates safely and appropriately, (ii) recognition of the agent within the limitations of routine testing, and (iii) rapid notification of the proper authorities about a potential outbreak.

The recognition of the initial signs of a viral epidemic within a population will vary depending on a number of factors, including mode of transmission, incubation period, and clinical presentation of the infection. Depending on the type of infection, viral shedding may occur before onset of infection and can continue through the course of the illness. The ability of the laboratory to recover and/or identify the virus is usually enhanced if patient specimens are collected early in the course of infection.

Transmission occurs either through direct contact with an infected individual (or animal), through exposure to contaminated fomites, or through the bite of an insect vector. An epidemic propagated by contact may spread more slowly through a population. Viral exposure through a common source such as contaminated food, water, or aerosolized product will result in concurrent infection of a larger number of individuals.

The laboratory must be alert to the possible presence of an unknown virus in a variety of specimen sources. Some viral infections disseminate from a primary infection site to a secondary location within the host. For example, the virus causing smallpox is shed from the upper respiratory mucosa during the very early stages of infection, but is found in larger concentrations in the characteristic skin lesions that develop during progression of the disease.

Although the clinical symptoms and laboratory diagnosis of some bioterrorism-targeted viral agents are well described, a genetically altered or manufactured agent may produce unexpected findings. The documentation of a carefully obtained clinical history will provide invaluable information that will help both the physician and the laboratory in determining the recognition of an unknown viral infection.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

1. The virus may remain unrecognized in a patient at the time of initial specimen collection. Depending on the manifestations of the illness, specimen sources will include respiratory secretions, blood and other body fluids, vesicle material, feces, or CSF. Adherence to standard precautions is mandatory during the specimen collection and transport process. Medical and laboratory personnel must consider every specimen a potential source of a highly infective agent.
2. If an agent of bioterrorism is suspected, do not attempt to collect specimens for diagnostic testing. Immediately inform the appropriate personnel designated by the hospital's bioterrorism readiness protocol so that they may contact the appropriate government agencies for further instructions. If no protocol exists, medical personnel should initiate direct communication with the local and/or state department of health.

B. Specimen transport and storage

1. Store specimens from patients with suspected virus in a tightly sealed protective container under refrigeration prior to receiving transport instructions.
2. Retain frozen specimens in the freezer, preferably at $-70^{\circ}C$.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

3. Retain cell culture tubes suspected of containing a biothreat virus at 35°C while awaiting instructions for transport to the Laboratory Response Network (LRN) reference facility. For longer-term storage, add a suitable cryopreservative and store at ≤ -70°C.
4. Store paraffin blocks or formalin-fixed tissues at room temperature. Do not freeze.

C. Transport

Consult government health officials for transport instructions. Packaging and shipping of specimens must follow the current guidelines for biohazardous materials (infectious substances). Package each patient's specimen separately to avoid cross contamination.

ANALYTICAL CONSIDERATIONS

III. PROCEDURES

It is imperative that these cultures be handled in a biological safety cabinet.

A. Biosafety

1. A biosafety cabinet (BSC) which meets BSL 2 specifications provides adequate protection for processing any specimens not initially recognized as harboring an unknown virus. Refer to the procedures in this handbook for instructions concerning the proper use of the BSC. Failure to follow BSC safety protocols can result in exposure to the biothreat virus.
2. Wear a gown and gloves while using the BSC.
3. Before disposal, disinfect all material within the BSC using a freshly prepared 1:100 dilution of sodium hypochlorite.
4. Air dry and perform acetone fixation of smears within the BSC.
5. Only perform centrifugation using polypropylene tubes with properly secured lids. A safety shield must cover the rotor during centrifugation. After centrifugation, remove the safety shield under the protection of a BSC.
6. As soon as preliminary information is received concerning the nature of the epidemic and/or virus, transport any remaining specimens or culture isolates to a BSL 4 facility for further evaluation.

B. Methods of identification

1. General information

   Specimens sent for routine viral or bacterial culture, electron microscopy, or histopathology may harbor virus from a clinically unrecognized case. Differentiation of the unknown virus from other pathogens (Table 16.11–1) is important for protection of the employee and rapid detection of an infection. In the event of a recognized epidemic, laboratorians are advised against initiating diagnostic testing without the consent of public health officials.
2. Nonculture methods
   - **a.** Electron microscopy and histopathology

     Histological or electron microscopy preparations are more likely to provide evidence of the presence of a viral agent. If a suspicious agent is detected using either of these methods, store clinical specimens, stained sections, and/or electron micrographs for further future analysis.
   - **b.** Antigen detection

     Unless antigenic cross-reactivity occurs between an unknown agent and a more commonly encountered virus, direct detection assays would yield only the identity of the virus to which the assay detection antibodies are directed.
3. Molecular methods
   - **a.** A variety of molecular techniques are described for viral detection, including PCR and electrophoretic analysis of viral proteins or endonuclease cleavage profiles, restriction maps, and nucleotide sequences.

Table 16.11–1 Differential diagnosis of an unknown viral infection

Influenza	Measles, rubella	Chickenpox
Sepsis	Miscellaneous viral exanthemas[a]	Erythema multiforme
CNS[b] infection	Drug eruptions	Stevens-Johnson syndrome
Appendicitis	Syphilis	Scabies
Pneumonia	Erythema multiforme	Impetigo
Leukemia	Insect bites	Drug eruptions
Enteric fever	Pemphigus	Miscellaneous viral exanthemas

[a] A miscellaneous viral exanthema (skin rash) would be caused by infections with viruses such as herpes simplex virus, varicella-zoster virus, enteroviruses or other poxviruses such as the agents of vaccinia and molluscum contagiosum, or poxviruses that can be passed from animals to humans.

[b] CNS, central nervous system.

II. PROCEDURES *(continued)*

b. Investigation of an unknown agent by any of these methods requires diagnostic techniques unavailable in most clinical laboratories. Store any specimens received for these types of analyses either at $-70°C$ (tissues) or refrigerated (whole blood). Check with the nearest LRN reference laboratory for precise instructions.

4. Antibody detection

a. Assays that measure antibody response are helpful for diagnosis of acute infection or past viral exposure.

b. Collect a serum specimen during both the acute and convalescent phases of the infection. Store sera at -20 to $>-70°C$ until testing.

C. Culture

1. Cytopathic effect (CPE) produced by viruses often suggests a specific viral type. Laboratories then confirm the identity of the virus using a neutralization or antigen detection assay.

2. Before production of CPE, hemadsorption of specific types of human or animal erythrocytes to the cell monolayer will determine the presence of a hemagglutinin-producing virus.

3. If the identity still remains unknown after testing, freeze cells and supernatant from the culture using a cryopreservative agent and keep at $-70°C$.

POSTANALYTICAL CONSIDERATIONS

IV. REPORTING RESULTS

Immediate communication with local, state, and federal health officials is critical in expediting identification of an unknown virus and initiation of outbreak protocols. Clinical laboratories may be limited in their ability to confirm the presence of the virus in clinical samples without assistance from government reference laboratory facilities.

V. INTERPRETATION OF RESULTS

The documentation of a carefully obtained clinical history will provide invaluable information that will help both the physician and the laboratory in determining the etiologic agent of an infection and for implementing infection control procedures. Acquisition of some viral infections requires animal exposure or insect exposure. Additionally, the travel history of the patient is important in determining the source of the zoonosis. This information, in conjunction with the sequence of prodromal symptoms, will allow rapid presumptive differentiation of infections.

SUPPLEMENTAL READING

Centers for Disease Control and Prevention. 2001. Recognition of illness associated with the intentional release of a biologic agent. *MMWR Morb. Mortal. Wkly. Rep.* **50:**893–897.

Franz, D. R., P. B. Jahrling, A. M. Friedlander, et al. 1997. Clinical recognition and management of patients exposed to biological warfare agents. *JAMA* **278:**399–411.

Henderson, D. A., T. V. Inglesby, J. G. Bartlett, M. S. Ascher, E. Eitzen, P. B. Jahrling, J. Hauer, M. Layton, J. McDade, M. T. Osterholm, T. O'Toole, G. Parker, T. Perl, P. K. Russell, and K. Tonat. 1999. Smallpox as a biological weapon: medical and public health management. *JAMA* **281:**2127–2137.

16.12 Q Fever—*Coxiella burnetii*

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Coxiella burnetii is the etiologic agent of Q fever. It is a pleomorphic coccobacillus that is gram negative, obligately intracellular, and 0.3 to 0.7 μm long. There is a spore-like form, the small cell variant, which is remarkably stable in extracellular environments. A large cell variant also exists that is the vegetative, metabolically active form. Mixtures of both forms are found in phagolysosomes. There is phase variation, similar to that in *Salmonella*, in which the lipopolysaccharide (LPS) varies chemically as either the virulent, phase I "smooth"-type LPS or the phase II "rough" LPS, associated with avirulent *C. burnetii*. *C. burnetii* is phylogenetically related to *Pseudomonas*, *Francisella*, and *Legionella*, within the *Legionella* group of the γ-*Proteobacteria* subdivision. It is more distantly related to *Rickettsia* (7).

Q fever is a zoonotic disease, especially of parturient goats, sheep, or cattle and, occasionally, domestic cats. There is also a substantial reservoir among wildlife, including mammals, birds, and arthropods (10). Ticks that are infected with *C. burnetii*, however, do not represent a major vector of human disease. Human-to-human transmission is rare. Infection may be acquired through ingestion of contaminated milk, and though the disease acquired is usually subclinical, it does result in seroconversion. Bulk milk supplies commonly contain *C. burnetii* (8). Active surveillance of Q fever has been inconsistent, but where there has been active surveillance for the disease, it has been found (1, 2, 12). On a worldwide basis, *C. burnetii* is the leading cause of overt laboratory-acquired infections among viral, rickettsial, and chlamydial agents. Most of these have involved work with pregnant sheep in research laboratories (6).

The potential for use of *C. burnetii* as an agent of bioterrorism derives from its combined properties of the source of infection being aerosolized contaminated dust particles, resistance to inactivation in the environment, and the fact that the infectious dose is very low. It is classified as a category B agent by the CDC. The symptoms of Q fever are generally nonspecific. There are multiple presentations, most commonly pneumonia (47 to 63%), hepatitis (60%), or fever only (14%) (11, 13, 14). It is estimated that self-limited febrile illness, in fact, may be the most common form of the disease. The incubation period is 2 to 3 weeks. The organisms proliferate in the lung following inhalation of contaminated aerosols and then invade the bloodstream. Acute Q fever is characterized by sudden onset of high fever, headache, myalgias, arthralgias, cough, and, less frequently, rash or a meningeal syndrome. In addition to radiographic manifestations of pneumonia, patients often have elevated liver enzyme levels and erythrocyte sedimentation rates and thrombocytopenia. Development of chronic Q fever is a more serious disease, which can occur up to 20 years after the initial infection. The major complication of chronic Q fever is endocarditis. Overall, the mortality rate of Q fever is low, approximately 2.4% (19), but it may be as high as 65% among those with chronic Q fever (13). Q fever-associated abdominal aortic aneurysms with or without simultaneous endocarditis have been reported for patients with *C. burnetii* infection (16).

Several potential bioterrorism agents could present as community-acquired pneumonias, including those causing tularemia, plague, anthrax, or Q fever (3). The milder form of pneumonic tularemia would be the most likely one to be clinically indistinguishable from Q fever, whereas plague or anthrax would typically follow a more rapidly fulminant course. In addition to atypical pneumonia as part of a clinical algorithm leading to consideration of Q fever, the following should suggest Q fever (4): influenza-like illness during periods of low influenza activity; hepatitis without markers for hepatitis A, B, or C; and fever of unknown origin in children.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

A. ◩ **NOTE:** Sentinel (formerly level A) laboratories should not accept environmental or animal specimens; such specimens should be forwarded directly to the nearest Laboratory Response Network (LRN) reference laboratory (usually the state health department laboratory).

1. Serum

Collect serum (red-top or serum separator tube, tiger-top tube) as soon as possible after onset of symptoms (acute phase) and with a follow-up specimen (convalescent phase) at >14 days for serological testing.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

2. Blood
   Collect blood in EDTA (lavender) or sodium citrate (blue) and maintain at 4°C for storage and shipping for PCR or special cultures. If possible, collect specimens prior to antimicrobial therapy. Either biopsy samples or blood samples are suitable for PCR.
3. Tissue, body fluids, and others, including cell cultures and cell supernatants
   Specimens can be maintained at 2 to 8°C if transported within 24 h. Store frozen at −70°C or on dry ice.

B. Specimens other than serum

1. In conjunction with instructions from the state public health laboratory, arrange for immediate shipment at 2 to 8°C to an appropriate higher-level LRN laboratory.
2. Follow infectious-substance regulations for packing and shipping.

C. Rejection criteria

1. Incomplete documentation
   All specimens must include the sender's name and telephone number to contact for the preliminary report and additional information.
2. Improper packaging/shipping
3. Lack of prior approval
   Do not ship specimens to an LRN reference laboratory without preliminary arrangements.

III. MATERIALS

Separate and store serum frozen until testing.

ANALYTICAL CONSIDERATIONS

IV. PROCEDURES

A. General

1. Since this organism is an obligate intracellular organism, it cannot be cultured on routine bacteriological media. *C. burnetii* can be cultured in various cells, including human embryo fibroblasts, L cells, and green monkey kidney cells. Culture procedures must be performed in BSL 3 facilities.
2. The laboratory diagnosis of Q fever is based mainly on serological testing.
3. Antibody responses are measured against phase I and II antigens of *C. burnetii*. Patients with acute Q fever typically produce an antibody response primarily to *C. burnetii* phase II antigen, while chronic *C. burnetii* infections typically elicit a higher antibody response to phase I antigen.

B. Precautions

1. Because of the highly infectious nature of this organism, specimens from suspected cases of Q fever should be immediately forwarded to the nearest LRN reference laboratory (BSL 3) for isolation and identification.
2. Due to the extreme infectivity of *C. burnetii*, sentinel laboratories should not attempt to culture this organism, but should be aware of the potential for inadvertent isolation of *C. burnetii* in cell culture systems designed for virus isolation. *C. burnetii* can be inadvertently isolated in conventional cell cultures in a wide variety of cell lines, including all fibroblast lines. After an incubation period of 5 to 15 days, *C. burnetii*-infected cells are detectable as cytoplasmic inclusions (Fig. 16.12–1).
3. Biohazardous waste should be decontaminated by autoclaving. Contaminated equipment or instruments can be decontaminated with approved disinfectants. The spore-like form of *C. burnetii* may be resistant to disinfection using dilute bleach, UV radiation, or heat achieved by autoclaving or boiling for less than 10 min. Special decontamination procedures are necessary for

Figure 16.12–1 Fibroblast L929 line infected with *C. burnetii*. (Courtesy of Didier Raoult.)

IV. PROCEDURES *(continued)*

surfaces potentially contaminated with *C. burnetii*. Household bleach solutions may be ineffective. Minor spills should be covered with absorbent paper, such as paper towels, and then flooded with 70% ethanol or 5% MicroChem-Plus (a dual quaternary ammonium compound), which should be allowed to act for 30 min before cleanup. Spills that involve samples with high concentrations of organisms, involve organic matter, or occur in areas of lower temperatures (e.g., refrigerators or freezers) should be exposed to disinfectant solution for 1 h before cleanup.

V. RESULTS

A. Confirmatory diagnosis

The diagnosis of Q fever can be confirmed by the following.

1. Demonstration of fourfold or greater changes in antibody titer between paired acute- and convalescent-phase serum samples by immunofluorescence antibody (IFA) testing; comparable sensitivity and specificity can be achieved with EIA when testing for immunoglobulin G (IgG) (18).
2. Detection of *C. burnetii* by PCR. Real-time PCR assays have been developed that are sensitive and specific, based on targets of the IS*1111* sequence present in multiple copies in the *C. burnetii* genome (9, 15).
3. Immunohistochemical staining of biopsy material from affected organs (11)
4. Culture of biopsy or blood samples (4, 5)

B. Presumptive diagnosis

The demonstration of a single positive IgG or IgM titer (as defined by the testing laboratory) in clinically compatible cases defines a probable case of Q fever. It is not possible to determine a standard cutoff value of EIA or IFA, and equivocal results may occur in some cases (17). Interpretive criteria in use by some laboratories are shown in Table 16.12–1.

C. Clues from ancillary tests suggestive of Q fever

Histological appearance of bone marrow or liver in which a fibrin ring is associated with granuloma formation (doughnut granulomas) (11). A clue to the diagnosis of chronic Q fever is culture-negative endocarditis (4).

Table 16.12–1 Typical Q fever diagnostic titers with indirect immunofluorescence (10)

Stage of disease	Phase I antibodies (IgG)	Phase II antibodies (IgG)
Acute	Positive but lower than titers of antibody to phase II *C. burnetii*	>1/200[a]
Chronic	>1/800	Positive but same as or lower than titers of antibody to phase I *C. burnetii*

[a] Fourfold rise in antibody titer between acute- and convalescent-phase samples is the strongest predictor of acute Q fever.

VI. QUALITY CONTROL

Commercial reference laboratories offer serological testing; PCR and culture are generally performed only in research laboratories.

POSTANALYTICAL CONSIDERATIONS

VII. LIMITATIONS

A. Laboratory diagnosis is difficult due to lack of specific criteria leading to the suspected clinical diagnosis of Q fever.

B. Cultures are difficult and hazardous to perform, leading to reliance on serological means of diagnosis in most cases of Q fever.

VIII. NOTIFICATIONS

A. Sentinel laboratories should consult with the state public health laboratory director (or designate) prior to or concurrent with testing if the attending physician suspects *C. burnetii*.

B. Serology is available through commercial reference as well as public health laboratories. Report positive results to the patient's physician and hospital infection control and public health officials.

REFERENCES

1. **Centers for Disease Control and Prevention.** 2002. Q fever: California, Georgia, Pennsylvania and Tennessee, 2000–2001. *MMWR Morb. Mortal. Wkly. Rep.* **51:**924–927.
2. **D'Angelo, I. J., L. J. Baker, and E. F. Schlosser.** 1979. Q fever in the United States. *J. Infect. Dis.* **139:**613–615.
3. **Daya, M., and Y. Nakamura.** 2005. Pulmonary disease from biological agents: anthrax, plague, Q fever, and tularemia. *Crit. Care Clin.* **21:**747–763.
4. **Fournier, P.-E., T. G. Marrie, and D. Raoult.** 1998. Diagnosis of Q fever. *J. Clin. Microbiol.* **36:**1823–1834.
5. **Gouriet, F., F. Fenollar, J.-Y. Patrice, M. Drancourt, and D. Raoult.** 2005. Use of shell-vial cell culture assay for isolation of bacteria from clinical specimens: 13 years of experience. *J. Clin. Microbiol.* **43:**4993–5002.
6. **Hall, C. J., S. J. Richmond, E. O. Caul, N. H. Pearce, and I. A. Silver.** 1982. Laboratory outbreak of Q fever acquired from sheep. *Lancet* **i:**1004–l006.
7. **Heinzen, R. H.** 1997. Intracellular development of *Coxiella burnetii*, p. 99–130. *In* B. Anderson, H. Friedman, and M. Bendinelli (ed.), *Rickettsial Infection and Immunity.* Plenum Press, New York, NY.
8. **Kim, S. G., E. H. Kim, C. J. Lafferty, and E. Dubovi.** 2005. *Coxiella burnetii* in bulk tank milk samples, United States. *J. Emerg. Infect. Dis.* **11:**619–620.
9. **Klee, S. R., J. Tyczka, H. Ellerbrok, T. Franz, S. Linke, G. Balijer, and B. Appel.** 2006. Highly sensitive real-time PCR for specific detection and quantification of Coxiella burnetii. *BMC Microbiol.* **6:**2.
10. **Madariaga, M. G., K. Rezai, G. T. Trenholme, and R. A. Weinstein.** 2003. Q fever: a biological weapon in your backyard. *Lancet Infect. Dis.* **3:**709–721.
11. **Maurin, M., and D. Raoult.** 1999. Q fever. *Clin. Microbiol. Rev.* **12:**518–553.
12. **McQuiston, J. H., J. E. Childs, and H. A. Thompson.** 2002. Q fever. *J. Am. Vet. Med. Assoc.* **221:**796–799.
13. **Raoult, D., T. Marrie, and J. Mege.** Natural history and pathophysiology of Q fever. *Lancet Infect. Dis.* **5:**219–226.
14. **Raoult, D., J.-L. Mege, and T. Marrie.** 2001. Q fever: queries remaining after decades of research, p. 29–56. *In* W. M. Scheld, W. A. Craig, and J. M. Hughes (ed.), *Emerging Infections 5.* ASM Press, Washington, DC.

REFERENCES *(continued)*

15. **Rolain, J.-M., D. Raoult, B. P. Marmion, R. J. Harris, P. Storm, and J. G. Ayres.** 2005. Molecular detection of *Coxiella burnetii* in blood and sera during Q fever. *QJM* **98:**615–620.
16. **Sessa, C.** 2005. Abdominal aortic aneurysm and *Coxiella burnetii* infection: report of three cases and review of the literature. *J. Vasc. Surg.* **42:**153–158.
17. **Setiyono, A., M. Ogawa, Y. Cai, S. Shiga, T. Kishimoto, and I. Kurane.** 2005. New criteria for immunofluorescence assay for Q fever diagnosis in Japan. *J. Clin. Microbiol.* **43:**5555–5559.
18. **Slabá, K., L. Skultéty, and R. Toman.** 2005. Efficiency of various serological techniques for diagnosing *Coxiella burnetii* infection. *Acta Virol.* **49:**123–127.
19. **Tissot-Dupont, H., D. Raoult, P. Brouqui, F. Janbon, D. Peyramond, P. J. Weiller, C. Chicheportiche, M. Nezri, and R. Poirier.** 1992. Epidemiologic features and clinical presentation of acute Q fever in hospitalized patients—323 French cases. *Am. J. Med.* **93:**427–434.

16.13 Clinical Laboratory Bioterrorism Readiness Plan

PREANALYTICAL CONSIDERATIONS

I. INTRODUCTION

The purpose of this template is to provide a model for laboratories to use for developing a bioterrorism (BT) preparedness plan. The components of this template can be used to develop a readiness plan to meet the needs of the institution. It is not meant to be all-inclusive. Rather, it is to serve as an aid in the process of developing a specific plan for each institution.

The laboratory BT preparedness plan should be integrated into the institutional BT preparedness plan.

Some of the specific laboratory protocols for the BT agents included in this template contain flowcharts. Ideally, these flowcharts should be integrated into laboratory procedures so that technologists have ready access to this information.

The format of a possible BT preparedness plan is outlined below.

◩ **NOTE:** It is quite possible that the laboratory will not be contacted in advance and informed that one of the potential agents of BT is suspected. As a result, it is essential that appropriate safeguards be taken, including subculture of all blood cultures in a biosafety cabinet or behind a safety shield, following the flowcharts for suspicious agents, and always considering the possibility of BT agents.

II. BT PREPAREDNESS PLAN

A. Purpose

1. The purpose of this protocol is to provide a formal description of how the laboratory will respond to a suspected or confirmed BT event. The laboratory may be called upon to assist in the diagnosis and management of patients who have been overtly or covertly exposed to a BT agent. The laboratory needs to promptly assist clinicians by providing them with accurate information on the selection, collection, and transport of specimens. In addition, the laboratory must handle these specimens in a manner that will result in the greatest probability of success in establishing a diagnosis and minimize the exposure of hospital personnel and patients to infectious agents.
2. In a suspected or confirmed BT event, immediate and effective communication with all appropriate institutional and medical personnel and with public health officials is imperative. If there is a specific, designated individual serving as the contact person for the institution's BT plan, this is the person who should be contacted.
3. An additional and very real possibility is that the laboratory will be the first to recognize that an organism isolated is a possible agent of BT.

B. Contact protocol and chain of command

1. If a possible BT agent is grown in the laboratory or detected by other laboratory means (as outlined in the laboratory protocols included in this procedure), place phone calls to the responsible physician and the following individuals noted below immediately. Contacting these individuals and the procedures required in the laboratory are *not* one-person tasks. Additional assistance from other technologists and laboratory support personnel is essential.

   or

II. BT PREPAREDNESS PLAN *(continued)*

2. If a specimen is submitted for detection of a BT agent as the result of a possible BT event, place phone calls to the following individuals or departments immediately: microbiology laboratory supervisor, microbiology manager, microbiology laboratory director, infection control officer, infectious disease physician, local health department, state health department, laboratory director on call, clinical pathologist on call, chief of infectious diseases, chief of pathology, and any other individuals deemed prudent by the laboratory. (Include contacts pertinent to your institution in a predetermined order, and delete those who are not to be contacted in your institution.)

◩ **NOTE:** Certain geographic areas are known to have natural human cases of infection due to BT agents (e.g., tularemia in Nantucket and Martha's Vineyard, MA, as well as Missouri, Oklahoma, and neighboring areas, and plague in much of the southwestern United States, especially New Mexico).

C. Laboratory Response Network

1. Introduction to the LRN

The Laboratory Response Network (LRN) is a consortium and partnership of laboratories that provide immediate and sustained laboratory testing and communication in support of public health emergencies, particularly in response to acts of BT. The LRN is currently comprised of local, state, and federal public health laboratories in addition to private and commercial clinical laboratories and selected food, water, agricultural, military, and veterinary testing laboratories. Other key federal partners include the Federal Bureau of Investigation, the Department of Defense, the Environmental Protection Agency, the Department of Agriculture, the Department of Justice, the Department of Energy, the Food and Drug Administration (FDA), the Association of Public Health Laboratories, NIH, the American Association of Veterinary Laboratory Diagnosticians, and the American Society for Microbiology (ASM). All laboratories are regarded as partners and, in some cases, registered members of the LRN. Preliminary testing and screening are performed primarily in a distributed rather than a centralized fashion to ensure a prompt and rapid initial response; a system of triage and referral of specimens ensures transfer of appropriate materials to specialty laboratories where sophisticated equipment, technologies, and expertise are applied to specimen analysis.

2. Goals of the LRN

a. Ensure that the nation's public health, clinical, and other select laboratories are prepared to detect and respond to a BT or chemical terrorism event in an appropriate and integrated manner.

b. Ensure that all member reference laboratories collectively maintain state-of-the-art biodetection and diagnostic capabilities and surge capacity as well as secure electronic communication of test results for the biological and chemical agents likely to be used in the commission of a biocrime or BT act.

c. Work with other departments and agencies to ensure a successful federal response to an act of BT and to facilitate and optimize the ability of states to competently respond independently to biocrimes or public health emergencies in the state.

d. Promote the CDC's and Department of Health and Human Services' (HHS) BT research agenda and the CDC's internal response needs.

e. Enlist an optimal number of registered participating LRN laboratories throughout the United States as determined by the LRN working group.

II. BT PREPAREDNESS PLAN
(continued)

3. Functions of the LRN
- **a.** A registry and linkage of clinical and private laboratories in the United States that would include sentinel and reference laboratories
- **b.** Complete, accurate, and standardized protocols for all levels of testing for agents deemed critical and likely to be used in the commission of biocrimes or acts of BT
- **c.** Secure but accessible supply of standardized reagents and diagnostic technologies produced and maintained by the CDC
- **d.** Secure electronic laboratory reporting that integrates with key epidemiological, surveillance, and emergency response components
- **e.** Training and proficiency testing essential to the diagnostic process

4. LRN structure for BT agent testing

LRN laboratories are designated as sentinel, reference, and national laboratories (Fig. 16.13–1).

- **a.** Sentinel laboratories

  Depending on the level of diagnostic testing, there are two kinds of sentinel laboratories. The advanced sentinel laboratories function at the local front line and have the most capability. Laboratories with less analytical capability that are also in a position to handle specimens that might contain agents of BT or emerging infectious disease are referred to as basic sentinel laboratories. Even though many sentinel laboratories are capable of providing a "presumptive identification" of some of the targeted organisms, they must refer isolates to an LRN reference laboratory. Characteristics of the two kinds of sentinel laboratories are as follows.

  (1) Basic sentinel laboratory

  (a) The laboratory is certified by the Centers for Medicare & Medicaid Services (CMS) for general laboratory testing, including microbiology.

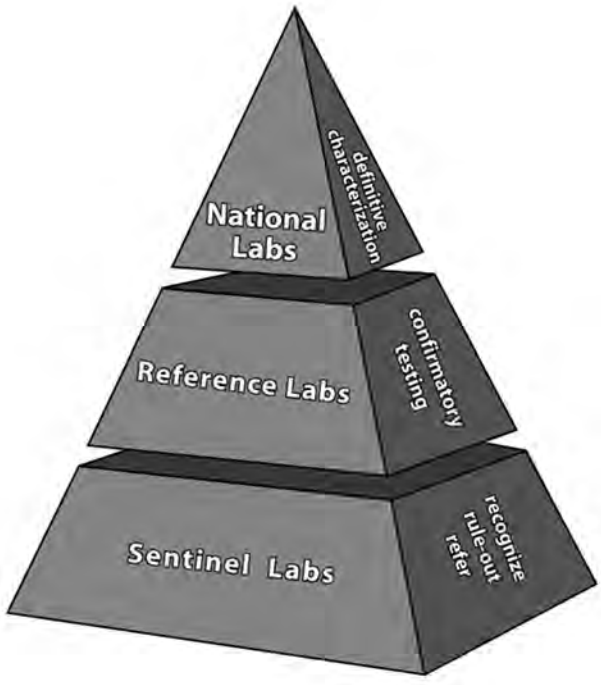

Figure 16.13–1 LRN structure for BT agent testing.

II. BT PREPAREDNESS PLAN
(continued)

(b) The laboratory is inspected successfully in accordance with the Clinical Laboratory Improvement Amendments (CLIA) or by an agency that has been given deemed status by the CMS. The laboratory has policies and procedures for referral of diagnostic specimens to an advanced sentinel laboratory.

(2) Advanced sentinel laboratory

(a) The laboratory is certified by the CMS as a high-complexity laboratory performing the specialty of microbiology.

(b) The laboratory is inspected successfully in accordance with CLIA or by an agency that has been given deemed status by the CMS.

(c) Has a class II or higher certified biological safety cabinet

(d) Complies with BSL 2 practices

(e) Has policies and procedures for use of additional respiratory protection, including definition of when necessary, as well as documentation of safe use (e.g., N-95 fit testing)

(f) Has policies and procedures that include the *Sentinel Level Clinical Microbiology Laboratory Guidelines*, which are available and can be downloaded from the ASM website (http://www.asm.org/Policy/index.asp?bid=6342)

(g) All personnel have been trained and demonstrated competency and are fully aware of the details contained within each sentinel level clinical microbiology laboratory guideline.

(h) Personnel have been trained and certified in guidelines for packing and shipping of infectious substances.

(i) Tracks and accounts for decontamination of laboratory waste (specimens and cultures); at a minimum, ensures that the contract with the waste/disposal agency be available for inspection in the laboratory safety or waste disposal manual.

It is further highly recommended that the laboratory comply with the following:

(j) The microbiology laboratory operates under negative pressure as recommended by the American Institute of Architects (AIA) *Guidelines for Construction of Healthcare Facilities.* If a microbiology laboratory is planning to remodel or construct a new facility, it should operate under negative air pressure as recommended by the AIA. This only applies to new construction or remodeling.

(k) There is on-site terminal decontamination capability for disposal of wastes categorized as BSL 3 or select agent (e.g., autoclaving).

b. Reference laboratories

LRN reference laboratories are local and state public health laboratories, selected academic or university-based laboratories, and designated specialty laboratories (veterinary, water, food, chemical, military, and agricultural) that possess the reagents and technology for definitive confirmation of identification, including toxin testing, of organisms referred by sentinel laboratories. LRN reference laboratories follow BSL 3 containment and practice guidelines. Contact your nearest LRN reference laboratory for instructions and guidance regarding the submission of suspicious agents for confirmatory testing.

II. BT PREPAREDNESS PLAN *(continued)*

c. National laboratories
LRN national laboratories are federal laboratories that have BSL 4 containment facilities and practice guidelines. The primary laboratory at this level is located at the CDC and specializes in the isolation and identification of BSL 4 agents such as Ebola, Marburg, and smallpox virus. This laboratory also possesses the capability of advanced genetic characterization and archiving of all BT agents. The U.S. Army Medical Research Institute for Infectious Diseases (USAMRIID) and the Naval Medical Research Center (NMRC) are also LRN national laboratories.

D. Responsibility of the clinical laboratory
As members of the LRN, sentinel laboratories have access to the network and serve as "sentinels" for the early detection of and raising suspicion regarding a suspicious agent that cannot be ruled out as a possible BT-associated organism. Sentinel laboratories do not have access to the CDC secure website for reference laboratory testing protocols or reagents. Instead, sentinel laboratories must utilize standardized testing protocols (ASM *Sentinel Clinical Microbiology Laboratory Guidelines*) that have been designed to utilize conventional tests to facilitate the "rule-out" or "referral" of a suspicious isolate to an LRN reference laboratory. The sentinel laboratory is *not* responsible for and *should not* make the decision that a BT event has occurred; that responsibility rests with local, state, and federal health and law enforcement officials. A designated individual within the facility (preferably the infection control officer) should be notified of a suspicious agent; this person, in turn, notifies the local public health officials. Under no circumstances should the laboratory contact law enforcement or public health officials. The exception is the need to contact the LRN reference laboratory for guidance in the disposition of the suspicious agent prior to referral for confirmatory testing.

◩ **NOTE:** In no case should the sentinel laboratory accept environmental (powders, letters, or packages), animal, food, or water specimens for examination, culture, or transport for BT-associated agents. Such specimens should be submitted directly to the nearest LRN reference laboratory.

E. Sentinel-level clinical microbiology laboratory guidelines for BT agents
The most recent sentinel-level clinical laboratory guidelines can be downloaded from the ASM website (http://www.asm.org/Policy/index.asp?bid = 6342).

1. Anthrax *(Bacillus anthracis)*
2. Avian influenza virus A H5N1
3. Botulism toxin
4. Brucellosis (*Brucella* species)
5. Plague *(Yersinia pestis)*
6. Tularemia *(Francisella tularensis)*
7. Q fever *(Coxiella burnetii)*
8. *Burkholderia mallei* and *Burkholderia pseudomallei*
9. Unknown viruses
10. Staphylococcal enterotoxin B

F. Packing and shipping instructions
Current packing and shipping instructions can be downloaded from the ASM website (http://www.asm.org/Policy/index.asp?bid = 6342).

G. Information checklist
Appendix 16.13–1 shows a checklist that may help in the gathering of information in a suspected BT event. The checklist is to be filled out by the shift operations manager, shift supervisor, or other designated personnel.

II. BT PREPAREDNESS PLAN *(continued)*

H. Handling of possible BT agent

◩ **NOTE:** Under no circumstances are viral cultures to be set up if smallpox, Ebola virus, or another of the viral agents of BT is suspected.

1. A lead BT technologist should be appointed and be notified immediately that a suspected BT specimen or agent is in the laboratory. Laboratory workers are to be informed promptly of the name and medical record number of the person(s) with the suspected infection and, if appropriate, to treat other specimens from the patient(s) appropriately. This must be done in a manner that is in compliance with the Health Insurance Portability and Accountability Act.
2. All suspected BT specimens are to be processed in the biological safety cabinet (whenever possible, this should be done in a room that is under negative pressure) while wearing PPE, such as gown, gloves, and mask.
3. Each of the plates, tubes, and blood culture bottles for which this applies must be labeled prominently: "Possible highly infectious agent: [fill in name of agent]."
4. All plates that have been streaked for culture or subculture will be sealed with shrink seal or the equivalent and labeled as in step 3 above.
5. Any growth from specimens is to be manipulated in the biological safety cabinet while wearing appropriate PPE, such as gown, gloves, and mask.
6. As the culture is being worked up, the technologist(s) working on the culture(s) must be in close touch with the microbiology supervisor and medical director.
7. An identification of the organism is *not* the role of the sentinel microbiology laboratory. An organism that is consistent with, for example, *Yersinia pestis* will be forwarded to an LRN reference or higher laboratory for definitive identification. Do not perform any more manipulation of the cultures than is absolutely essential.

I. Policy sign-off list

See Appendix 16.13–2.

J. Training sign-off list

See Appendix 16.13–3.

K. Handling of BT agents

See Appendix 16.13–4.

L. Therapy of BT agents

Specific treatment or prophylaxis for known or suspected exposure to BT agents will, in the setting of a BT event, likely be forthcoming from public health authorities. In the absence of this information on the specific therapy for a given outbreak, a good source for information on treatment and prophylaxis is **D. N. Gilbert, R. C. Moellering, and M. A. Sande.** *The Sanford Guide to Antimicrobial Therapy,* 33rd ed., Table 1B: prophylaxis and treatment of organisms of potential use as biological weapons, p. 46. Antimicrobial Therapy, Inc., Hyde Park, VT (http://www.sanfordguide.com/). Please note that the table is pathogen based and does not give information based upon a clinical syndrome in the absence of knowledge of the pathogen.

III. ACKNOWLEDGMENT

I thank the Oregon State Public Health Laboratory (http://www.ohd.hr.state.or.us/phl.index.cfm) for information from its website that was useful in the drafting of this procedure.

REFERENCES

1. **Department of Health and Human Services.** 1999. *Biosafety in Microbiological and Biomedical Laboratories,* 4th ed. U.S. Government Printing Office, Washington, DC.
2. **Department of Health, Education, and Welfare.** 1974. *Biohazards Safety Guide.* Department of Health, Education, and Welfare, Bethesda, MD.
3. **Pike, R. M.** 1976. Laboratory-associated infections. Summary and analysis of 3921 cases. *Health Lab. Sci.* **13:**105–114.

APPENDIX 16.13–1

Information Checklist

Step	Task/data	Date/time completed	Signature
1.	Name of patient(s), medical record number(s), patient location(s) and other pertinent information. A. B. C. D. E. F. G. H.		
2.	Who contacted lab about possibility of bioterrorism?		
3.	Person's (in step 2) phone number		
4.	Suspected bioterrorism agent(s) (e.g., anthrax, plague, etc., or unknown)	1. 2. 3. 4. 5.	
5.	Contacted microbiology personnel	☐ Yes ☐ No Who	
6.	Contacted clinical pathologist on call	☐ Yes ☐ No	
7.	Contacted ID physician (if instructed) and/or IC practitioner (if instructed)	☐ Yes ☐ No	
8.	If instructed to contact others *within* facility, write who and whether the person was available.	Who: Contacted: ☐ Yes ☐ No Who: Contacted: ☐ Yes ☐ No	

continued

APPENDIX 16.13–1 *(continued)*

Information Checklist *(continued)*

Step	Task/data	Date/time completed	Signature
9.	If instructed to contact others *outside* facility, write who and whether the person was available.	Who: Contacted: ☐ Yes ☐ No Who: Contacted: ☐ Yes ☐ No	
10.	Specimens for suspected bioterrorism agents placed in the biological safety cabinet in the ____ part of the laboratory.	☐ Yes ☐ No	

APPENDIX 16.13–2

Policy Sign-Off List

Date reviewed	Employee signature

APPENDIX 16.13–3

Training Sign-Off List

Date	Employee signature	Signature of trainer

APPENDIX 16.13–4

CDC BSL designations for laboratories

BSL	Agents	Practices	Safety equipment	Facility
1	Not known to cause disease in healthy adults	Standard microbiological procedures	None required	Open bench-top sink required
2	Associated with human disease. Hazard = autoinoculation, ingestion, or mucous membrane exposure	BSL 1 practice plus limited access, biohazard warning signs, sharps precautions, and a biosafety manual defining waste decontamination or medical surveillance policies	Class I or II BSC,[a] splash guards and other devices to prevent splashes or aerosols. PPE (lab coats, gloves, and face protection) as needed	BSL 1 plus autoclave available
3	Indigenous or exotic agents with potential aerosol transmission. Disease may have serious or lethal consequences.	BSL 2 practice plus controlled access, decontamination of all waste, decontamination of clothing before laundering, baseline serum	BSL 2 safety equipment plus respiratory protection as needed	BSL 2 plus physical separation from access corridors, self-closing double-door access, exhausted air not recirculated, negative airflow into the lab
4	Dangerous and exotic agents that pose high risk of life-threatening disease; aerosol transmitted	BSL 3 practices plus clothing change before entering, shower on exit, all materials decontaminated on exit from facility	All procedures conducted in class III BSC or class I or II BSC in combination with full-body, air-supplied, positive-pressure personnel suit	BSL 3 plus separate building or isolated zone, dedicated supply/exhaust, vacuum and decontamination system

[a] BSC, biological safety cabinet.

Recommended BSL for BT agents

Agent	BSL		Specimen exposure risk	Recommended laboratories precautions	
	Specimen handling	Culture handling		BSL 2	BSL 3
Alphaviruses	2	3	Blood, CSF. Tissue culture and animal inoculation studies should be performed at BSL 3 and are *not sentinel (level A) laboratory procedures.*	Activities involving clinical material collection and transport	
Bacillus anthracis	2	3	Blood, skin lesion exudates, CSF, pleural fluid, sputum, and, rarely, urine and feces	Activities involving clinical material collection and diagnostic quantities of infectious cultures	Activities with high potential for aerosol or droplet production
Brucella spp.[a]	2	3	Blood, bone marrow, CSF, tissue, semen, and, occasionally, urine	Activities limited to collection, transport, and plating of clinical material	All activities involving manipulations of cultures
Burkholderia pseudomallei	2	3	Blood, sputum, CSF, tissue, abscesses, and urine	Activities limited to collection, transport, and plating of clinical material	All activities involving manipulations of cultures
Burkholderia mallei	2	3	Blood, sputum, CSF, tissue, abscesses, and urine	Activities limited to collection, transport, and plating of clinical material	All activities involving manipulations of cultures

(continued)

APPENDIX 16.13–4 *(continued)*

Recommended BSL for BT agents *(continued)*

Agent	BSL		Specimen exposure risk	Recommended laboratories precautions	
	Specimen handling	Culture handling		BSL 2	BSL 3
Coxiella burnetii[b]	2	3	Blood, tissue, body fluids, feces. Manipulation of tissues from infected animals and tissue culture should be performed at BSL 3 and are *not sentinel laboratory procedures.*	Activities limited to collection and transport of clinical material, including serological examinations	
Clostridium botulinum[c]	2	3	Toxin may be present in food specimens, clinical material (serum, gastric, and feces). *Toxin is extremely poisonous!*	Activities with materials known to be or potentially containing toxin must be handled in a biological safety cabinet (class II) with a lab coat, disposable surgical gloves, and a face shield (as needed).	Activities with high potential for aerosol or droplet production
Francisella tularensis[d]	2	3	Skin lesion exudates, respiratory secretions, CSF, blood, urine, tissues from infected animals, and fluids from infected arthropods	Activities limited to collection, transport, and plating of clinical material	All activities involving manipulations of cultures
Yersinia pestis[e]	2	3	Bubo fluid, blood, sputum, CSF, feces, and urine	Activities involving clinical material collection and diagnostic quantities of infectious cultures	Activities with high potential for aerosol or droplet production
Smallpox[f]	4	4	Lesion fluid or crusts, respiratory secretions, or tissue	Packing and shipping. Do *not* put in cell culture.	
Staphylococcal enterotoxin B	2	2	Toxin may be present in food specimens, clinical material (serum, gastric, urine, respiratory secretions, and feces), and isolates of *Staphylococcus aureus.*	Activities involving clinical material collection and diagnostic quantities of infectious cultures	
VHF[g]	4	4	Blood, urine, respiratory, and throat secretions, semen, and tissue	Packing and shipping. Do *not* put in cell culture.	

[a] Laboratory-acquired brucellosis has occurred by "sniffing" cultures; aerosols generated by centrifugation; mouth pipetting; accidental parenteral inoculations; sprays into eyes, nose, and mouth; direct contact with clinical specimens; and when no breach in technique could be identified.

[b] Laboratory-acquired infections have been acquired from virulent phase I organisms due to infectious aerosols from cell culture and the use of embryonated eggs to propagate *C. burnetii.*

[c] Exposure to toxin is the primary laboratory hazard, since absorption can occur with direct contact with skin, eyes, or mucous membranes, including the respiratory tract. The toxin can be neutralized by 0.1 M sodium hydroxide. *C. botulinum* is inactivated by a 1:10 dilution of household bleach. Contact time is 20 min. If material contains both toxin and organisms, the spill must be sequentially treated with bleach and sodium hydroxide for a total contact time of 40 min.

[d] Laboratory-acquired tularemia infection has been more commonly associated with cultures than with clinical materials or animals. Direct skin/mucous membrane contact with cultures, parenteral inoculation, ingestion, and aerosol exposure have resulted in infection.

[e] Special care should be taken to avoid the generation of aerosols.

[f] Ingestion, parenteral inoculation, and droplet or aerosol exposure of mucous membranes or broken skin with infectious fluids or tissues are the primary hazards to laboratory workers.

[g] Respiratory exposure to infectious aerosols, mucous membrane exposure to infectious droplets, and accidental parenteral inoculation are the primary hazards to laboratory workers.

APPENDIX 16.13–4 *(continued)*

Specimen selection for suspected BT agents[a]

Disease/agent	Specimen selection		Transport and storage	Specimen plating and processing				
				SBA	CHOC	MAC	Stain	Other
Anthrax *(Bacillus anthracis)*	Possible *Bacillus anthracis* exposure in an asymptomatic patient	Swab of anterior nares: only to be collected if so advised by local public health authorities	≤24 h, RT	No	No	No	None	Follow public health instructions on anterior nares; swab *only* if advised to collect such samples.
	Cutaneous	Vesicular stage: collect fluid from intact vesicles on sterile swab(s). The organism is best demonstrated in this stage.	≤24 h, RT	X	X	X	Gram stain	
		Eschar stage: without removing eschar, insert swab moistened in sterile saline beneath the edge of eschar, rotate, and collect lesion material.	≤24 h, RT	X	X	X	Gram stain	
		Vesicular stage and eschar stage: collect 2 punch biopsy samples. Place one sample in 10% formalin to be sent to the CDC for histopathology, immunohistochemical staining, and PCR.	One punch biopsy in 10% formalin. Once in formalin, can be stored until transported to the CDC	No	No	No	Performed at the CDC	Arrange for transport to the CDC.
		Submit second biopsy sample in an anaerobic transport vial for culture.	Second punch biopsy sample in anaerobic transport vial (≤24 h, RT)	X	X	X	Gram stain	
		Blood cultures: collect 2 sets (1 set is 2 bottles) per institutional procedure for routine blood cultures.	Transport at RT. Incubate at 35–37°C per blood culture protocol.	Blood culture bottles			Positive in some cases during late stages of disease	
		Purple-top tube (EDTA): for inpatients only, collect for direct Gram stain.	≤2 h, RT	No	No	No	Gram stain	
		Red-top or blue-top tubes for serology; white tube for PCR	≤24 h, 4°C	No	No	No	No	Arrange for transport to the CDC.
	Gastrointestinal	Stool: collect 5–10 g in a clean, sterile, leakproof container.	≤24 h, 4°C	Inoculate routine stool plating media plus CNA or PEA.				Minimal recovery
		Blood cultures: collect 2 sets (1 set is 2 bottles) per institutional procedure for routine blood cultures.	Transport at RT. Incubate at 35–37°C per blood culture protocol.	Blood culture bottles			Positive in late stages of disease	

(continued)

APPENDIX 16.13–4 *(continued)*

Specimen selection for suspected BT agents[a] *(continued)*

Disease/agent	Specimen selection		Transport and storage	Specimen plating and processing				
				SBA	CHOC	MAC	Stain	Other
Anthrax *(Bacillus anthracis) (cont.)*	Gastrointestinal *(cont.)*	Purple-top tube (EDTA): for inpatients only, collect for direct Gram stain.	≤2 h, RT	No	No	No	Gram stain	
		Red-top or blue-top tubes for serology; white tube for PCR	≤24 h, 4°C	No	No	No	No	
	Inhalation	Sputum: collect expectorated specimen into a sterile, leakproof container.	≤24 h, 4°C	X	X	X	Gram stain	Minimal recovery
		Pleural fluid: collect specimen into sterile, leakproof container.	≤24 h, 4°C	X	X	X	Gram stain	Save excess (if any) for PCR.
		Blood cultures: collect 2 sets (1 set is 2 bottles) per institutional procedure for routine blood cultures.	Transport at RT. Incubate at 35–37°C per blood culture protocol.	Blood culture bottles			Positive in late stages of disease	
		Purple-top tube (EDTA): for inpatients only, collect for direct Gram stain.	≤2 h, RT	No	No	No	Gram stain	
		Red-top or blue-top tubes for serology; white tube for PCR	≤24 h, 4°C	No	No	No	No	
	Meningitis	CSF culture: aseptically collect CSF per institutional procedure.	≤24 h, RT	X	X		Gram stain	May be seen in late stages of disease; consider adding broth medium such as BHI
		Blood cultures: collect 2 sets (1 set is 2 bottles) per institutional procedure for routine blood cultures.	Transport at RT. Incubate at 35–37°C per blood culture protocol.	Blood culture bottles			Positive in late stages of disease	
Brucellosis *(Brucella melitensis, B. abortus, B. suis, B. canis)*	Acute, subacute, or chronic	Serum: collect 10–12 cc (ml) of acute-phase specimen as soon as possible after disease onset. Follow with a convalescent-phase specimen obtained 21 days later.	Transport in ≤2 h at RT. Store at −20°C.	Specimen should be stored and shipped frozen at −20°C to state laboratory or other LRN reference (level B or C) laboratory.			Serological diagnosis 1. Single titer: ≥1:160 2. Fourfold rise 3. IgM *Note: B. canis* does not cross-react with standard serological reagents.	

APPENDIX 16.13–4 *(continued)*

Specimen selection for suspected BT agents[a] *(continued)*

Disease/agent	Specimen selection		Transport and storage	Specimen plating and processing				
				SBA	CHOC	MAC	Stain	Other
Brucellosis *(Brucella melitensis, B. abortus, B. suis, B. canis) (cont.)*	Acute, subacute, or chronic *(cont.)*	Blood: collect 2 sets (1 set is 2 bottles) per institutional procedure for routine blood cultures.	Transport at RT. Incubate at 35–37°C per blood culture protocol.	Blood culture bottles: Subculture at 5 days and hold for 21 days.				Blood culture isolation rates vary from 15–70% depending on methods and length of incubation. Cultures should be manipulated in a biological safety cabinet. PPE includes gloves, gown, mask, and protective face shield. All cultures should be taped shut during incubation.
		Bone marrow, spleen, or liver: collect per institution's surgical/pathology procedure.	≤24 h RT	X	X		Gram stain	Cultures should be manipulated in a biological safety cabinet. PPE includes gloves, gown, mask, and protective face shield. All cultures should be taped shut during incubation.
				Hold cultures for at least 7 days.				
	Meningitis	CSF culture: aseptically collect CSF per institutional procedure.	≤24 h, RT	X	X		Gram stain	Cultures should be manipulated in a biological safety cabinet. PPE includes gloves, gown, mask, and protective face shield. All cultures should be taped shut during incubation. Consider adding broth medium such as BHI.
				Hold cultures for at least 7 days.				
		CSF for antibody testing	−20°C	Specimen should be stored and shipped frozen at −20°C or lower temp to state laboratory or other LRN reference laboratory.			None	

[a] CNA, colistin-nalidixic acid agar; PEA, phenylethyl alcohol blood agar; RT, room temperature; X, yes.

APPENDIX 16.13–4 *(continued)*

Specimen selection: bioterrorism agents[a]

Disease/agent	Specimen selection					Transport and storage	Specimen handling
	Specimen type	Clinical syndrome					Specimen(s) of choice for confirming botulism 1. Serum 2. Wound/tissue 3. Stool 4. Incriminated food
		Foodborne	Infant	Wound	Intentional release (airborne)		
Botulism (botulinum toxin)	Enema fluid, 20 ml	X	X		X	4°C	Purge with a minimal amount of sterile nonbacteriostatic water to minimize dilution of toxin.
	Food sample, 10–50 g	X	X		X	4°C	Foods that support *C. botulinum* growth will have a pH of 3.5–7.0; the most common pH is 5.5–6.5. Submit food in original container, placing individually in leakproof sealed transport devices.
	Gastric fluid, 20 ml	X, A				4°C	Collect up to 20 ml.
	Intestinal fluid, 20 ml	A	A			4°C	Autopsy: intestinal contents from various areas of the small and large intestines should be provided.
	Nasal swab (anaerobic swab)				X	RT	For aerosolized botulinum toxin exposure, obtain nasal cultures for *C. botulinum* and serum for mouse toxicity testing.
	Serum, 15–20 ml	X, A		X	X	4°C	Serum should be obtained as soon as possible after the onset of symptoms and before antitoxin is given. Whole blood (30 ml [3 red-top or gold-top tubes]) is required for mouse toxicity testing. For infants, serum is generally not useful, since the toxin is quickly absorbed before serum can be obtained.
	Stool, >25 g	X	X	X	X	4°C	Botulism has been confirmed in infants with only "pea-size" stools. Please note: anticholinesterase given orally, as in patients with myasthenia gravis, has been shown to interfere with toxin testing.
	Vomitus, 20 ml	X				4°C	Collect up to 20 ml.
	Wound, tissue—anaerobic swab or transport system					Anaerobic swab or transport system. Transport at RT.	Exudate, tissue, or swabs must be collected and transported in an anaerobic transport system. Samples from an enema or feces should also be submitted, since the wound may not be the source of botulinum toxin.

[a] A, autopsy; RT, room temperature; X, yes.

APPENDIX 16.13–4 *(continued)*

Specimen selection: bioterrorism agents[a]

Disease/agent	Specimen selection			Transport and storage	Specimen handling
	Specimen type	Foodborne	Airborne (intentional release)		
Staphylococcal enterotoxin B (from *Staphylococcus aureus*)	Serum, 10 ml	X	X	2–8°C	1. Obtain as soon as possible after the onset of symptoms to detect the toxin. 2. Also collect 7–14 days after onset of illness to compare acute- and convalescent-phase antibody titers. 3. Do not send whole blood, since hemolysis during transit will compromise the quality of the specimen.
	Nasal swab—Dacron or rayon swab		X	2–8°C	Collect a nasal swab within 24 h of exposure by rubbing a dry, sterile swab (Dacron or rayon) on the mucosa of the anterior nares. Place in protective transport tube.
	Induced respiratory secretions		X	2–8°C	Collect sputum induced by instilling 10–25 ml of sterile saline into nasal passages into a sterile screw-top container.
	Urine, 20–30 ml	X	X	2–8°C	Collect into a sterile, leakproof container with screw-top lid.
	Stool or gastric aspirate, 10–50 g	X	X	2–8°C	Collect into a sterile, leakproof container with screw-top lid.
	Postmortem, 10 g	X	X	2–8°C	Obtain specimens of the intestinal contents from different levels of the small and large bowel. Place 10 g of specimen into a sterile, leakproof container with screw-top lid. Obtain serum as previously described.
	Culture isolate	X	X	2–8°C	Send *S. aureus* isolate for toxin testing on appropriate agar slant.
	Food specimen	X	X	2–8°C	Food should be left in its original container if possible or placed in sterile unbreakable containers and labeled carefully. Place containers individually in leakproof containers (i.e., sealed plastic bags) to prevent cross-contamination during shipment. Empty containers with remnants of suspected contaminated foods can be examined.

[a] X, yes.

APPENDIX 16.13–4 *(continued)*

Specimen selection: bioterrorism agents[a]

Disease/agent	Specimen selection		Transport and storage	Specimen plating and processing				
				BAP	CHOC	MAC	Stain	Other
Plague *(Yersinia pestis)*	Possible *Y. pestis* exposure in asymptomatic patient	No cultures or serology indicated						Follow public health instructions if advised to collect specimens.
	Bubonic	Blood cultures: collect 2 sets (1 set is 2 bottles) per institutional procedure for routine blood cultures.	Transport at RT. Incubate at 35–37°C per blood culture protocol.	Blood culture bottles			Gram stain of positive cultures	If suspicion of plague is high, obtain an additional set for incubation at RT (22–28°C) without shaking.
		Tiger-top, red-top, or gold-top tube: for serology (acute and, if needed for diagnosis, convalescent-phase serum in 14 days) Green-top (heparin) tube: for PCR	≤24 h, 4°C	No			No	Patients with negative cultures having a single titer, ≥1:10, specific to F1 antigen by agglutination would meet presumptive criteria.
		Lymph node (bubo) aspirate: flushing with 1.0 ml of sterile saline may be needed to obtain material.	Transport at RT or 4°C if transport is delayed. Store for ≤24 h at 4°C.	X	X	X	Gram, Giemsa, Wright's stain	Contact LRN reference lab or above laboratory to prepare smears for DFA.
		Tissue: collect in sterile container with 1 or 2 drops of sterile, nonbacteriostatic saline.	Transport at RT or 4°C if transport is delayed. Store for ≤24 h at 4°C.	X	X	X	Gram, Giemsa, Wright's stain	Contact LRN reference lab or above laboratory to prepare smears for DFA.
		Throat: collect routine throat culture using a swab collected into a sterile, leakproof container.	≤24 h, 4°C	X	X	X	Gram stain	Contact LRN reference lab or above laboratory to prepare smears for DFA.
	Pneumonic	Sputum/throat: collect routine throat culture using a swab or expectorated sputum collected into a sterile, leakproof container.	≤24 h, 4°C	X	X	X	Gram stain	Contact LRN reference lab or above laboratory to prepare smears for DFA.
		Bronchial/tracheal wash: collect per institution's procedure in an area dedicated to collecting respiratory specimens under isolation/containment circumstances, i.e., isolation chamber/ "bubble."	≤24 h, 4°C	X	X	X	Gram stain	Contact LRN reference lab or above laboratory to prepare smears for DFA.
		Blood cultures: collect 2 sets (1 set is 2 bottles) per institutional procedure for routine blood cultures.	Transport at RT. Incubate at 35–37°C per blood culture protocol.	Blood culture bottles			Gram stain of positive cultures	If suspicion of plague is high, obtain an additional set for incubation at RT (22–28°C) without shaking.

APPENDIX 16.13–4 *(continued)*

Specimen selection: bioterrorism agents[a]

Disease/agent	Specimen selection		Transport and storage	Specimen plating and processing				
				BAP	CHOC	MAC	Stain	Other
Plague *(Yersinia pestis) (cont.)*	Pneumonic *(cont.)*	Tiger-top, red-top, or gold-top tube: for serology (acute-phase and, if needed for diagnosis, convalescent-phase serum in 14 days) Green-top (heparin) tube: for PCR	≤24 h, 4°C	No			No	Patients with negative cultures having a single titer, ≥1:10, specific to F1 antigen by agglutination would meet presumptive criteria.
	Meningitis	Blood cultures: collect 2 sets (1 set is 2 bottles) per institutional procedure for routine blood cultures.	Transport at RT. Incubate at 35–37°C per blood culture protocol.	Blood culture bottles			Gram stain of positive cultures	If suspicion of plague is high, obtain an additional set for incubation at RT (22–28°C) without shaking.
		Tiger-top, red-top, or gold-top tube: for serology (acute-phase and, if needed for diagnosis, convalescent-phase serum in 14 days) Green-top (heparin) tube: for PCR	≤24 h, 4°C	No			No	Patients with negative cultures having a single titer, ≥1:10, specific to F1 antigen by agglutination would meet presumptive criteria.
		CSF	Transport at RT. Store incubated at 35–37°C.	X	X		Gram stain	Can add broth culture at RT (22–28°C) without shaking.
Tularemia *(Francisella tularensis)*	Possible *F. tularensis* exposure in asymptomatic patient	No cultures or serology indicated						Follow public health instructions if advised to collect specimens.
	Oculoglandular	Conjunctival scraping	≤24 h, 4°C	X	X	X	Gram stain; prepare smears for DFA referral.	Add a BCYE plate and a plate selective for *Neisseria gonorrhoeae* such as modified Thayer-Martin agar. Manipulate cultures in a biological safety cabinet. PPE includes gloves, gown, mask, and protective face shield. All cultures should be taped shut during incubation.
		Lymph node aspirate: flushing with 1.0 ml of sterile saline may be needed to obtain material.	Transport at RT or 4°C if transport is delayed. Store for ≤24 h at 4°C.	X	X	X	Gram stain; prepare smears for DFA referral.	Add a BCYE plate and a plate selective for *N. gonorrhoeae*, such as modified Thayer-Martin. Manipulate cultures in a biological safety cabinet. PPE includes gloves, gown, mask, and protective face shield. All cultures should be taped shut during incubation.

(continued)

APPENDIX 16.13–4 *(continued)*

Specimen selection: bioterrorism agents[a] *(continued)*

Disease/agent	Specimen selection		Transport and storage	Specimen plating and processing				
				BAP	CHOC	MAC	Stain	Other
Tularemia *(Francisella tularensis) (cont.)*	Oculoglandular *(cont.)*	Blood cultures: collect 2 sets (1 set is 2 bottles) per institutional procedure for routine blood cultures. Growth is more likely from aerobic bottle.	Transport at RT. Incubate at 35–37°C per blood culture protocol.	Blood culture bottles; subculture the broth to BCYE plate and incubate aerobically.			Cultures should be manipulated in a biological safety cabinet. PPE includes gloves, gown, mask, and protective face shield. All cultures should be taped shut during incubation.	
	Ulceroglandular	Blood cultures: collect 2 sets (1 set is 2 bottles) per institutional procedure for routine blood cultures. Growth is more likely from aerobic bottle.	Transport at RT. Incubate at 35–37°C per blood culture protocol.	Blood culture bottles; subculture the broth to BCYE plate and incubate aerobically.			Cultures should be manipulated in a biological safety cabinet. PPE includes gloves, gown, mask, and protective face shield. All cultures should be taped shut during incubation.	
		Ulcer or tissue: collect biopsy sample (best specimen), scraping, or swab.	≤24 h, 4°C	X	X	X	Gram stain	Add a BCYE plate and a plate selective for *N. gonorrhoeae* such as modified Thayer-Martin agar. Prepare smears for DFA referral. Manipulate cultures in a biological safety cabinet. PPE includes gloves, gown, mask, and protective face shield. All cultures should be taped shut during incubation.
		Lymph node aspirate: flushing with 1.0 ml of sterile saline may be needed to obtain material.	Transport at RT or 4°C if transport is delayed. Store at ≤24 h and 4°C.	X	X	X	Gram stain; prepare smears for DFA referral	
	Pneumonic	Sputum/throat: collect routine throat culture using a swab or expectorated sputum collected into a sterile, leakproof container.	≤24 h, 4°C	X	X	X	Gram stain	Add a BCYE plate and a plate selective for *N. gonorrhoeae* such as modified Thayer-Martin agar. Prepare smears for DFA referral. Manipulate cultures in a biological safety cabinet. PPE includes gloves, gown, mask, and protective face shield. All cultures should be taped shut during incubation.
		Bronchial/tracheal wash: collect per institution's procedure in an area dedicated to collecting respiratory specimens under isolation/containment circumstances, i.e., isolation chamber/ "bubble."	≤24 h, 4°C	X	X	X	Gram stain	
		Blood cultures: collect 2 sets (1 set is 2 bottles) per institutional procedure for routine blood cultures. Growth is more likely from aerobic bottle.	Transport at RT. Incubate at 35–37°C per blood culture protocol.	Blood culture bottles; subculture the broth to BCYE plate and incubate aerobically.			Cultures should be manipulated in a biological safety cabinet. PPE includes gloves, gown, mask, and protective face shield. All cultures should be taped shut during incubation.	
		2 red-top or gold-top tubes: for PCR and serology (acute-phase and, if needed for diagnosis, convalescent-phase serum in 14 days)	≤2 h, RT ≤24 h, 4°C	No			Positive serology test would meet presumptive criteria. Confirmation requires culture identification or a fourfold rise in titer.	

[a] BYCE, buffered charcoal-yeast extract agar; DFA, direct fluorescent antibody; RT, room temperature; X, yes.

APPENDIX 16.13–4 *(continued)*

Specimen selection: bioterrorism agents[a]

Disease/agent	Specimen selection and transport			Specimen handling
Smallpox (variola virus)	See CDC document *Specimen Collection and Transport Guidelines* for detailed instructions (http://www.bt.cdc.gov/agent/smallpox/response-plan/index.asp#guidec; click on Guide D) *Note:* Only recently and successfully *vaccinated* personnel (within 3 years) wearing appropriate barrier protection (gloves, gown, and shoe covers) should be involved in specimen collection for suspected cases of smallpox. Respiratory protection is not needed for personnel with recent successful vaccination. Masks and eyewear or face shields should be used if splashing is anticipated. If unvaccinated personnel must be utilized to collect specimens, only those without contraindications to vaccination should be utilized, as they would require immediate vaccination if the diagnosis of smallpox is confirmed. Fit-tested N-95 masks should be worn by unvaccinated individuals caring for suspected patients.			
	Rash	Biopsy specimens	See CDC document *Specimen Collection and Transport Guidelines* for detailed instructions (Guide D).	1. *A suspected case of smallpox should be reported* immediately *to the respective local and state health departments for review.* 2. And if, after review, smallpox is still suspected, one of the following should be contacted immediately. CDC Emergency Response Hotline (24 h): (770) 488-7100 Poxvirus Section, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases (NCID), CDC, Atlanta, GA, Laboratory: (404) 639-4931 Bioterrorism Preparedness and Response Program, NCID, CDC: (404) 639-0385 or (404) 639-2468 (8 a.m. to 5 p.m. weekdays) *NOTE: Approval must be obtained prior to the shipment of potential smallpox patient clinical specimens to the CDC.* 3. At this time, review the packaging/shipping requirements with the CDC and request assistance in coordinating a carrier for transport/shipment. 4. *Hand carry all specimens and do not send specimens via pneumatic tube system.* 5. Do not attempt viral cultures: this is a BSL 4 agent, and this could result in a very unsafe situation in which there is a significant amount of infectious virus.
		Scabs		
		Vesicular fluid		
	Posterior tonsillar tissue swab	Swab		
	Blood	Use plastic tubes.		
	Autopsy	Portions of skin containing lesions, liver, spleen, lung, lymph nodes, and/or kidney		
VHF (various viruses, including Ebola, Marburg, Lassa, Machupo, Junin, Guanarito, Sabia, Crimean-Congo hemorrhagic fever, Rift Valley fever, Omsk hemorrhagic fever, and Kyasanur Forest disease viruses, among others)	Serum for antibody testing: collect blood in red-top or gold-top tubes. Obtain convalescent-phase serum at least 14 days after the acute-phase specimen is obtained. Use a Vacutainer or other sealed sterile dry tube for blood collection.		Transport within ~2 h at RT. Store at −20°C to −70°C.	Specific handling conditions are currently under development. Contact the CDC to discuss proper collection and handling. 1. Double-bag each specimen. 2. Swab the exterior of the outside bag with disinfectant *before* removal from the patient's room. 3. Do not use glass tubes. 4. Hand carry all specimens and do not send specimens via pneumatic tube system. *Note:* Disposable equipment and sharps go into rigid containers containing disinfectant that are then autoclaved or incinerated. Double-bag refuse. The exterior of the outside bag is to be treated with disinfectant and then autoclaved or incinerated. *Do not attempt tissue culture isolation.* This is only to be done in a BSL 4 facility. In laboratory: 1. Strict barrier precautions are to be used. PPE includes gloves, gown, mask, shoe covers, and protective face shield.
	Viral culture, blood: collect serum, heparinized plasma (green-top tube), or whole blood during acute febrile illness.		Transport at RT. Store at 4°C or frozen on dry ice or liquid nitrogen.	
	Throat wash specimens: mix with equal volume of viral transport medium.		Transport on wet ice. Store at −40°C or colder.	
	Urine: mix with equal volume of viral transport medium.		Transport on wet ice. Store at −40°C or colder.	
	CSF, tissue, other specimens		As per discussion with the CDC	

(continued)

APPENDIX 16.13–4 *(continued)*

Specimen selection: bioterrorism agents[a] *(continued)*

Disease/agent	Specimen selection and transport		Specimen handling
VHF (various viruses, including Ebola, Marburg, Lassa, Machupo, Junin, Guanarito, Sabia, Crimean-Congo hemorrhagic fever, Rift Valley fever, Omsk hemorrhagic fever, and Kyasanur Forest disease viruses, among others) *(cont.)*	CSF, tissue, other specimens *(cont.)*	As per discussion with the CDC *(cont.)*	2. Handle specimens in a biological safety cabinet if possible. 3. Consider a respiratory mask with a HEPA filter. 4. Specimens should be centrifuged at low speed.
	Blood cultures: if clinical and travel history warrants, collect 2 sets (1 set is 2 bottles) of blood cultures per institutional procedure for routine blood cultures.	Transport at RT. Incubate at 35–37°C per blood culture protocol.	Bacteremia with disseminated intravascular coagulation and malaria due to *Plasmodium falciparum* are two life-threatening and treatable clinical entities that can present with prominent clinical findings of hemorrhage and fever in a patient with a history of travel to areas with VHF. Handle with precautions noted above. Continue to use the same precautions as above.
	Malaria smear of peripheral blood: if clinical and travel history warrants	Lavender-top tube at RT	
Q fever *(Coxiella burnetii)*	Serum: collect 10 ml of serum (red-top, tiger-top, or gold-top tube) as soon as possible after onset of symptoms (acute phase) and with a follow-up specimen (convalescent phase) at ≥14 days for serological testing.	Transport within ~6 h at 4°C. Store at −20 to −70°C.	*Do not attempt tissue culture isolation,* as that could result in a very unsafe situation in which there is a significant amount of infectious organism. Sentinel laboratories should consult with state public health laboratory director (or designate) prior to or concurrent with testing if *C. burnetii* is suspected by the attending physician. Serology is available through commercial reference as well as public health laboratories.
	Blood: collect blood in EDTA (lavender) or sodium citrate (blue) and maintain at 4°C for storage and shipping for PCR or special cultures. If possible, collect specimens prior to antimicrobial therapy.	Transport within ~6 h at 4°C. Store at 4°C.	
	Tissue, body fluids, others, including cell cultures and cell supernatants: specimens can be kept at 2–8°C if transported within 24 h. Store frozen at −70°C or on dry ice.	Transport within <24 h at 2–8°C. Store at −70°C or on dry ice.	
Alphaviruses (include eastern equine, western equine, and Venezuelan equine encephalitis viruses, among others)	Serum: collect 10 ml of serum (red-top, tiger-top, or gold-top tube) as soon as possible after onset of symptoms (acute phase) and with a follow-up specimen (convalescent phase) at ≥14 days for serological testing.	Transport within ~6 h at 4°C. Store at −20°C to −70°C.	*Do not attempt tissue culture isolation,* as that could result in a very unsafe situation in which there is a significant amount of infectious organism.
	Blood: collect blood in EDTA (lavender) or sodium citrate (blue) and maintain at 4°C for storage and shipping for PCR or special studies.	Transport within ~6 h at 4°C. Store at 4°C.	
	CSF: specimens (greater than 1 ml) can be kept at 2–8°C if transported within 24 h. If frozen, store at −70°C and transport on dry ice.	Transport on wet ice. If already frozen, store at −70°C and transport on dry ice.	
	Tissue, body fluids, others, including cell cultures and cell supernatants: specimens can be kept at 2–8°C if transported within 24 h. If frozen, store at −70°C and transport on dry ice.	Transport on wet ice. If already frozen, store at −70°C and transport on dry ice.	

[a] RT, room temperature; VHF, viral hemorrhagic fever.

APPENDIX 16.13–4 *(continued)*

Specimen selection: bioterrorism agents[a]

Disease/agent	Specimen selection		Transport and storage	Specimen plating and processing					
				BAP	CHOC	MAC	PC	Stain	Other
Melioidosis and glanders *(Burkholderia pseudomallei* and *Burkholderia mallei)*	Possible *B. pseudomallei* or *B. mallei* exposure in asymptomatic patient	No cultures or serology indicated							Follow public health instructions if advised to collect specimens.
	Clinical illness	Bone marrow	Transport within ≤2 h at RT. Store for ≤24 h at 4°C.		X			Gram stain	*B. pseudomallei* is a small gram-negative bacillus that may demonstrate bipolar morphology on stain. *B. mallei* is a small gram-negative coccobacillus. Incubation should be at 35–37°C, ambient atmosphere; CO_2 incubation is acceptable.
		Blood cultures: collect 2 sets (1 set is 2 bottles) per institutional procedure for routine blood cultures *or* collect lysis-centrifugation (e.g., Isolator) blood cultures.	Transport at RT. Incubate at 35–37°C per blood culture protocol.	Blood culture bottles *or* collect lysis-centrifugation (e.g., Isolator) blood cultures and plate to:					Cultures should be manipulated in a biological safety cabinet. PPE includes gloves, gown, mask, and protective face shield. All cultures should be taped shut during incubation. Incubation should be at 35–37°C, ambient atmosphere; CO_2 incubation is acceptable. *Burkholderia cepacia* selective agar medium has been shown to be useful in isolation of *B. pseudomallei* for specimens in which indigenous microflora is likely to be encountered. Ashdown medium is a selective medium specifically designed for recovery of *B. pseudomallei*. This medium is not likely to be available in most sentinel laboratories. Incubation should be at 35–37°C, ambient atmosphere; CO_2 incubation is acceptable.
					X				
		Respiratory specimens, abscess material, wound specimens, urine	Transport within ≤2 h at RT. Store for ≤24 h at 4°C.	X	X	X	X	Gram stain	
		Serum: red-top or gold-top tube for both acute- and convalescent-phase samples (latter obtained 14 days after the acute-phase specimen)	Transport within ~6 h at 4°C. Store at −20 to −70°C.	Obtain if serological diagnosis of *B. pseudomallei* infection is being considered.					

[a] PC, selective medium for *B. cepacia*; RT, room temperature; X, yes.

APPENDIX 16.13–4 *(continued)*

BT agent characteristics summary: microorganisms[a]

Characteristic	*B. anthracis*	*Y. pestis*	*B. pseudomallei* and *B. mallei*	*F. tularensis*	*Brucella* spp.	Variola virus (smallpox)
Gram stain morphology	Large gram-positive rod Nonmotile From blood agar: no capsule, central to subterminal spores that do not enlarge the cell From blood: capsule, no spores	Plump gram-negative rod Gram stain: ± bipolar or "safety pin" appearance Wright-Giemsa: bipolar or safety pin appearance	*B. pseudomallei:* small gram-negative rod *B. mallei:* small gram-negative coccobacillus Gram stain: ± bipolar or safety pin appearance *(B. pseudomallei)* Wright-Giemsa: bipolar or safety pin appearance *(B. pseudomallei)*	Minute GNCB Poorly staining Smaller than *Haemophilus influenzae* Pleomorphic	Tiny GNCB Faintly staining	
Growth	Standard conditions Extremely rapid	28°C optimal, without agitation 35–37°C more slowly	35–37°C Ambient atmosphere, though CO_2 is acceptable	Aerobic conditions Growth is best on media containing cysteine, such as BCYE, but will often grow initially on CHOC or BAP	Grows in blood culture media Can require blind subculturing	Grows in most cell lines Unusual or unrecognizable CPE
Colonial morphology (BAP)	Nonhemolytic Ground glass Irregular/wavy edges Tenacious "Beaten egg whites" when touched with loop	Pinpoint at 24–48 h "Fried egg" or "hammered copper" or shiny at 48–72 h Nonhemolytic	*B. pseudomallei:* BAP: small, smooth creamy colonies in first 1–2 days, gradually changing after a few days to dry, wrinkled colonies similar to *Pseudomonas stutzeri* *B. mallei:* BAP: smooth, gray, translucent colonies without pigment	Does not pass well on BAP	Small colonies Punctate after 48 h Nonhemolytic	
Tests	Cat +	Cat + Ox − Urease − MAC: Lac − Indole −	Cat + Colistin (10 μg) and polymyxin B (300 U) (R) Motility + *(B. pseudomallei)* Motility − *(B. mallei)* Indole − Oxidase + *(B. pseudomallei)* Oxidase ± *(B. mallei)* MAC: Lac − *(B. pseudomallei)* MAC: Lac − or NG *(B. mallei)*	Cat wk + Ox − Urease − β-Lac − Satellite − MAC: NG	Ox + Urease +, though some are negative Satellite − MAC: poor to NG	CPE can be passed

[a] GNCB, gram-negative coccobacillus; CPE, cytopathic effect; Cat, catalase; Ox, oxidase; (R), resistant; NG, no growth; Lac, lactose.

APPENDIX 16.13–4 *(continued)*

BT agent clinical summary

Disease	Virulence factor(s)	Infective dose	Incubation period	Duration of illness	Person-to-person transmission[e]	Isolation precautions for hospitalized[f]	Persistence of organism
Inhalation anthrax	Exotoxin[a] capsule	Lower limit unknown, ID2 estimated at 9 spores[b]	1–6 days	3–5 days	No	Standard	>40 yr
Brucellosis	LPS[c]; PMN survival	10–100 organisms	5–60 days (usually 1–2 mo)	Weeks to months	Via breast milk[g] and sexually[h] (rare)	Standard	Water/soil, ~10 wk
Botulism	Neurotoxin	0.001 μg/kg is LD_{50} for type A	6 h–10 days (usually 1–5 days)	Death in 24–72 h; lasts months if not lethal	No	Standard	Food/water, ~weeks
Glanders	Little studied, possible antiphagocytic capsule	Low	10–14 days via aerosol	Death in 7–10 days in septicemic form	Yes (low)	Standard	Very stable
Melioidosis	Possibly LPS, exotoxin, intracellular survival, antiphagocytic capsule	Low	2 days–26 yr	Days to months	Yes (rare)[i]	Standard	Very stable in water/soil
Pneumonic plague	V and W antigens LPS (endotoxin) F1 antigen[d]	<100 organisms	2–3 days	1–6 days	Yes (high)	Droplet[f]	Soil, up to 1 yr
Q fever	Intracellular survival LPS (endotoxin)	1–10 organisms	10–40 days	~2 wk (acute), months to years (chronic)	Rare[j]	Standard	Very stable
Smallpox		10–100 particles	7–17 days	~4 wk	Yes (high)	Airborne[f]	Very stable
Staphylococcal enterotoxin B	Superantigen	0.0004 μg/kg causes incapacitation; LD_{50} is 0.02 μg/kg	3–12 h after inhalation	Hours	No	Standard	Resistant to freezing
Tularemia	Intracellular survival	10–50 organisms	2–10 days	≥2 wk	Single case report during autopsy	Standard	Moist soil, ~months
VHF	Varies with virus	1–10 particles	4–21 days	7–16 days	Yes (moderate)	Airborne and contact[f]	Unstable

[a] *B. anthracis* exotoxin or exotoxins consist of three components: the edema factor and lethal factor exert their effect within cells by interacting with a common transport protein designated "protective antigen" (so named because, when modified, it contributes to vaccine efficacy). Expression of toxic factors is mediated by one plasmid, and that of the capsule (D-glutamic acid polypeptide) is mediated by a second plasmid. Strains repeatedly subcultured at 42°C become avirulent as a result of losing virulence-determining plasmids, which is thought to be the basis for Pasteur's attenuated anthrax vaccine used at Pouilly-le-Fort in 1881.

[b] The estimate that nine inhaled spores would infect 2% of the exposed human population is based on data from *Science* **266:**1202–1208, 1994. The dose needed to infect 50% of the exposed human population may be 8,000 or higher.

[c] The major virulence factor for brucellosis appears to be an endotoxic lipopolysaccharide (LPS) among smooth strains. Pathogenicity is related to an LPS containing poly *N*-formyl perosamine O chain, Cu-Zn superoxide dismutase, erythrulose phosphate dehydrogenase, intracellular survival stress-induced proteins, and adenine and guanine monophosphate inhibitors of phagocyte functions.

[d] The V and W antigens and the F1 capsular antigens are only expressed at 7°C and not at the lower temperature of the flea (20 to 25°C).

[e] Periods of communicability are as follows: for inhalation anthrax and botulism, none (no evidence of person-to-person transmission); pneumonic plague, 72 h following initiation of appropriate antimicrobial therapy or until sputum culture is negative; smallpox, approximately 3 weeks (usually corresponds with the initial appearance of skin lesions to their final disappearance and is most infectious during the first week of rash via inhalation of virus released from oropharyngeal lesion secretions of the index case); VHF, varies with virus, but at minimum, all for the duration of illness, and for Ebola/Marburg, transmission through semen may occur up to 7 weeks after clinical recovery.

[f] Guidelines for isolation precautions in hospitals can be found in *Infect. Control Hosp. Epidemiol.* **17:**53–80, 1996, in addition to the standard precautions that apply to all patients.

[g] Published reports of possible transmission of brucellosis via human breast milk may be found in *Int. J. Infect. Dis.* **4:**55–56, 2000; *Ann. Trop. Paediatr.* **10:**305–307, 1990; *J. Infect.* **26:**346–348, 1993; and *Trop. Geogr. Med.* **40:**151–152, 1988.

[h] Published reports of possible sexual transmission of brucellosis can be found in *Lancet* **i:**773, 1983; *Aten Primaria* **8:**165–166, 1991; *Lancet* **337:**848–849, 1991; *Lancet* **347:**1763, 1996; *Lancet* **337:**14–15, 1991; *Infection* **11:**313–314, 1983; and *Lancet* **348:**615, 1996.

[i] See *Lancet* **337:**1290–1291, 1991.

[j] Published reports of possible sexual transmission of Q fever can be found in *Clin. Infect. Dis.* **22:**1087–1088, 1996, and *Clin. Infect. Dis.* **33:**399–402, 2001.

APPENDIX 16.13–4 *(continued)*

Alternative names for BT

Agent(s)	Other information that may appear on requisition
Bacillus anthracis	Anthrax, cutaneous anthrax, gastrointestinal anthrax, inhalation anthrax, anthrax meningitis, patient with hemorrhagic mediastinitis
Brucella melitensis, B. suis, B. abortus, B. canis	Brucellosis; history of ingestion of goat's milk; history of consumption of Mexican cheese; slaughterhouse worker; history of consumption of unpasteurized milk or cheese; contact with goats, sheep, cattle, or camels; laboratory worker with accident
Burkholderia mallei	*Pseudomonas mallei*, glanders, laboratory worker with accident
Burkholderia pseudomallei	*Pseudomonas pseudomallei*, melioidosis
Clostridium botulinum toxin	Botulism, botulinum toxin, botulism toxin, infant botulism, wound botlism, food from patient with botulism
Coxiella burnetii	Q fever, pneumonia and sheep exposure, pneumonia and goat exposure, culture-negative endocarditis
Crimean-Congo hemorrhagic fever virus	Congo-Crimean hemorrhagic fever virus, CCHF, viral hemorrhagic fever, VHF, hemorrhagic fever
Ebola virus	Ebola, viral hemorrhagic fever, VHF, hemorrhagic fever
Francisella tularensis	Tularemia, *Pasteurella tularensis*, rabbit fever, deerfly fever, history of skinning animals, history of rabbit contact, tularemic pneumonia, typhoidal tularemia, oculoglandular tularemia, ulceroglandular tularemia, glandular tularemia, pharyngeal tularemia
Guanarito virus	Venezuelan hemorrhagic fever virus, viral hemorrhagic fever, VHF, hemorrhagic fever
Hantaviruses (one causes a VHF)	Korean hemorrhagic fever, Sin Nombre virus, hantavirus pulmonary syndrome, viral hemorrhagic fever, VHF, hemorrhagic fever
Junin virus (a VHF)	Argentinian hemorrhagic fever virus, viral hemorrhagic fever, VHF, hemorrhagic fever
Lassa fever virus	Viral hemorrhagic fever, VHF, hemorrhagic fever
Machupo virus	Bolivian hemorrhagic fever virus, viral hemorrhagic fever, VHF, hemorrhagic fever
Marburg virus	Marburg, viral hemorrhagic fever, VHF, hemorrhagic fever
Nipah virus	Hendra-like virus, pig contact with encephalitis
Smallpox virus	Variola, smallpox
Staphylococcal enterotoxin B	*Staphylococcus aureus* enterotoxin B, *Staphylococcus aureus* enterotoxin, staphylococcal enterotoxin, food from patient with food poisoning
Viral hemorrhagic fever	Hemorrhagic fever, VHF
Yersinia pestis	Plague, bubonic plague, pneumonic plague, septicemic plague, bubo, *Pasteurella pestis*, plague meningitis

16.14 Avian Influenza

PREANALYTICAL CONSIDERATIONS

I. PRINCIPLE

Influenza A virus is a member of the family *Orthomyxoviridae.* Influenza viruses are enveloped, with a segmented, single-stranded RNA genome. This family also contains influenza B and C viruses. Point mutations in the envelope protein hemagglutinin (H), referred to as antigenic drift, result in the emergence of new strains of influenza A and B viruses and the resultant annual outbreaks and epidemics. New influenza A virus subtypes emerge as the result of reassortment of H and neuraminidase (N) sequences from two different subtypes, referred to as antigenic shift. These new subtypes are responsible for influenza pandemics. There are currently 16 recognized H subtypes and 9 recognized N subtypes. While virtually all combinations of influenza A subtypes naturally infect waterfowl and shorebirds, certain subtypes infect poultry and mammalian species. Subtypes H1N1, H3N2, H2N2, and H1N2 have circulated, or are currently circulating widely, among humans. Subtype H5N1, causing highly pathogenic avian influenza, was identified in 1996 in southern China. Influenza A H5N1 is significant, though not unique, in its ability to cross normal species barriers and directly infect humans; avian subtypes H9N2 and H7N7 are also known to cause infection in humans. For this reason, testing for H5N1 virus alone is not recommended, and any unusual influenza viruses that cannot be subtyped should be referred to a public health laboratory or the CDC. Among pathogenic avian influenza virus strains, the wide geographical distribution of H5N1 in avian species and the number and severity of human infections are unprecedented. If, or when, the virus evolves into a strain transmitted readily among humans, and unless there is a dramatic decrease in the pathogenicity of the resulting virus, the result will likely be an influenza pandemic with mortality rates not seen since the 1918 pandemic.

The classical epidemiological cycle of influenza A virus includes wild waterfowl and shorebirds, which are naturally infected; domestic waterfowl and poultry, which acquire virus from wild birds; pigs, which serve as "mixing vessels" for both avian and mammalian adapted strains; and humans, who are susceptible to the reassorted viruses. Reassortment can also occur during human-to-human transmission. The H5N1 virus has bypassed this epidemiological cycle, has crossed normal species barriers, and is capable of being transmitted directly from poultry to humans. First identified as a cause of highly pathogenic avian influenza in southern China in 1996, the virus has since spread to Asia, Southeast Asia, Central Asia, the Middle East, Eastern and Western Europe, and Africa (http://gamapserver.who.int/mapLibrary/Files/Maps/Global_SubNat_H5N1inAnimalConfirmedCUMULATIVE_20081003.png).H5N1 has been found in domestic fowl and a variety of migratory and resident wild bird species. Avian influenza strains infect the intestinal tract and are shed at high titers in feces. Transmission rates are high among birds congregating at bodies of water. Human infections caused by H5N1 were first identified in 1997. Human H5N1 infections are the result of exposure to high viral titers in infected birds or feces. There is evidence of human-to-human transmission, yet secondary cases are very limited due to the avian host specificity of H5N1. Hundreds of confirmed human cases of avian influenza H5N1 have been reported to the World Health Organization (http://www.who.int/csr/disease/avian_influenza/country/en/), and the mortality rate has exceeded 60%. Human cases have been reported from Azerbaijan, Bangladesh, Cambodia, China, Djibouti, Egypt, Indonesia, Iraq, Lao People's Democratic Republic, Myanmar, Nigeria, Pakistan, Thailand, Turkey, and Vietnam.

Influenza pandemics result from the emergence of antigenically distinct subtypes of influenza A virus. During the 20th century, influenza pandemics occurred in 1918, 1957, and 1968. The subtypes causing these pandemics all had avian origins and adapted to high transmissibility among humans. Pathogenicity among the pandemic virus strains varied. In order to cause a pandemic, an influenza virus strain must be sufficiently distinct antigenically from circulating strains, there must be an immunologically naïve host population, the virus must replicate efficiently in humans, and there must be efficient human-to-human transmission.

Influenza virus is considered a category C biothreat agent. Agents in this category are emerging, readily available, and easily disseminated. In addition, they are capable of causing high morbidity and mortality rates (http://www.bt.cdc.gov/agent/agentlist-category.asp).

Influenza caused by H5N1 shares features with the influenza of the Spanish pandemic of 1918. Morbidity and mortality are severe in previously healthy, young and middle-aged persons. The innate immune response is in part responsible for

pathogenesis, causing fluid accumulation in the lungs. While influenza caused by H5N1 is notable for its aggressive course and high mortality rates, evidence indicates that mild disease and asymptomatic infections occur. Symptomatic cases are characterized by high fever, cough, and lower respiratory tract symptoms (shortness of breath and pulmonary infiltrates) in virtually 100% of patients. Diarrhea occurs more frequently than with influenza caused by human-adapted subtypes. The frequencies of pneumonia and diarrhea distinguish avian from seasonal influenza. Over 60% of reported H5N1 influenza cases were fatal. Death is primarily due to respiratory or multiorgan failure. Unlike human-adapted subtypes, H5N1 is found in relatively high titers in lower respiratory tract specimens, throat swabs, and stool. Although it is not known if infections with a pandemic strain will yield frequently demonstrable viremia, the current H5N1 virus has been isolated from serum. The CDC has developed a case definition and risk assessment for human influenza caused by influenza A H5N1 (http://www2a.cdc.gov/han/archivesys/ViewMsgV.asp?AlertNum=00246). When there is a suspected case, clinicians and infection control practitioners should work closely with state health department epidemiologists to perform a case risk assessment. Specimens from suspected avian influenza cases should be referred to the local or state public health laboratory, or another Laboratory Response Network (LRN) reference laboratory.

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING

Observe standard precautions.

A. Specimen collection

Detection of influenza A H5N1 is more likely from specimens collected within the first 3 days of illness onset. If possible, serial specimens should be collected over several days from the same patient to increase clinical sensitivity.

Dacron- or rayon-tipped swabs should be used for specimen collection, as other materials may inhibit reverse transcriptase PCR (RT-PCR). Rapid antigen detection kits provide or specify swab types. Swabs placed in viral transport medium are generally suitable for RT-PCR testing. Specific specimen requirements are provided by the testing laboratory.

The collection of lower respiratory specimens generates aerosols and requires infection control precautions for influenza A H5N1, including the use of gloves, gown, eye protection, and a respirator rated at least N-95.

1. Respiratory specimens

Throat swabs and lower respiratory samples such as bronchoalveolar lavage samples and tracheal aspirates are the preferred specimens for detection of influenza A H5N1 virus. Nasal swabs and aspirates are acceptable, but they may contain lower titers than throat swabs. This is an important distinction between H5N1 and seasonal, human-adapted influenza A subtypes. Since in a given suspected case of influenza the subtype will be unknown, collection of paired throat and nasopharyngeal swabs (in the same tube of transport medium) will optimize detection of both H5N1 and human-adapted influenza viruses.

◩ **NOTE:** Collection of a nasopharyngeal swab and a throat swab from the same patient (submitted in the same tube of viral transport medium) would provide optimal specimens for both human-adapted and avian influenza strains.

2. Rectal specimens

In contrast to the case with seasonal human influenza, diarrhea is a common symptom of H5N1 infection. Influenza A H5N1 viral RNA has been detected in rectal swabs by RT-PCR. However, current CDC testing recommendations (http://www.hhs.gov/pandemicflu/plan/) do not include rectal or stool specimens. Additionally, rectal specimens are inappropriate for human-adapted influenza strains.

3. Serum

Influenza A H5N1-specific antibody can be detected in serum by the microneutralization assay. Paired specimens, the first collected during acute illness and the second collected 2 to 4 weeks later, are required for definitive diagnosis.

B. Specimen transport and storage

Specimens should be stored at refrigeration temperatures, unless specified otherwise by test procedures. For virus isolation, specimens should be stored at re-

II. SPECIMEN COLLECTION, TRANSPORT, AND HANDLING *(continued)*

frigeration temperatures no longer than 2 days, or frozen at ≤70°C, and shipped on dry ice. Follow current regulations for packaging and shipping hazardous materials; see the American Society for Microbiology (ASM) *Sentinel Laboratory Guidelines for Suspected Agents of Bioterrorism and Emerging Infectious Diseases/Packing and Shipping Infectious Substances* (http://www.asm.org/images/pdf/PackingandShipping1-08.pdf).

ANALYTICAL CONSIDERATIONS

III. PROCEDURES

NOTE: An influenza pandemic will likely result in a dramatic increase in testing orders and the potential for temporary deterioration of production and distribution systems. Therefore, laboratorians should plan for shortages of basic laboratory supplies.

It is imperative that these cultures be handled in a biological safety cabinet.

A. Biosafety

Do *not* perform culture on specimens if avian influenza A H5N1 is suspected, unless it can be performed under enhanced BSL 3 laboratory conditions. Very few laboratories have the capability to operate under enhanced BSL 3 conditions. Enhancements include use of respirators, decontamination of all waste (solid and liquid), and showering of personnel before exiting. Molecular testing, rapid antigen testing, and direct fluorescent-antibody staining can be performed on respiratory specimens under standard BSL 2 conditions in a class II biological safety cabinet. A complete description of enhanced BSL 3 requirements is available at http://www.hhs.gov/pandemicflu/plan/.

Only laboratories capable of performing culture under BSL 3 conditions with enhancements should perform culture to evaluate a suspected influenza H5N1 case. If these criteria are met, and culture is performed, consultation with the CDC and the state public health laboratory is recommended.

B. Methods of identification

1. Rapid antigen tests

Rapid antigen tests are among the least reliable for diagnosis of influenza and should not be used to rule out avian influenza in a suspected case, especially during the prepandemic phase. To date, approximately 17% of specimens from confirmed H5N1 cases have been positive by rapid antigen tests (2).

Because rapid influenza antigen tests provide a result in 30 min or less, they significantly impact patient treatment and management. These tests are widely used for diagnosis of influenza in central, point-of-care, and physician office laboratories. Several rapid antigen tests are commercially available, some of which are able to distinguish between influenza A and B types. Some of these tests are Clinical Laboratory Improvements Act waived. Rapid antigen tests are less sensitive than culture or RT-PCR. Manufacturer-claimed sensitivity ranges from 40 to 100% and specificity from 52 to 100% compared to viral culture. As with all laboratory tests, the positive predictive value of rapid influenza tests is low when disease prevalence is low. Therefore, positive results outside of the influenza "season" should be interpreted with caution and confirmed by additional tests.

While rapid antigen capture assays may detect avian influenza virus subtypes, including H5N1, currently available tests are not capable of distinguishing specific influenza A subtypes. Recent evidence indicates that currently available rapid antigen tests are extremely insensitive for H5N1 (1, 2) and should not be used to rule out avian influenza in a suspected case, especially during the prepandemic phase.

III. PROCEDURES *(continued)*

2. Fluorescent-antibody staining of antigens

The staining of influenza antigens with fluorescent antibody is an additional rapid test. When performed directly on cells from respiratory specimens, this method can provide results in less than an hour. The availability of fluorescent-antibody staining is restricted to laboratories with immunofluorescent microscopes and trained technologists able to accurately interpret fluorescent staining patterns. Since this is not a point-of-care test, the factors with the most significant impact on turnaround time of test results are specimen transport to the testing laboratory and batching of specimens. Fluorescently labeled antibodies specific for influenza A and B viruses are available. Some commercially available influenza virus antibodies are provided in pools with antibodies to other common respiratory viruses. Fluorescent-antibody staining is generally considered to be slightly more sensitive than rapid antigen tests. Specificity is high but depends on well-trained, experienced technologists.

Fluorescent-antibody staining reagents specific for influenza A virus will detect avian influenza A H5N1 (1). Currently, there are no H5N1-specific fluorescent-antibody reagents commercially available.

3. Nucleic acid amplification

Nucleic acid amplification methods such as RT-PCR and nucleic acid sequence-based amplification are becoming more commonly used for detection of influenza virus and other respiratory viruses. Using real-time, fluorescent detection of amplified product, laboratories are able to perform molecular tests in less than 3 h. These are consistently the most sensitive methods for detection of influenza virus, including H5N1. High specificity requires judicious selection of primers and probes, optimization of amplification conditions, and careful interpretation of results. Continuous adherence to laboratory protocol is essential to avoid false positives due to carryover contamination. Currently, the only Food and Drug Administration (FDA)-cleared nucleic acid amplification test for influenza H5N1 is the RT-PCR method developed by the CDC and used by LRN reference laboratories. There are also commercially developed, FDA-cleared PCR assays that detect influenza A and B viruses (one of which identifies influenza H1 and H3 subtypes), but none have performance claims for H5N1 or other novel subtypes. In addition to LRN reference laboratories, some commercial and hospital laboratories offer nucleic acid amplification testing for influenza A H5N1. Unlike the LRN method, these tests are developed, verified, and validated in-house by each laboratory. Consequently, the performance characteristics of the tests may vary among laboratories. The lack of access to clinical specimens from H5N1-infected patients, or to the infectious virus itself, severely limits the extent to which many laboratories can verify and validate test performance.

RT-PCR testing only for the H5N1 subtype is not recommended. Specimens from suspected cases should be tested for both influenza A and B and currently circulating influenza A subtypes in addition to H5N1.

Initial specimen processing, including addition of lysis buffer, can be performed within a biological safety cabinet in a BSL 2 laboratory. Some specimen lysis buffers do not inactivate viruses. If lysis buffer that is known to inactivate the virus is used, further processing can be performed outside the biological safety cabinet.

4. Culture

Culture provides highly specific laboratory diagnosis of influenza but requires fresh, refrigerated specimens for optimal sensitivity. Specimens in viral transport medium must be kept at 2 to 8°C and processed within 48 h

III. PROCEDURES *(continued)*

to avoid an excessive decrease in viral titer. With proper specimen handling, culture is more sensitive than antigen detection methods. Historically, culture methods have been considered too slow to impact patient management. Incubation for at least 5 days is generally required to detect influenza virus in tube cultures. Tubes are generally held for 14 days prior to reporting a negative result. Influenza virus is detected in tube cultures by the presence of cytopathic effect, adsorption or agglutination of RBCs, and fluorescently labeled antibodies specific for influenza A and B viruses. Spin-amplified shell vial cultures have reduced the time to detection to 1 to 3 days. However, this is considered by many to be too slow to impact patient treatment or isolation decisions. Culture is valuable for detecting influenza virus infection missed by rapid testing, for confirmation of nonculture results when disease prevalence is low, and to obtain isolates for characterization and surveillance.

Influenza A H5N1 grows in cell lines commonly used for isolation of human-adapted influenza virus, including primary monkey kidney, Madin-Darby canine kidney, A-549, and others. Clinical laboratories must be cognizant that H5N1 and other highly pathogenic novel subtypes can be cultivated unknowingly from unrecognized human cases of avian influenza. Do not perform culture on specimens if avian influenza A H5N1 is suspected, unless culture is performed under enhanced BSL 3 laboratory conditions. Contact a local or state public health laboratory for instructions on handling and shipping of specimens from suspected avian influenza cases.

Instructions for clinical microbiology laboratories that set up viral cultures under BSL 2 conditions on a specimen or patient later determined to be positive for influenza A H5N1 are as follows.

- **a.** Consult with the public health laboratory or CDC. These laboratories may request specimens or cultures.
- **b.** Isolate specimens, cell culture vessels, and supplies potentially contaminated with influenza A H5N1 virus. Sterilize by autoclaving, and discard.
- **c.** Disinfect work area.
- **d.** Monitor potentially exposed staff for symptoms. Public health or medical experts may recommend quarantine and prophylactic treatment.
- **e.** Highly pathogenic avian influenza viruses, including H5N1, are select agents regulated by the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture. If live H5N1 virus is isolated from a clinical specimen, the CDC or APHIS must be notified, and the agent must be transferred or destroyed. Documentation of transfer or destruction must be maintained for 3 years.

5. Serology

Serologic test methods to detect influenza virus-specific antibodies are available and are generally performed in reference and public health laboratories. These methods include indirect fluorescent-antibody testing (IFA), complement fixation, hemagglutination inhibition (HI), and neutralization. HI and neutralization are often used with specific antigens to identify influenza virus type- and subtype-specific antibody titers. The diagnostic utility of serology is limited by the need, generally, to collect both acute- and convalescent-phase sera to identify either seroconversion or a fourfold rise in antibody titer. As a result, serologic methods that detect immunoglobulin G (IgG) responses have relatively little impact on patient management. IFA and other tests that detect IgM antibodies can detect acute infection, but sensitivity is reduced because serum IgM levels are often low due to repeated exposure to vaccine or circulating virus.

POSTANALYTICAL CONSIDERATIONS

IV. REPORTING AND INTERPRETATION OF RESULTS

If a clinical microbiology laboratory presumptively identifies or cannot rule out influenza A H5N1 from a patient, immediate communication with the public health department is essential. Positive results for influenza A H5N1, or other novel strains of influenza virus, should be considered presumptive, pending confirmation by an LRN reference laboratory. Specimens from suspected avian influenza cases should be referred to the local or state public health laboratory or another LRN reference laboratory.

Carefully obtained travel and clinical histories will provide invaluable information that will help both the physician and the laboratory in determining the etiologic agent, implementing infection control procedures, and determining necessary confirmatory testing.

REFERENCES

1. **Fedorko, D. P., N. A. Nelson, J. M. McAuliffe, and K. Subbarao.** 2006. Performance of rapid tests for detection of avian influenza A virus types H5N1 and H9N2. *J. Clin. Microbiol.* **44:**1596–1597.
2. **Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of Human Infection with Avian Influenza A (H5N1) Virus.** 2008. Update on avian influenza A (H5N1) virus infection in humans. *N. Engl. J. Med.* **358:**261–273.

SUPPLEMENTAL READING

Atmar, R. L. 2007. Influenza viruses, p. 1340–1351. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.

de Jong, M. D., V. C. Bach, T. Q. Phan, M. H. Vo, T. T. Tran, B. H. Nguyen, M. Beld, T. P. Le, H. K. Truong, V. V. Nguyen, T. H. Tran, Q. H. Do, and J. Farrar. 2005. Fatal avian influenza A (H5N1) in a child presenting with diarrhea followed by coma. *N. Engl. J. Med.* **352:**686–691.

de Jong, M. D., C. P. Simmons, T. T. Thanh, V. M. Hien, G. J. Smith, T. N. Chau, D. M. Hoang, N. Van Vinh Chau, T. H. Khanh, V. C. Dong, P. T. Qui, B. Van Cam, Q. Ha do, Y. Guan, J. S. Peiris, N. T. Chinh, T. T. Hien, and J. Farrar. 2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat. Med.* **12:**1203–1207.

de Jong, M. D., T. T. Tran, H. K. Truong, M. H. Vo, G. J. Smith, V. C. Nguyen, V. C. Bach, T. Q. Phan, Q. H. Do, Y. Guan, J. S. Peiris, T. H. Tran, and J. Farrar. 2005. Oseltamivir resistance during treatment of influenza A (H5N1) infection. *N. Engl. J. Med.* **353:**2667–2672.

Nyoman Kandun, I., H. Wibisono, E. R. Sedyaningsih, Yusharmen, W. Hadisoedarsuno, W. Purba, H. Santoso, C. Septiawati, E. Tresnaningsih, B. Heriyanto, D. Yuwono, S. Harun, S. Soeroso, S. Giriputra, P. J. Blair, A. Jeremijenko, H. Kosasih, S. D. Putnam, G. Samaan, M. Silitonga, K. H. Chan, L. L. M. Poon, W. Lim, A. Klimov, S. Lindstrom, Y. Guan, R. Donis, J. Katz, N. Cox, M. Peiris, and T. M. Uyeki. 2006. Three Indonesian clusters of H5N1 virus infection in 2005. *N. Engl. J. Med.* **355:**2186–2194.

Oner, A. F., A. Bay, S. Arslan, H. Akdeniz, H. A. Sahin, Y. Cesur, S. Epcacan, N. Yilmaz, I. Deger, B. Kizilyildiz, H. Karsen, and M. Ceyhan. 2006. Avian influenza A (H5N1) infection in Eastern Turkey in 2006. *N. Engl. J. Med.* **355:**2179–2185.

Yuen, K. Y., P. K. Chan, M. Peiris, D. N. Tsang, T. L. Que, K. F. Shortridge, P. T. Cheung, W. K. To, E. T. Ho, R. Sung, and A. F. Cheng. 1998. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. *Lancet* **351:**467–471.

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